Cannabinoid Signalling

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Abstract

The general aim of the study was to investigate the signalling pathways utilised by cannabinoids. Cannabinoid CB_1 receptor stimulation in DDT_1 MF-2 smooth muscle cells induces a rise in $[Ca^{2+}]_i$, which is dependent on extracellular Ca^{2+} and modulated by thapsigargin-sensitive stores and MAP kinase suggesting capacitative Ca^{2+} entry (CCE). Non-capacitative calcium entry (NCCE) stimulated by arachidonic acid (AA) partly mediates histamine H_1 receptor-evoked increases in $[Ca^{2+}]_i$ in DDT_1 MF-2 cells. In the current study both Ca^{2+} entry mechanisms and a possible link between MAP kinase activation and increasing $[Ca^{2+}]_i$, were investigated. In the whole-cell patch clamp configuration, the cannabinoid receptor agonist CP 55,940 evoked a transient Ca^{2+}-dependent K^+ current, which was not blocked by inhibitors of CCE, 2-APB and SKF 96365, although SKF 96365 did inhibit the outward current evoked by the refilling component of the response to histamine. AA but not its metabolites evoked a transient outward current and inhibited the response to CP 55,940 in a concentration-dependent manner. CP 55,940 induced a concentration-dependent release of AA, which was inhibited by the CB_1 receptor antagonist SR 141716A. The non-specific Ca^{2+} channel blockers, La^{3+} and Gd^{3+}, inhibited the CP 55,940-induced current at concentrations that had no effect on thapsigargin-evoked CCE. La^{3+} also inhibited AA-mediated currents. The effect of CP 55,940 on AA release was abolished by phospholipase A_2 inhibition with quinacrine. This compound also inhibited outward currents mediated by CP 55,940. The data supports the possibility that in DDT_1 MF-2 cells AA is an integral component of the CB_1 receptor signalling pathway, upstream of NCCE and, via PLA_2, downstream of MAP kinase.

In a parallel line of work the present study aimed to identify the signalling events that might mediate a cannabinoid-induced inhibition of neurotransmission in the myenteric plexus, leading to a reduction in intestinal motility. Myenteric neurons were grown in primary culture enabling electrophysiological recordings to be made from individual cells to study the effects of cannabinoids on ion conductance. Immunohistochemistry validated these neurons as a model for those *in situ*, demonstrating that all CB_1 receptor-positive cells express the cholinergic marker choline acetyltransferase. CP 55,940 was not shown to activate G-protein inwardly rectifying K^+ channels but did inhibit evoked Ca^{2+} currents in myenteric cultures, a signalling mechanism that may underlie the CB_1 receptor-mediated inhibition of neurotransmitter release from presynaptic sites.

Nicotinic ACh (nACh) receptors are also expressed on cultured myenteric neurons. Stimulation of these receptors by nicotine evoked a transient inward current, which was inhibited by CP 55,940 and the endogenous cannabinoid anandamide, in an SR 14716A-insensitive manner. In fact, SR 141716A alone inhibited currents mediated by nACh receptors. PEA, a cannabinoid ligand whose effects are thought to occur independently of CB_1/ CB_2 receptor activation, also inhibited nicotine-induced currents. Pertussis toxin, a $G_{i/o}$ inhibitor, did not reverse the cannabinoid-induced inhibition of nicotinic currents. In addition, CP 55,940 inhibited the sustained inward current evoked by 5-HT application in cultured myenteric neurons. The results suggest that cannabinoids inhibit nACh channels through a CB_1 receptor-independent pathway in myenteric neurons, which would lead to a reduction in excitatory neurotransmission in the intact myenteric plexus. The inhibitory effect on the 5-HT-induced sustained inward current also suggests a cannabinoid-evoked inhibition of currents possibly mediated by the $5\text{-}HT_{1P}$ receptor.

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Contents

Abbreviations

AA	arachidonic acid
AAI	aminoalkylindoles
AC	adenylyl cyclase
ACh	acetylcholine
AEA	anandamide
2-AG	2-arachidonyl glycerol
AHP	after-hyperpolarisation
AIDS	Acquired Immunodeficiency Syndrome
AMT	anandamide membrane transporter
ANOVA	analysis of variance
2-APB	2-aminoethoxydiphenylborane
ATK	arachidonoyl trifluoromethyl ketone
ATP	adenosine triphosphate
BCA	bicinchoninic acid
4-BPB	4-bromophenacyl bromide
BSA	bovine serum albumin
C6	hexamethonium
$[Ca^{2+}]_i$	intracellular Ca^{2+} concentration
cAMP	cyclic adenosine monophosphate
CB_1	cannabinoid CB_1 receptor
CB_2	cannabinoid CB_2 receptor
CeA	central amygdala
CC	classical cannabinoid
CCE	capacitative Ca^{2+} entry
ChAT	choline acetyltransferase
CHO	chinese hamster ovary
Cm	membrane capacitance
CM	circular muscle
DAG	diacylglycerol
DAGL	diacylglycerol lipase
DHβE	dihydro-β-erythroidine
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DRG	dorsal root ganglion
DSE	depolarisation-induced suppression of excitation
DSI	depolarisation-induced suppression of inhibition
ECL	enhanced chemo luminescence
ECS	extracellular solution
EDHF	endothelium-derived hyperpolarising factory
Em	membrane potential
FAAH	fatty acid amide hydrolase
FCS	fetal calf serum
FDA	Food and Drug Administration
fEPSP	fast excitatory postsynaptic potential
GABA	γ-aminobutyric acid
GDP	guanosine diphosphate
GIRK	G-protein inwardly rectifying K^+ channel

GPCR	G-protein-coupled receptor
GTP	guanosine triphosphate
H_1	histamine H_1 receptor
HEK	human embryionic kidney
5-HT	5-hydroxytryptamine
I	current
I_A	A-type K^+ current
I_{ARC}	arachidonate regulated Ca^{2+} current
ICC	interstitial cells of Cajal
ICS	intracellular solution
i.c.v.	intracerebroventricular
I_D	D-type K^+ current
IFN	interferon
$I_{K,Ca}$	Ca^{2+}-dependent K^+ current
IL	interleukin
I_M	M-type K^+ current
$InsP_3$	inositol triphosphate
$InsP_4$	inositol tetrakisphosphate
i.p.	intraperitoneal
IPAN	intrinsic primary afferent neuron
i.v.	intravenous
K_{ir}	inwardly rectifying K^+ channel
LM	longitudinal muscle
LTP	long-term potentiation
M	muscarinic
MAFP	methyl arachidonyl fluorophosphonate
MAGL	monoacylglycerol lipase
MAP kinase	mitogen-activated protein kinase
MDCK	Madin-Darby canine kidney
MLA	methyllycaconitine citrate
MP	myenteric plexus
MPLM	myenteric plexus longitudinal muscle
NAc	nucleus accumbens
nACh	nicotinic acetylcholine receptor
NADA	*N*-arachidonoyl-dopamine
NANC	non-adrenergic, non-cholinergic
NArPE	*N*-arachidonoylphosphatidylethanolamine
NCC	non-classical cannabinoid
NCCE	non-capacitative Ca^{2+} entry
NF	neurofilament
NKA	neurokinin A
NK_x	neurokinin NK_x receptor
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NOS	nitric oxide synthase
P_2	purine P_2 receptor
PACAP	pituitary adenylyl cyclase activating peptide
PAG	periaqueductal grey
PBS	phosphate buffered saline
PE	phosphatidylethanolamine

PEA	palmitoylethanolamide
PFC	prefrontal cortex
PI	phosphatidylinositol
PI3K	phosphatidylinositol-3-kinase
PIP_2	phosphatidylinositol diphosphate
PKA	protein kinase A
PKB	protein kinase B
PLA_1	phospholipase A_1
PLA_2	phospholipase A_2
$cPLA_2$	cytosolic phospholipase A_2
$iPLA_2$	Ca^{2+}-independent phospholipase A_2
$sPLA_2$	secretory phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
P/N	positive/ negative
PTX	pertussis toxin
PVDF	polyvinylidene diflouride
R	resistance
R_m	membrane resistance
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	standard error of the mean
sEPSP	slow excitatory postsynaptic potential
SERCA	sarcoplasmic/ endoplasmic reticulum Ca^{2+} ATPase
SOCCs	store-operated Ca^{2+} channels
SOM	somatostatin
SP	submucosal plexus
Sub P	substance P
TEA	tetraethylammonium
Δ^9-THC	Δ^9-tetrahydrocannabinol
TM	transmembrane
TNF	tumour necrosis factor
TK	tachykinin
TTX	tetrodotoxin
V	voltage
VIP	vasoactive intestinal polypeptide
VOCCs	voltage-operated Ca^{2+} channels
VR_1	vanilloid VR_1 receptor
VTA	ventral tegmental area
WDR	wide dynamic range

INTRODUCTION

1.1 Background

The hemp plant *Cannabis sativa* has been used for over 4000 years as both a therapeutic and recreational drug. The beneficial effects of these compounds were documented as early as the 4th Century B.C. where it was used for the treatment of medical ailments such as malaria, constipation, rheumatic pains and female disorders. At present, there are a number of proven and potential therapeutic actions of cannabinoids (the active constituents of cannabis) such as anti-emetics (Lewis *et al.*, 1994; Abrahamov *et al.*, 1995), analgesics (Pertwee, 2000; Iversen and Chapman, 2002), anti-anxiolytics (Gaetani *et al.*, 2003) and anti-convulsants (Wallace *et al.*, 2001). They may also have a use as anti-inflammatory agents (Zurier *et al.*, 1998), immunosuppressive agents (Cabral *et al.*, 1998) and in diseases such as glaucoma (Song and Slowey, 2000), Alzheimer's disease (Milton, 2002), multiple sclerosis (Baker *et al.*, 2000) and in motor disorders such as Huntington's and Parkinson's disease (Van der Stelt and Di Marzo, 2003).

To date, only two cannabinoid-based medicines have been given Food and Drug Administration (FDA) approval, dronabinol and nabilone (Beal *et al.*, 1995; Palmer *et al.*, 2002). Dronabinol is prescribed for patients with wasting syndromes such as Acquired Immunodeficiency Syndrome (AIDS) patients, to stimulate appetite, while nabilone is used to control the nausea produced by cancer chemotherapy. The major problem with developing cannabinoids as therapeutic agents is separating the beneficial effects from the unwanted side effects. This includes sedation, cognitive dysfunction and ataxia as well as the psychotropic effects. Therefore there is a great need to understand the pharmacology of the endogenous cannabinoid system, including the receptors at which they exert their effects. Only then will we be able to

exploit cannabinoids to their full potential, as therapeutic agents, while at the same time reducing the unwanted side effects.

1.2 Cannabinoid receptors

1.2.1 CB_1 receptor

The resin secreted by the plant *C. sativa* contains about 60 active compounds of which Δ^9- tetrahydrocannabinol (Δ^9–THC) is the principal psychoactive component (Gaoni and Mechoulam, 1964). Due to its highly lipophilic nature it was initially believed that Δ^9–THC exerted its effects by interacting with the plasma membrane, stimulating or inhibiting membrane-associated enzymes and altering the physical state of ion channels (Hillard *et al.*, 1985; Martin, 1986). Speculation about the cellular actions of cannabinoids was finally resolved when functional inhibition of adenylyl cyclase, and hence a reduction in cyclic adenosine monophosphate (cAMP; the second messenger produced by adenylyl cyclase), was observed following the addition of Δ^9–THC to neuroblastoma cells (Howlett and Fleming, 1984). The inhibition was blocked with pertussis toxin (PTX) suggesting the involvement of a $G_{i/o}$ protein (Howlett *et al.*, 1986). These actions, as well as studies demonstrating stereoselectivity of the (–)-enantiomers of Δ^9–THC (Dewey, 1986) and specific binding of radiolabeled agonists in rat brain membranes (Devane *et al.*, 1988), indicated that most of the central cannabinoid effects were mediated by a specific membrane receptor protein, the CB_1 receptor. The CB_1 receptor was eventually cloned from rat cerebral cortex (Matsuda *et al.*, 1990) and then later from human brain and testis (Gerard *et al.*, 1991) and mouse brain (Chakrabarti *et al.*, 1995). A splice variant

of the CB_1 receptor was also isolated from human lung and was designated CB_{1A} (Shire *et al.*, 1995).

The CB_1 receptor is expressed in high abundance within certain regions of the brain that correlate well with the observed effects of cannabinoids, including impairments in cognition, memory, learning and motor coordination (Abood and Martin, 1992). Hence, CB_1 receptors have been isolated in the hippocampus, basal ganglia, cerebral cortex, amygdala and cerebellum (Herkenham *et al.*, 1990; Glass *et al.*, 1997; Tsou *et al.*, 1998a). Peripherally, CB_1 receptors have been identified in the spleen and tonsils (Galiegue *et al.*, 1995), the guinea-pig small intestine (Pertwee *et al.*, 1996a), the mouse urinary bladder (Pertwee and Fernando, 1996), the mouse vas deferens (Pertwee *et al.*, 1996b), sympathetic nerve terminals (Ishac *et al.*, 1996; Vizi *et al.*, 2001), hamster smooth muscle cells (Filipeanu *et al.*, 1997), cat vascular smooth muscle cells (Gebremedhin *et al.*, 1999) and at very low levels in adrenal gland, heart, prostate, uterus and ovary (Galiegue *et al.*, 1995).

Recent studies used *in vivo* imaging techniques (positron emission tomography) to identify CB_1 receptor occupancy in mouse brain (Gifford *et al.*, 2002). The technique requires the use of radioisotopes that specifically bind to the target receptor. Interestingly, concentrations of cannabinoids that produced a profound sedation and inhibition of locomotor activity in mice did not reduce CB_1 receptor binding by radioisotopes in the cerebellum and hippocampus (Gifford *et al.*, 2002). This suggests that the occupancy of the CB_1 receptor necessary for the behavioural effects of cannabinoids is very low.

1.2.2 CB_2 receptor

The second cannabinoid receptor, the CB_2 receptor, was later cloned from human promyelocytic leukaemia cells (HL-60 cells) (Munro *et al.*, 1993). This receptor shares with the CB_1 receptor the structural feature typified by seven transmembrane spanning domains and is also coupled to a PTX-sensitive G protein. The clone has 68% amino acid sequence identity to the CB_1 receptor, within the transmembrane domains, and only 44% identity throughout the total protein (Munro *et al.*, 1993). CB_2 receptors are restricted to the periphery where they have been observed in the marginal zone of the spleen (Munro *et al.*, 1993; Schatz *et al.*, 1997), in tonsils and on immune cells (B-cells, monocytes, T-cells) (Munro *et al.*, 1993; Galiegue *et al.*, 1995; Schatz *et al.*, 1997). The localisation of CB_2 receptors in immune tissues implies that cannabinoid-induced immunosuppression involves a receptor-mediated process (see section 1.4.6.7).

1.2.3 CB_x receptor

New data suggests the presence of novel, as yet, uncloned cannabinoid receptors. Using the brains of CB_1 receptor knockout mice ($CB_1^{-/-}$) it was shown that there was significant (though reduced) binding of the cannabinoid agonist [^{3}H]WIN 55,212-2 (Breivogel *et al.*, 2001). Moreover, both WIN 55,212-2 and the endogenous cannabinoid anandamide were still able to stimulate some [^{35}S]guanosine triphosphate (GTP)γS binding (an indicator of G-protein-coupled receptor activation) in $CB_1^{-/-}$ brain, which was not blocked by the cannabinoid CB_1 receptor antagonist SR 141716A. Significant levels of stimulation were observed in the cortex and

hippocampus. In accordance with this finding Monory *et al.* (2002) showed that WIN 55,212-2 was able to stimulate [^{35}S]GTPγS binding in the cerebellum of $CB_1^{-/-}$ mice. SR 141716A could not reverse this effect (Monory *et al.*, 2002). Another study also using $CB_1^{-/-}$ mice found that WIN 55,212-2 was able to inhibit excitatory glutamatergic postsynaptic currents in the hippocampus (Hajos *et al.*, 2001). The cannabinoid-mediated inhibition was sensitive to SR 141716A and was also inhibited by the vanilloid VR_1 receptor antagonist capsazepine (Hajos *et al.*, 2001; Hajos and Freund, 2002a, b). Consistent with these observations WIN 55,212-2 attenuated the release of [^{3}H]glutamate from $CB_1^{-/-}$ mouse hippocampal synaptosomes (Kofalvi *et al.*, 2003). However, in contrast to the elctrophysiological studies, SR 141716A and capsazepine did not antagonise the effect of WIN 55,212-2 (Kofalvi *et al.*, 2003). In fact SR 141716A was shown to potentiate the inhibitory effect of WIN 55,212-2 (Kofalvi *et al.*, 2003). In the basolateral amygdala of anaesthetised rats WIN 55,212-2 inhibited neuronal firing in an SR 141716A- and capsazepine-sensitive manner (Pistis *et al.*, 2004). HU-210, another potent CB_1 receptor agonist, could not mimic the effects of WIN 55,212-2 (Pistis *et al.*, 2004). Another study looking at a cannabinoid-mediated inhibition of cAMP formation in mouse astrocytes reported an SR 141716A-insensitive action of WIN 55,212-2 (Sagan *et al.*, 1999). Immunohistochemical staining confirmed that these astrocytes did not express CB_1 receptors, although the inhibitory actions of WIN 55,212-2 could be blocked by PTX suggesting the involvement of a G-protein-coupled receptor (GPCR) (Sagan *et al.*, 1999).

In the periphery the endogenous cannabinoid anandamide induced mesenteric vasodilatation in $CB_1^{-/-}$ mice, which was SR 141716A-sensitive (Jarai *et al.*, 1999). This novel receptor differs from those in the brain as WIN 55,212-2 was not able to

produce vasodilatation (Jarai *et al.*, 1999) and capsazepine was unable to inhibit vasodilatation (Jarai *et al.*, 1999). Instead a nonpsychoactive synthetic cannabinoid analogue, abnormal-cannabidiol, was found to selectively stimulate the endothelial receptor.

1.3 Cannabinoid receptor ligands

1.3.1 Cannabinoid agonists

Cannabinoid agonists can be divided into four main groups (see Palmer *et al.*, 2002 for review):-

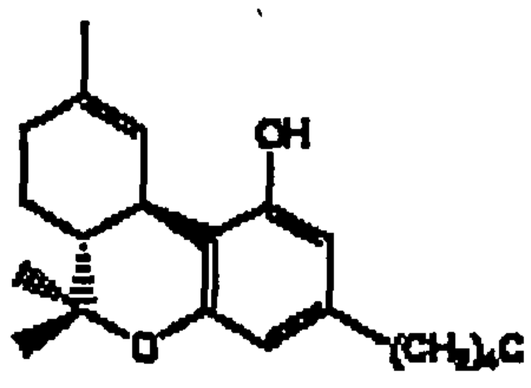
i) classical cannabinoids

ii) non-classical cannabinoids

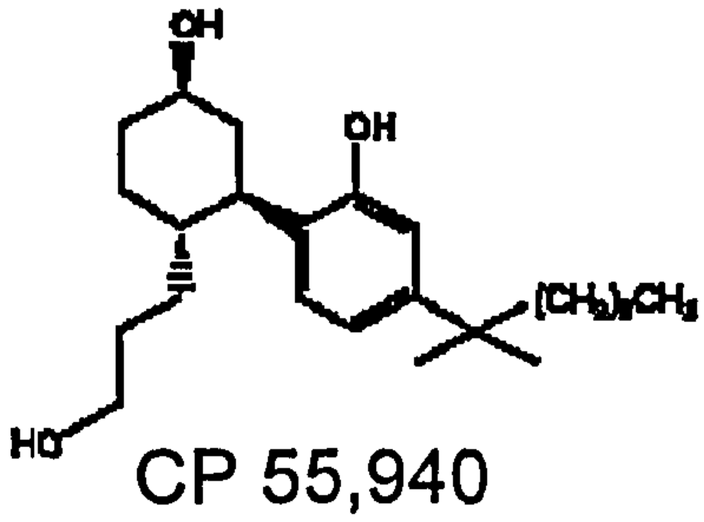
iii) aminoalkylindole group

iv) eicosanoid group

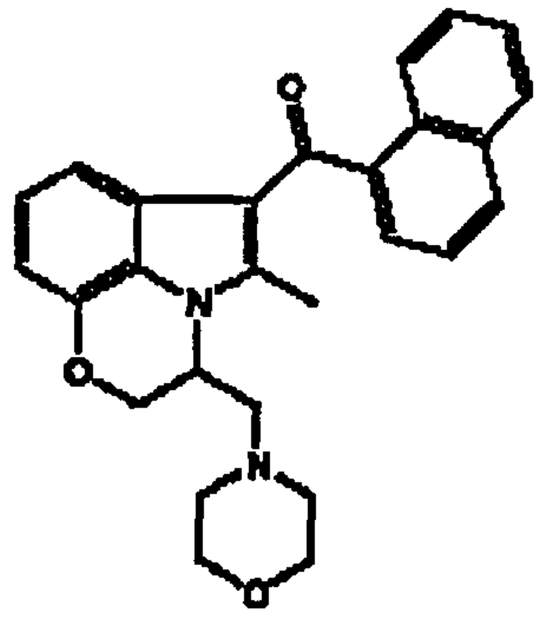
The classical cannabinoid (CC) compounds are tricyclic terpenoid derivatives bearing a benzopyran moiety, of which Δ^9–THC (Fig. 1.1) and its analogue HU-210 are examples. The only two licensed cannabinoid-based drugs (dronabinol and nabilone) are derived from this group. The CC structural features that seem to be important for cannabinoid activity are the phenolic hydroxyl group and the five-carbon alkyl chain (Goutopoulos and Makriyannis, 2002). Another CC receptor agonist worth mentioning is 0-1057. This stands out from established agonists in being readily soluble in water (Pertwee *et al.*, 2000). The development of these new water-soluble



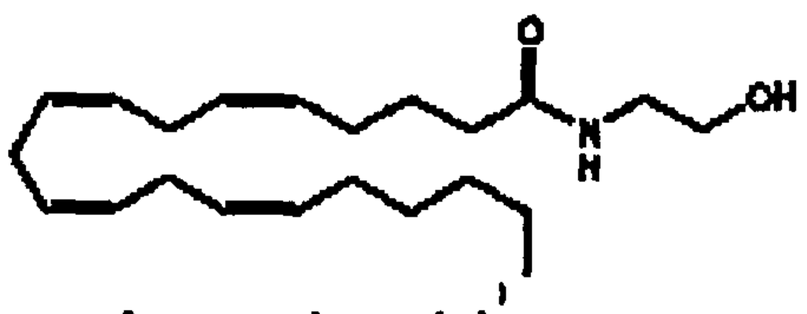
Δ^9-THC



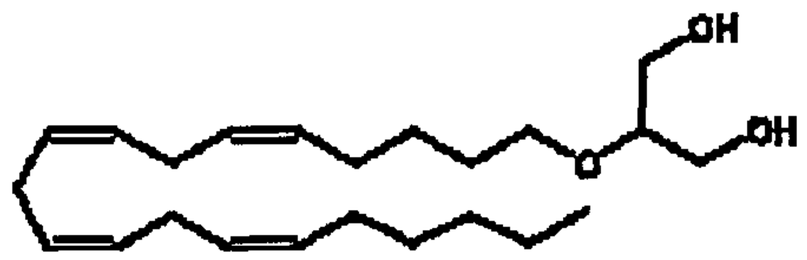
CP 55,940



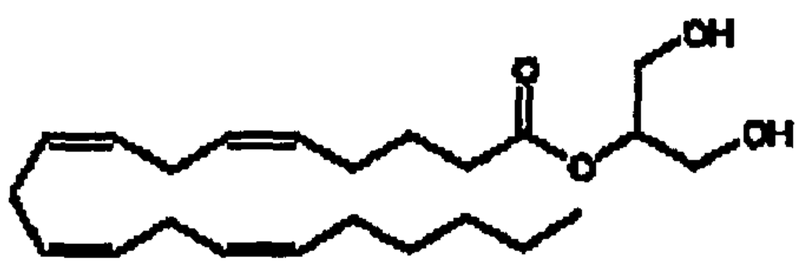
WIN 55,212-2



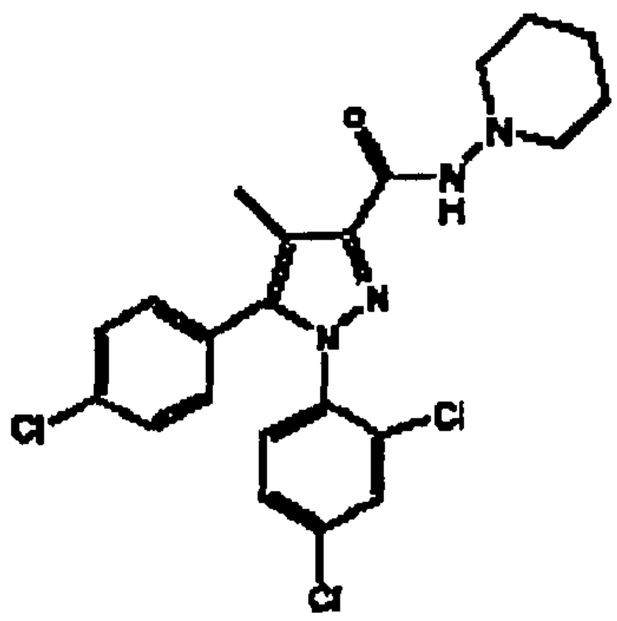
Anandamide



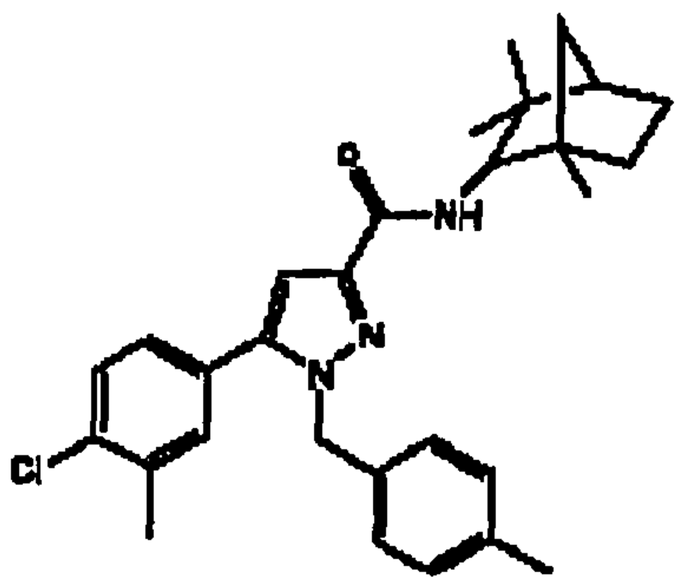
Noladin ether



2-Arachidonyl glycerol



SR 141716A



SR 144528

Figure 1.1 Chemical structures of cannabinoid ligands.

cannabinoids could facilitate drug delivery in the future e.g. the administration of cannabinoids by aerosol inhalation.

The second non-classical cannabinoid (NCC) group, developed by Pfizer (USA), consists of bicyclic and tricyclic analogues of Δ^9–THC lacking the pyran ring of CCs. The side chain and phenolic hydroxyl of the NCCs are crucial for activity. CP 55,940 is a member of this group, exhibiting high affinity, efficacy and stereoselectivity for both cannabinoid receptors (Fig. 1.1). [^{3}H]CP 55,940 was used to identify cannabinoid binding sites in rat brain, which led to the discovery of the CB_1 receptor (Devane *et al.*, 1988).

The third group, developed by Sterling Winthrop (USA), is made up of aminoalkylindoles (AAIs) and includes the potent CB_1/ CB_2 receptor agonist WIN 55,212-2 (Fig. 1.1). The structures of these compounds are quite different from those in the first two groups. The 3-aroyl moiety and the 1-chain, which must contain nitrogen, are important for cannabinergic activity (Goutopoulos and Makriyannis, 2002).

The final group of compounds contains arachidonic acid (AA) derivatives and includes the extensively researched endogenous cannabinoids (or endocannabinoids) anandamide and 2-arachidonyl glycerol (2-AG) (Fig. 1.1). The structure of anandamide consists of AA coupled to ethanolamine through an amide linkage (Devane *et al.*, 1992), whereas 2-AG is an arachidonylester rather than an amide (Stella *et al.*, 1997). The endocannabinoid system is discussed in more detail later on (see section 1.4).

1.3.2 Cannabinoid antagonists

To pharmacologically identify the receptor subtypes mediating cannabinoid effects, receptor-specific antagonists were developed. The first was WIN 56,098, which was shown to antagonise the effects of WIN 55,212-2 through the CB_1 receptor (Pacheco *et al.*, 1991). However, this antagonism was rather weak. More potent antagonists were developed including 6-bromopravadoline and 6-iodopravadoline (AM630) but they showed partial agonist behaviour in some preparations (Pertwee, 1997).

The most potent and well-characterised CB_1 receptor antagonist is SR 141716A (Rinaldi-Carmona *et al.*, 1994), developed by Sanofi (France) (Fig. 1.1). This compound readily displaces [^{3}H]CP 55,940 from specific binding sites and has been shown to prevent cannabinoid-mediated effects, both *in vivo* and *in vitro*. Another compound also developed by Sanofi, SR 144528 (Fig. 1.1), exhibited greater selectivity for the CB_2 receptor (Rinaldi-Carmona *et al.*, 1998).

There is convincing evidence that SR 141716A and SR 144528 produce opposite effects to those seen with CB_1 receptor agonists. For example, in CB_1 receptor-transfected rat superior cervical ganglion neurons WIN 55,212-2 inhibited evoked Ca^{2+} currents (Pan *et al.*, 1998). However, SR 141716A alone increased Ca^{2+} currents in a dose-dependent manner. Another study looked at the effects of cannabinoids on cAMP production in human brain regions, induced by the adenylyl cyclase activator forskolin (Mato *et al.*, 2002). WIN 55,212-2 inhibited cAMP accumulation in the frontal cortex, hippocampus, cerebellum and striatum. Alone, SR 141716A evoked a concentration-dependent increase in basal cAMP with the highest increases observed in the frontal cortex and cerebellum. Furthermore, *in vivo*, SR

141716A has been shown to increase locomotor activity (Compton *et al.*, 1996), produce hyperalagesia (Richardson *et al.*, 1997) and improve short-term memory (Terranova *et al.*, 1995) in mice. In CB_2-transfected Chinese hamster ovary (CHO) cells SR 144528 has also been shown to stimulate forskolin-induced adenylyl cyclase activity (Rinaldi-Carmona *et al.*, 1998) and inhibit GTPγS binding (Ross *et al.*, 1999).

While some of these observations could be attributable to a direct antagonism of responses evoked at cannabinoid receptors by released endocannabinoids, the results together suggest that these compounds are acting as inverse agonists. Other CB_1 receptor antagonists, AM251 and AM281, have also been developed, which are analogues of SR 141716A (Palmer *et al.*, 2002). However, they share the same inverse agonist properties as the SR compounds.

Recently, a novel cannabinoid ligand has become available, O-2050. The ligand was shown to act as a CB_1 receptor antagonist in the mouse vas deferens but did not exhibit any inverse agonism, even at high concentrations (Martin *et al.*, 2002). Further work is required to establish if the same 'silent' antagonist properties are observed in other assays. The development of these 'silent' antagonists will help to further define cannabinoid-mediated effects in the future.

1.4 The endogenous cannabinoid system

1.4.1 The discovery of endocannabinoids

The discovery of a specific cannabinoid receptor in the brain suggested that an endogenous agonist must be present to stimulate it. The hypothesis that such an endocannabinoid should be lipophilic, like the classical exogenous cannabinoids, led Devane *et al.* (1992) to search for such a ligand in the hydrophobic fractions of

porcine brain extracts. Arachidonylethanolamide (anandamide) was eventually isolated from porcine brain providing the first evidence of an endogenous cannabinoid system. It was shown to bind and functionally stimulate not only the CB_1 receptor (Devane *et al.*, 1992; Felder *et al.*, 1993; Vogel *et al.*, 1993; Childers *et al.*, 1994) but also the CB_2 receptor (Slipetz *et al.*, 1995; Shire *et al.*, 1996). Together these studies showed that anandamide was able to displace cannabinoid agonist binding, dose-dependently inhibit electrically-evoked contractions in the mouse vas deferens, inhibit adenylyl cyclase and inhibit N-type Ca^{2+} currents. These actions, like other cannabinoid agonists, were PTX-sensitive implicating the activation of $G_{i/o}$-proteins. Anandamide was also able to mimic the effects of Δ^9-THC in the mouse tetrad, an assay of cannabimetic activity including hypothermia, catalepsy, depression of motor activity and analgesia (Fride and Mechoulam, 1993; Smith *et al.*, 1994).

The relatively low affinity of anandamide for the CB_1 receptor suggested that other more potent endogenous agonists might exist. A second type of endocannabinoid was later isolated from canine gut (Mechoulam *et al.*, 1995) and rat brain (Sugiura *et al.*, 1995), 2-AG. It too was able to inhibit forskolin-stimulated cAMP production, inhibit electrically-evoked contractions of the mouse vas deferens and produce the typical tetrad of effects in mice normally observed to Δ^9-THC (Mechoulam *et al.*, 1995; Sugiura *et al.*, 1996b). Furthermore, brain tissue concentrations of 2-AG were approximately 200-fold higher than those of anandamide (Stella *et al.*, 1997). Evidence also exists which strongly suggests that 2-AG and not anandamide is the natural physiological ligand for both CB_1 and CB_2 receptors. In neuroblastoma x glioma hybrid NG108-15 cells and HL-60 cells, expressing CB_1 and CB_2 receptors respectively, cannabinoids evoked an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Sugiura *et al.*, 1999, 2000). 2-AG induced

the greater agonistic activity when compared to anandamide and was also found to be more potent than synthetic cannabinoid agonists such as CP 55,940 and WIN 55,212-2.

In the last few years more candidates for the role of endogenous cannabinoid ligands have been proposed, all sharing the typical AA backbone. These include 2-arachidonyl glyceryl ether (noladin ether), *0*-arachidonoyl-ethanolamine (virodhamine) and *N*-arachidonoyl-dopamine (NADA). Noladin ether, like anandamide, was also isolated from porcine brain (Hanus *et al.*, 2001) while virodhamine was identified in rat brain, human hippocampus and in some peripheral tissues expressing the CB_2 receptor (Porter *et al.*, 2002). NADA was detected in both rat and bovine brain, with the highest levels found in the striatum and hippocampus (Huang *et al.*, 2002).

Noladin ether was shown to bind to the CB_1 receptor with nanomolar affinity and to the CB_2 receptor with low micromolar affinity and exhibited cannabimetic activity in the mouse tetrad (Hanus *et al.*, 2001). Virodhamine was found to act as a partial agonist with *in vivo* antagonist activity at the CB_1 receptor, although at the CB_2 receptor it acted as a full agonist (Porter *et al.*, 2002). Interestingly, virodhamine is chemically very unstable and it is rapidly converted to anandamide in aqueous environments (Porter *et al.*, 2002). As a potential endogenous antagonist at the CB_1 receptor, virodhamine adds a new form of regulation to the endocannabinoid system.

NADA exhibited greater affinity for the CB_1 receptor over the CB_2 receptor and was found to be more potent and efficacious than anandamide as a CB_1 receptor agonist, as assessed by measuring the stimulatory effect on Ca^{2+} release in N18TG2 neuroblastoma cells (Bisogno *et al.*, 2000).

1.4.2 Neuromodulatory action of endocannabinoids

The main function of the endocannabinoid system is to regulate synaptic neurotransmission. Both anandamide and 2-AG serve as neurotransmitters in their own right, acting as retrograde messengers to inhibit neurotransmitter release through cannabinoid receptors located on presynaptic sites. Gill *et al.* (1970) described one of the first examples of a cannabinoid inhibiting neurotransmitter release. These authors found that Δ^9-THC inhibited electrically-evoked contractions of the guinea-pig ileum but did not affect the response to exogenously applied acetylcholine (ACh) suggesting that the cannabinoid-induced inhibition was a presynaptic event. Depolarisation of cultured striatal and cortical neurons evoked a release of anandamide (Di Marzo *et al.*, 1994), which was shown to inhibit the electrically-evoked release of dopamine in rat striatal slices (Cadogan *et al.*, 1997). During the past three decades cannabinoid-mediated inhibition of transmitter release has been identified in many experimental models including those using human tissue such as hippocampus, heart and ileum (see Schlicker and Kathmann, 2001 for review).

The hippocampus is a prime example where extensive research has been conducted to elucidate the neuromodulatory actions of endocannabinoids. Depolarised rat hippocampal neurons rapidly release anandamide and 2-AG (Stella *et al.*, 1997; Di Marzo *et al.*, 1998), which may modulate long-term potentiation (LTP) (Terranova *et al.*, 1995), a phenomenon whereby synapses become increasingly sensitive so that a constant level of presynaptic stimulation becomes converted into a larger postsynaptic output. The depolarisation is associated with an inhibition of the glutamatergic or GABAergic inputs received by these cells (Hajos *et al.*, 2001; Wilson and Nicoll, 2001) and is known as depolarisation-induced suppression of inhibition (DSI) and

excitation (DSE) respectively. The inhibitory effect on γ-aminobutyric acid (GABA) release was mimicked by WIN 55,212-2 and blocked by the CB_1 receptor antagonist SR 141716A (Wilson and Nicoll, 2001; Ohno-Shosaku *et al.*, 2001). In $CB_1^{-/-}$ mice WIN 55,212-2 was also shown to inhibit glutamatergic transmission in the hippocampus, albeit through a novel cannabinoid receptor, whereas DSI was totally abolished (Hajos *et al.*, 2001; Hajos and Freund, 2002a, b). Moreover, acute administration of cannabinoids reversibly impairs cognitive functions both in animals and humans (Sullivan, 2000), an effect probably mediated through the inhibition of glutamate release and a reduction in LTP. CB_1 receptor-mediated DSE and DSI have also been demonstrated in the cerebellum of rats (Kreitzer and Regehr, 2001a, b). This accumulated data suggests that the retrograde messenger in DSI and DSE is likely to be an endogenous cannabinoid. However, more *in vivo* studies are required to assess the physiological relevance of DSI and DSE. Work presented by Hampson *et al.* (2003) set out to define the specific conditions that elicit DSI at GABAergic synapses in rat CA1 hippocampal pyramidal neurons. Attempts were made to elicit DSI with trains of pulses that mimicked hippocampal cell firing patterns *in vivo*, for instance when animals performed a short-term memory test. However, the authors concluded that, under their experimental conditions, the normal firing patterns of hippocampal neurons that occur *in vivo* do not appear to evoke DSI.

Cannabinoid agonists including CP 55,940 and WIN 55,212-2 have also been shown to inhibit ACh release in the hippocampus (Gifford and Ashby, 1996; Gessa *et al.*, 1997). This inhibitory effect on ACh release may also contribute to the negative effects on learning and memory associated with cannabinoids.

CB_1 receptors are widely distributed in both the CNS and PNS (Herkenham *et al.*, 1990; Tsou *et al.*, 1998a; Ralevic, 2003) strongly suggesting that retrograde

inhibition by endogenous cannabinoids is an important mechanism in these areas, modulating the release of many neurotransmitters including those at excitatory and inhibitory synapses.

1.4.3 Endocannabinoid synthesis

Due to their lipophilicity endocannabinoids could not be stored in vesicles as they could diffuse freely across membranes. Instead it is believed that they are produced 'upon demand' and released from neurons immediately after synthesis (Cadas *et al.*, 1997; Mechoulam *et al.*, 1998; Giuffrida *et al.*, 1999). The biosynthesis and release of anandamide and 2-AG is coupled to postsynaptic depolarisation and the influx of Ca^{2+} into neurons (Di Marzo *et al.*, 1994; Stella *et al.*, 1997; Stella and Piomelli, 2001), although other stimuli including glutamate- (Hansen *et al.*, 1999; Maejima *et al.*, 2001) and dopamine- (Giuffrida *et al.*, 1999) induced stimulation of neurons has also been shown to evoke endocannabinoid synthesis.

It is thought that anandamide synthesis (Fig. 1.2A) involves phospholipase D-catalysed hydrolysis of a phospholipid precursor, *N*-arachidonoyl phosphatidylethanolamine (NArPE) (Di Marzo *et al.*, 1994). In support of this, chromatographic and mass spectrometric analyses have shown that NArPE is present in murine brain, testes and leukocytes, where it may serve as a physiological precursor for anandamide (Di Marzo *et al.*, 1994; Cadas *et al.*, 1996; Cadas *et al.*, 1997). Studies have also characterised the Ca^{2+}-dependent *trans*-acylase that catalyses NArPE formation (Di Marzo *et al.*, 1996a; Cadas *et al.*, 1997). The discovery of this

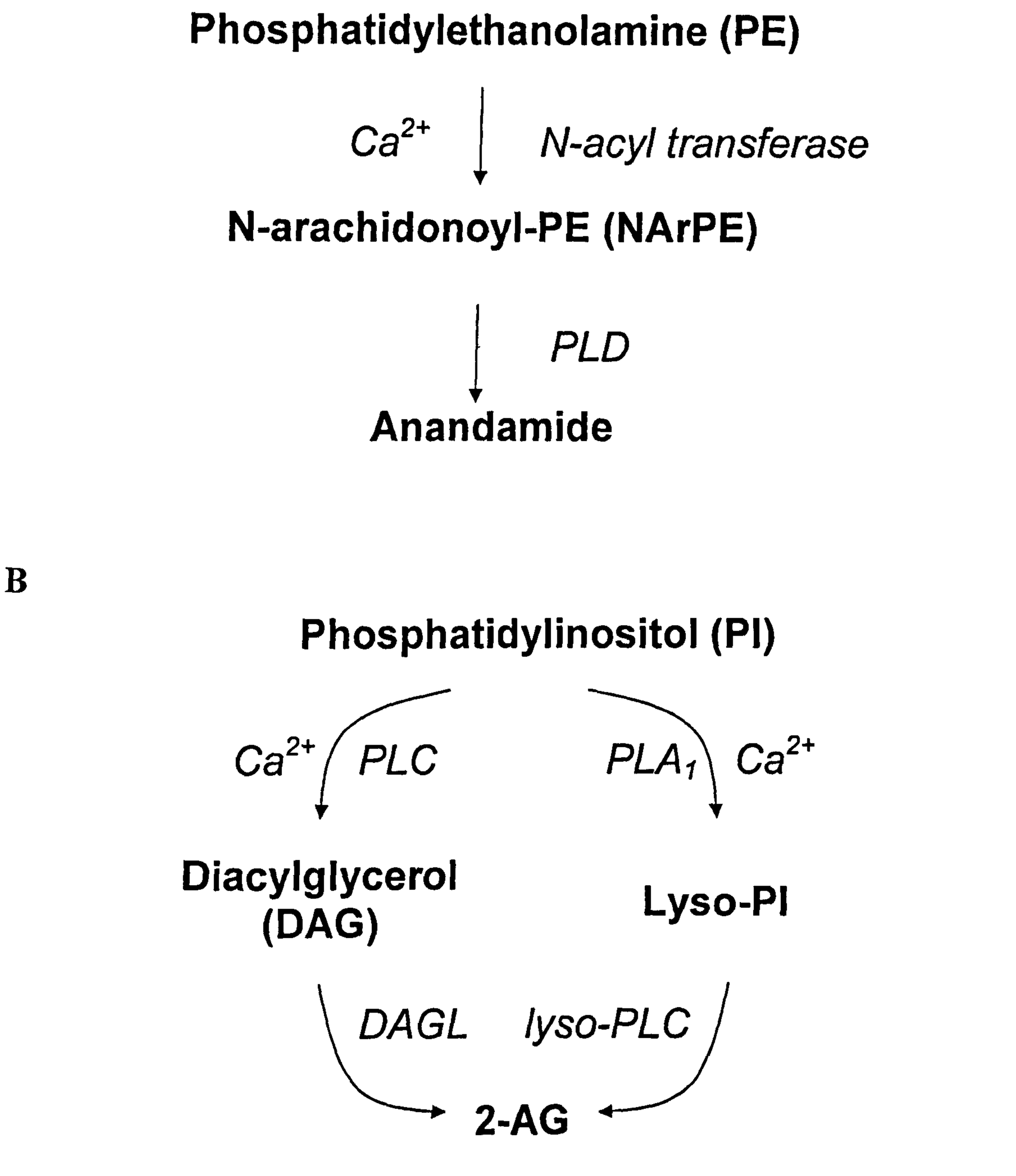


Figure 1.2 Pathways for the synthesis of anandamide and 2-AG.
A, anandamide may be synthesised from the phospholipase D (PLD)-induced conversion of NArPE, which is initially derived from PE by the actions of the Ca^{2+}-dependent *N*-acyl transferase (Di Marzo *et al.*, 1994; Cadas *et al.*, 1997). ***B***, a phospholipase C (PLC)-mediated hydrolysis of PI may produce DAG, which may be subsequently converted to 2-AG by DAG lipase (DAGL). Alternatively, phospholipase A_1 (PLA_1) may generate lyso-PI, which may be hydrolysed to 2-AG by lyso-PLC activity. 2-AG production is also Ca^{2+}-dependent (Stella *et al.*, 1997; Stella and Piomelli, 2001).

enzyme in brain areas containing high levels of anandamide (Cadas *et al.*, 1997), for example hippocampus, cortex and striatum, strongly support its role in the physiological biosynthesis of the endocannabinoid.

There are two possible routes for the biosynthesis of 2-AG in neurons (Fig. 1.2B). The first involves the phospholipase C (PLC)-dependent hydrolysis of membrane lipids to form diacylglycerol (DAG), which may be subsequently converted to 2-AG by DAG lipase (DAGL) (Di Marzo *et al.*, 1996b; Stella *et al.*, 1997). The alternative route involves a phospholipase A_1 (PLA_1)-mediated production of lysophospholipids, which could be hydrolysed to 2-AG by a lyso-PLC enzyme (Piomelli *et al.*, 1998). However, the fact that inhibitors of PLC and DAGL prevent the formation of 2-AG in cultures of cortical neurons suggests that the PLC/ DAGL pathway may be the predominant route through which 2-AG is synthesised (Stella *et al.*, 1997; Stella and Piomelli, 2001). Moreover, two DAGL isozymes have just been cloned and enzymatically characterised (Bisogno *et al.*, 2003). They were mostly found in the plasma membrane, were stimulated by Ca^{2+} and catalysed the Ca^{2+}-dependent formation of 2-AG.

Very little is known about the biosynthesis of the three most recently proposed endocannabinoids, noladin ether, virodhamine and NADA. In N18TG2 neuroblastoma cells, stimulation with ionomycin, an agent that evokes a release of Ca^{2+} from intracellular stores, did not produce noladin ether, under conditions where high levels of 2-AG are normally produced (Fezza *et al.*, 2002). This might suggest a Ca^{2+}-independent pathway for the formation of this endocannabinoid in neurons. NADA may be synthesised from AA and dopamine or tyrosine (Huang *et al.*, 2002).

1.4.4 Cellular reuptake of endocannabinoids

Classical neurotransmitters are usually inactivated by facilitated re-uptake from neurons and subsequent enzymatic degradation. This process may also mediate the clearance of lipid messengers. Being lipophilic in nature, endocannabinoids can diffuse through the plasma membrane if their concentration is greater in the extracellular milieu compared to the intracellular environment. However, if this process is to be rapid it needs to be driven by controllable and selective mechanisms such as a membrane transporter or fast intracellular enzymatic hydrolysis, or both. Cellular reuptake of anandamide may involve a membrane-bound transporter molecule(s), known as the anandamide membrane transporter (AMT) (Di Marzo *et al.*, 1994; Beltramo *et al.*, 1997). Its mechanism of transport differs from that of amine and amino acid transmitters in that it does not require cellular energy or external Na^+, implying that it may be mediated through facilitated diffusion, and it is saturable (Beltramo *et al.*, 1997; Bisogno *et al.*, 1997; Hillard *et al.*, 1997). The substrate selectivity of the AMT has also been investigated (Piomelli *et al.*, 1999). In human astrocytoma cells [^{3}H]anandamide uptake was shown not to be affected by lipids that bear close structural resemblance to anandamide including AA, ceramide, prostaglandins and leukotrienes. However, [^{3}H]anandamide uptake was competitively blocked by non-radioactive anandamide and the anandamide analogue AM404 (Beltramo *et al.*, 1997; Piomelli *et al.*, 1999).

The uptake of 2-AG may occur through the same transporter that targets anandamide. Anandamide and 2-AG can prevent each other's uptake, while the accumulation of either endocannabinoid is blocked with similar potencies by the transport inhibitor AM404 (Beltramo and Piomelli, 2001; Bisogno *et al.*, 2001). Thus,

AM404 was shown to inhibit [^{14}C]anandamide and [^{3}H]2-AG accumulation in C6 glioma cells (Bisogno *et al.*, 2001). Despite these similarities, differences in their uptake have also been documented. For instance [^{3}H]2-AG uptake by astrocytoma cells was reduced in the presence of AA, which had no effect on [^{3}H]anandamide uptake (Beltramo and Piomelli, 2001). An explanation for this could be that the fatty acid may indirectly prevent the facilitated diffusion of [^{3}H]2-AG by inhibiting its conversion to AA (possibly via product inhibition) in the intracellular compartment. Accordingly, interference of AA incorporation into phospholipids was shown to decrease [^{3}H]2-AG uptake in astrocytoma cells but not [^{3}H]anandamide (Beltramo and Piomelli, 2001). Hence, the rates of anandamide and 2-AG transport may differ in their sensitivity to intracellular degradation.

Evidence also exists which suggests that noladin ether, virodhamine and NADA also use the same membrane transporter. In rat C6 glioma cells noladin ether inhibited the uptake of both anandamide and 2-AG and *vice versa* (Fezza *et al.*, 2002). NADA and virodhamine were also able to inhibit anandamide uptake in C6 glioma cells (Huang *et al.*, 2002) and basophils (RBL-2H3 cells) (Porter *et al.*, 2002) respectively.

However, it must be emphasised that the AMT and associated protein(s), as yet, have not been isolated or cloned leading some authors to doubt their existence. Glaser *et al.* (2003) examined the initial rates of anandamide accumulation (<1 minute) in cultured N18TG2 neuroblastoma cells. These authors observed that anandamide uptake was not saturable but anandamide metabolism, measured by increasing ethanolamine concentration after five minutes pre-incubation was saturable. In addition, the supposed AMT inhibitor AM404 was shown to inhibit anandamide hydrolysis in rat brain homogenates by inhibiting fatty acid amide

hydrolase (FAAH; see section 1.4.5) (Glaser *et al.*, 2003). AM404 was also shown to inhibit the uptake of anandamide, preincubated for 5 minutes, in neuroblastoma cells but, in contrast, had no significant effect on the initial uptake of anandamide (25 second incubation). These results suggest that anandamide uptake is a process of simple diffusion, driven by metabolism and therefore the likely mechanism by which AM404 raises anandamide levels is by inhibiting FAAH.

1.4.5 Endocannabinoid hydrolysis

Once inside the cell both anandamide and 2-AG can undergo enzymatic degradation by the membrane bound protein FAAH (Ueda and Yamamoto, 2000; Deutsch *et al.*, 2001) and are immediately degraded to AA and ethanolamine or glycerol respectively (Deutsch and Chin, 1993; Di Marzo *et al.*, 1994). FAAH distribution in the brain is highest in areas rich in CB_1 receptors including the cortex, hippocampus and cerebellum (Thomas *et al.*, 1997; Romero *et al.*, 2002) and inhibitors of FAAH significantly potentiate anandamide effects in the behavioural 'tetrad' in mice (Compton and Martin, 1997). This strongly supports a role of FAAH as a cannabimetic neuromodulator. Mice knockouts have also been created in which the FAAH enzyme is dysfunctional (Cravatt *et al.*, 2001). These mice show a 15-fold higher level of anandamide in the brain compared to wild-types and also a reduced pain sensation, which is paralleled by the analgesic effect of FAAH inhibitors (Kathuria *et al.*, 2003). 2-AG levels were not increased in FAAH knockouts (Lichtman *et al.*, 2002). This is in agreement with previous reports on the existence of additional hydrolases for 2-AG degradation in porcine brain, rat platelets and macrophages (Di Marzo *et al.*, 1999; Goparaju *et al.*, 1999). This(ese) enzyme(s),

known as monoacylglycerol lipase(s) (MAGL), is(are) also expressed in rat brain regions with high CB_1 receptor density, such as the hippocampus (Dinh *et al.*, 2002), supporting the role of MAGL in the degradation of 2-AG.

Esterification may provide a means to inactivate noladin ether (Fezza *et al.*, 2002), while methylation of NADA has been observed by catecholamine *O*-methyl transferase (Huang *et al.*, 2002).

1.4.6 Physiological functions of endocannabinoids

1.4.6.1 Pain modulation

The analgesic effects of cannabinoids have been well documented (see Pertwee, 2001 for review) and the role of endocannabinoids in pain modulation is supported by the presence of CB_1 receptors in central and peripheral sites associated with the processing of nociceptive messages. This includes the periaqueductal gray (PAG) and the rostral ventral medulla (Herkenham *et al.*, 1991b; Tsou *et al.*, 1998a, b), brain areas that suppress spinal responses to noxious stimuli, and the superficial layers of the spinal dorsal horn and the dorsal root ganglion (DRG) (Sanudo-Pena *et al.*, 1999; Salio *et al.*, 2002).

Early studies demonstrated that WIN 55,212-2, administered intracerebroventricularly (i.c.v.) in rats, suppressed tail-flick responses (Martin *et al.*, 1993). In anaesthetised rats extracellular single-unit recordings were obtained from wide-dynamic range (WDR) neurons (Hohmann *et al.*, 1995), which respond to both touch and pain. The firing rate of WDR neurons increased in response to noxious mechanical pressure that was significantly reduced by WIN 55,212-2.

Endocannabinoids appear to participate in endogenous pain modulation by actions in the PAG. Intra-PAG microinjection of cannabinoid agonists CP 55,940 and HU-210 into rats were anti-nociceptive in the tail-flick test (Lichtman *et al.*, 1996) and reduced formalin-evoked nociceptive behaviour (Finn *et al.*, 2003) respectively. Moreover, Walker *et al.* (1999) used *in vivo* microdialysis to demonstrate that the PAG releases anandamide in response to pain stimuli (formalin injection) and electrical stimulation of the PAG, strongly suggesting that endocannabinoids do play a role in antinociception in this brain area.

Immunohistochemical studies by Tsou *et al.* (1998a, b) showed that FAAH is present in the ventral posterior lateral nucleus of the thalamus. This nucleus is the termination zone of the spinothalamic tract, a pathway that is a major source of nociceptive information to the brain (Walker *et al.*, 2002). In FAAH knockout mice the analgesic effect of exogenously applied anandamide was enhanced in a CB_1 receptor-dependent manner (Cravatt *et al.*, 2001).

Cannabinoids may also exert their analgesic effects through sites in the periphery. In mice anandamide and WIN 55,212-2 exhibited antinociceptive actions when injected into the hind paw together with formalin, in an SR 141716A-sensitive manner (Calignano *et al.*, 1998). Peripheral injection of anandamide significantly inhibited noxious mechanically-evoked responses of spinal neurons in rats with hindpaw inflammation (Sokal *et al.*, 2003). The inhibitory effect of anandamide was blocked by co-injection of the CB_2 receptor antagonist SR 144528. CB_2-mediated antinociceptive effects, especially involving inflammatory hyperalgesia, may result from an inhibitory effect on immune cells and hence a reduction in pro-inflammatory mediators (Malan *et al.*, 2003).

1.4.6.2 Motor control

The endocannabinoid system plays a regulatory role in movement and co-ordination (see Van der Stelt and Di Marzo, 2003 for review). Indeed, two out of the four symptoms in the mouse tetrad involve inhibitory effects on motor activity i.e. hypokinesia and catalepsy (Fride and Mechoulam, 1993; Pertwee, 1997). It is well known that CB_1 receptors are abundantly distributed in the basal ganglia (both internal and external segments of the globus pallidus, substantia nigra, striatum), an area of the brain involved in the control of movement, particularly presynaptically on GABAergic striatonigral and striatopallidal projection neurons (Herkenham *et al.*, 1991a; Tsou *et al.*, 1998a). CB_1 receptors are also likely to be located on subthalamopallidal and/ or subthalamonigral glutamatergic neurons, as revealed by CB_1 receptor mRNA in the subthalamic nucleus (Hohmann and Herkenham, 2000). In addition, both anandamide and 2-AG are present in the basal ganglia (Bisogno *et al.*, 1999). FAAH activity is also present in high levels in all regions of the basal ganglia, particularly in the globus pallidus and the substantia nigra (Desarnaud *et al.*, 1995), further supporting a functional role for the endocannabinoid system in the control of movement.

The effects associated with cannabinoids in the basal ganglia presumably occur through the modulation of neurotransmitters that are involved in the control of movement, mainly GABA, glutamate and dopamine. Stimulation of CB_1 receptors localised on axonal terminals of striatal GABAergic neurons has been shown to potentiate GABA transmission by inhibiting the uptake of the neurotransmitter (Maneuf *et al.*, 1996). This phenomenon could underlie the potentiation by cannabinoids of catalepsy in rats induced by the $GABA_A$ agonist muscimol (Wickens

and Pertwee, 1993). In contrast, some authors have reported an inhibitory effect of cannabinoids on GABA release including the substantia nigra (Chan *et al.*, 1998) and striatum (Szabo *et al.*, 1998).

Szabo *et al.* (2000) showed that WIN 55,212-2 could inhibit electrically-evoked excitatory postsynaptic currents in the substantia nigra. This inhibition of glutamatergic transmission was reversed by SR 141716A supporting an involvement of CB_1 receptors. Consistent with this finding, WIN 55,212-2 was shown to inhibit glutamate release from subthalamonigral neurons (Sanudo-Pena and Walker, 1997), leading to a reduction in motor activity (Miller *et al.*, 1998).

WIN 55,212-2 and CP 55,940 were unable to inhibit the electrically-evoked release of dopamine in the rat striatum (Szabo *et al.*, 1999) suggesting that endocannabinoids do not directly modulate dopaminergic transmission. Instead changes in dopamine transmission may result from the modulation of glutamate and GABA by endocannabinoids. For example, the CB_1 receptor-mediated reduction in glutamatergic input to the substantia nigra (Szabo *et al.*, 2000) would lead to a reduced activation of dopaminergic neurons in this region and a subsequent reduction of dopamine release in the striatum (Cadogan *et al.*, 1997).

1.4.6.3 Learning and memory

As described earlier, the endogenous cannabinoid system modulates neurotransmitter release in the hippocampus, an area of the brain associated with learning and memory (see section 1.4.2). As such, cannabinoids have been shown to disrupt or enhance the performance of working memory tasks. In mice, Δ^9-THC disrupted performance of working memory task (using the Morris water maze) at

doses lower than those required to elicit the classical tetrad of cannabimetic effects (Varvel *et al.*, 2001). The CB_1 receptor antagonist SR 141716A reversed these performance deficits. Conversely, administration of SR 141716A alone enhanced spatial memory tasks in rats as assessed by the radial-arm maze task (Lichtman, 2000).

In the hippocampus endocannabinoids were shown to interfere with the induction of LTP (Terranova *et al.*, 1995; Stella *et al.*, 1997), a possible candidate mechanism for learning and memory. This effect may occur through a cannabinoid-mediated reduction in glutamate release (Misner and Sullivan, 1999). However, a recent study by Diana *et al.* (2002) demonstrated that WIN 55,212-2 had no significant effect on LTP in the rat hippocampus, in experiments that controlled for basal glutamatergic synaptic transmission. This suggests that the negative effects in learning and memory, associated with cannabinoids, cannot be explained by a selective derangement of hippocampal LTP alone.

Another important brain structure in cognitive function is the prefrontal cortex (PFC), which exhibits a high density of CB_1 receptors (Herkenham *et al.*, 1990). Δ^9-THC increased presynaptic dopamine efflux and utilisation in the PFC (Chen *et al.*, 1990; Jentsch *et al.*, 1997) while, in rat PFC slices, it was demonstrated that cannabinoids influence glutamatergic synaptic transmission and plasticity (Auclair *et al.*, 2000). In this latter study the cannabinoid agonists CP 55,940 and WIN 55,212-2 reduced monosynaptic excitatory postsynaptic potentials in an SR 141716A-sensitive manner. Taken together these observations suggest that the endocannabinoid and dopamine systems are closely co-operating in the regulatory role of cognition in the PFC.

1.4.6.4 Brain reward

The psychotropic and addictive properties of cannabis suggest that endocannabinoids are involved in modulating reward pathways in the brain (see Lupica *et al.*, 2004 for review). The central neuronal circuits involved in mediating reward aspects of most drugs of abuse (mesolimbic system) originate in an area known as the ventral tegmental area (VTA). One of the hallmark actions for drugs of abuse, including amphetamine, cocaine and opioids like morphine and heroin, is their ability to increase dopamine transmission of VTA dopaminergic neurons (Di Chiara and Imperato, 1988).

Comparable to the dopaminergic nigrostriatal pathway, there is no co-localisation of CB_1 receptors in dopaminergic neurons of the VTA (Herkenham *et al.*, 1991b; Tsou *et al.*, 1998a), which seems to rule out a direct control of endocannabinoids on dopamine transmission. Nevertheless, increased dopamine release and firing of dopaminergic neurons in the rat VTA has been found after systemic administration of Δ^9-THC, WIN 55,212-2, HU-210 and CP 55,940 (French, 1997; Gessa *et al.*, 1998; Wu and French, 2000), as well as in brain slices containing the VTA (Cheer *et al.*, 2000). Also noteworthy was the observation that these cannabinoid effects could be blocked by SR 141716A. This suggests that, in a similar manner to the basal ganglia, CB_1 receptor-induced modulation of neurotransmission may modulate dopamine transmission. Szabo *et al.* (2002) showed that WIN 55,212-2 could inhibit the electrically-evoked release of GABA in rat brain slices containing the VTA. This effect was reduced by SR 141716A. Hence, activation of CB_1 receptors in the VTA may increase dopaminergic activity through a reduction in inhibitory tone brought about by the blockade of intrinsic GABA release.

1.4.6.5 Feeding and appetite

Cannabinoids enhance appetite and are clinically used for this purpose, particularly in AIDS and cancer patients (Beal *et al.*, 1995; Palmer *et al.*, 2002). Δ^9-THC and anandamide have been shown to increase food intake in rats (Williams *et al.*, 1998; Williams and Kirkham, 1999) while SR 141716A has been reported to inhibit the intake of palatable food and alcohol (Arnone *et al.*, 1997; Simiand *et al.*, 1998).

Food-deprived rats were found to exhibit enhanced levels of 2-AG in the hypothalamus, an area of the brain that is thought to control food intake (Kirkham *et al.*, 2002). Levels of 2-AG decreased when the animals were fed. Leptin is considered to be a key signal through which the hypothalamus senses the nutritional state of the body (Mechoulam and Fride, 2001). In knockout mice that lacked leptin, hypothalamic levels of 2-AG were higher than controls but returned to normal values when leptin was injected (Di Marzo *et al.*, 2001). $CB_1^{-/-}$ mice were also shown to eat less than wild-types and SR 141716A reduced food intake in wild-type but not knockout mice (Di Marzo *et al.*, 2001). These findings together indicate that endocannabinoids in the hypothalamus may tonically activate CB_1 receptors to maintain food intake, which are in turn modulated by the levels of leptin present.

1.4.6.6 Cardiovascular regulation

Endocannabinoids mediate changes within the cardiovascular system (see Randall *et al.*, 2002 for review). Early studies have shown that anandamide causes bradycardia (with brief secondary hypotension) then a transient pressor effect, which

is followed by a delayed, but maintained, depressor action in anaesthetised rats (Varga *et al.*, 1995, 1996; Lake *et al.*, 1997). The anandamide-induced stimulation of vanilloid VR_1 receptors, located on sensory nerves (see section 1.7.2), was thought to mediate the bradycardia as it was inhibited by the VR_1 receptor antagonist capsazepine but not SR 141716A (Malinowska *et al.*, 2001). The transient pressor effect induced by anandamide appeared to be independent of the central nervous system and CB_1 receptors as it was not blocked by cervical spinal cord transection, α-adrenoceptor blockade, or SR 141716A (Varga *et al.*, 1996; Lake *et al.*, 1997). The second depressor effect, which follows the transient pressor phase, may be mediated by presynaptic CB_1 receptor-evoked inhibition of sympathetic outflow (in the periphery) as the effect was attenuated by cervical spinal transection, α-adrenoceptor antagonists and SR 141716A, but not capsazepine (Varga *et al.*, 1995, 1996; Lake *et al.*, 1997; Malinowska *et al.*, 2001).

Interestingly, in humans Δ^9-THC induces a tachycardia, which can be inhibited by SR 141716A (Huestis *et al.*, 2001). Normally the heart is under dominant vagal tone and so the Δ^9-THC-induced tachycardia probably resulted from an inhibition of ACh release from cardiac vagal efferents via presynaptic CB_1 receptors (Szabo *et al.*, 2001).

Some authors have suggested that endothelium-derived hyperpolarising factor (EDHF) is an endocannabinoid released from the vascular endothelium onto CB_1 receptors located on smooth muscle cells. In particular Randall *et al.* (1996) showed that SR 141716A inhibits nitric oxide (NO)- and prostanoid-independent, endothelium-dependent relaxations mediated by EDHF in the rat mesentery. However, further studies have indicated that the vasodilator action of anandamide had both an endothelium-dependent and endothelium-independent component and only

the former was sensitive to inhibition by SR 141716A (Chaytor *et al.*, 1999; Mukhopadhyay *et al.*, 2002a). This argues against anandamide itself being EDHF but together with the observation that anandamide and not Δ^9-THC elicited vasodilatation in the rat mesenteric vascular bed (Wagner *et al.*, 1999), lead to the idea of a novel endothelial cannabinoid receptor distinct from CB_1 or CB_2 receptors (Jarai *et al.*, 1999).

Endocannabinoids may play a key role in producing the hypotension associated with haemorrhagic and endotoxic shock. Wagner *et al.* (1997) demonstrated in a rat model of haemorrhagic shock that activated macrophages release anandamide, which may contribute towards the hypotension. Following injection with SR 141716A the mortality of rats increased. Similarly in endotoxic shock the synthesis of 2-AG in platelets and anandamide in macrophages was increased (Varga *et al.*, 1998). This suggests that endocannabinoid-mediated vasodilation may improve tissue oxygenation by counteracting the excessive sympathetic vasoconstriction triggered by haemorrhage or myocardial infarction.

1.4.6.7 Immune modulation

As mentioned earlier the presence of both CB_1 and, more abundantly, CB_2 receptors within areas devoted to host immunity suggest that endocannabinoids modulate immune function (see Parolaro *et al.*, 2002 for review). In addition, immune cells such as macrophages and leukocytes have been found to both synthesise and degrade anandamide and 2-AG (Di Marzo *et al.*, 1996a; Bisogno *et al.*, 1997; Varga *et al.*, 1998; Di Marzo *et al.*, 1999). This strongly implicates immune cells in the

peripheral regulation of the endocannabinoid system and endocannabinoid homeostasis.

Schwarz *et al.* (1994) examined the immunoregulatory effects of cannabinoids on mitogen-induced T and B human lymphocyte proliferation. Anandamide and CP 55,940 caused significant inhibition of lymphocyte proliferation. Higher concentrations of cannabinoid were also shown to induce cell death by apoptosis (Schwarz *et al.*, 1994). In mouse splenocytes 2-AG also demonstrated inhibitory effects on lymphocyte proliferation (Lee *et al.*, 1995).

Immunosuppression may occur through a change in immune cell shape and hence its functionality. Acute exposure of macrophages and human monocytes to anandamide and 2-AG respectively resulted in the cells rounding up and becoming non-mobile (Stefano *et al.*, 1998, 2000). The CB_1 receptor antagonist SR 141716A blocked this action.

During inflammation, due to injury or pathogen infection, immune cells produce several cytokines such as interferons (IFNs), tumour-necrosis factor-α (TNFα) and interleukins (ILs), which are signalling proteins that regulate immune responses. Evidence exists to suggest that cannabinoids inhibit the production of these cytokines. Δ^9-THC reduced IFN levels and IL-2 production in mice infected with herpes simplex virus compared to controls (Ouyang *et al.*, 1998) and also inhibited TNFα production by cultured mouse peritoneal macrophages (Zheng *et al.*, 1992). Ihenetu *et al.* (2003) showed that WIN 55,212-2 inhibited IL-2 release from human mononuclear cells in a concentration-dependent manner. SR 144528 antagonised this effect, implicating the CB_2 receptor.

Generally, endocannabinoid signalling plays a negative role in the onset of the immune response but an exact role for endocannabinoids and cannabinoid receptors in the maintenance of immune system homeostasis still needs to be defined.

1.5 Signal transduction mechanisms of CB_1 receptors

1.5.1 $G_{i/o}$-protein coupling

The CB_1 cannabinoid receptor is a GPCR, comprising seven hydrophobic transmembrane (TM) helices (Fig. 1.3) and is a member of the rhodopsin subfamily of GPCRs. Cannabinoid agonists interact with the receptor within the pore formed within the TM helical cluster (Mukhopadhyay *et al.*, 2002b). The three cytosolic loops and a putative fourth loop formed by palmitoylation at the juxtamembrane C-terminal region contribute to the activation of G proteins. The proximal CB_1 receptor intracellular C-terminal domain (amino acids 401-417) is critical for G-protein coupling and the distal C-terminal tail domain (amino acids 418-472) modulates the magnitude and kinetics of signal transduction (Nie and Lewis, 2001).

As with all GPCRs, cannabinoid receptors are predicted to share a common three-dimensional fold (Wess, 1998). Cannabinoid binding causes conformational changes in the receptor protein that promotes the receptor's association with heterotrimeric G-proteins. These G-proteins consist of an α-subunit bound to $\beta\gamma$ complexes, which are attached to the intracellular surface of the cell membrane. Ligand binding triggers the exchange of GTP for guanosine diphosphate (GDP) on the α-subunit and results in both G-protein dissociation from the membrane and α-subunit dissociation from the $\beta\gamma$ complex. The released G-protein subunits are then

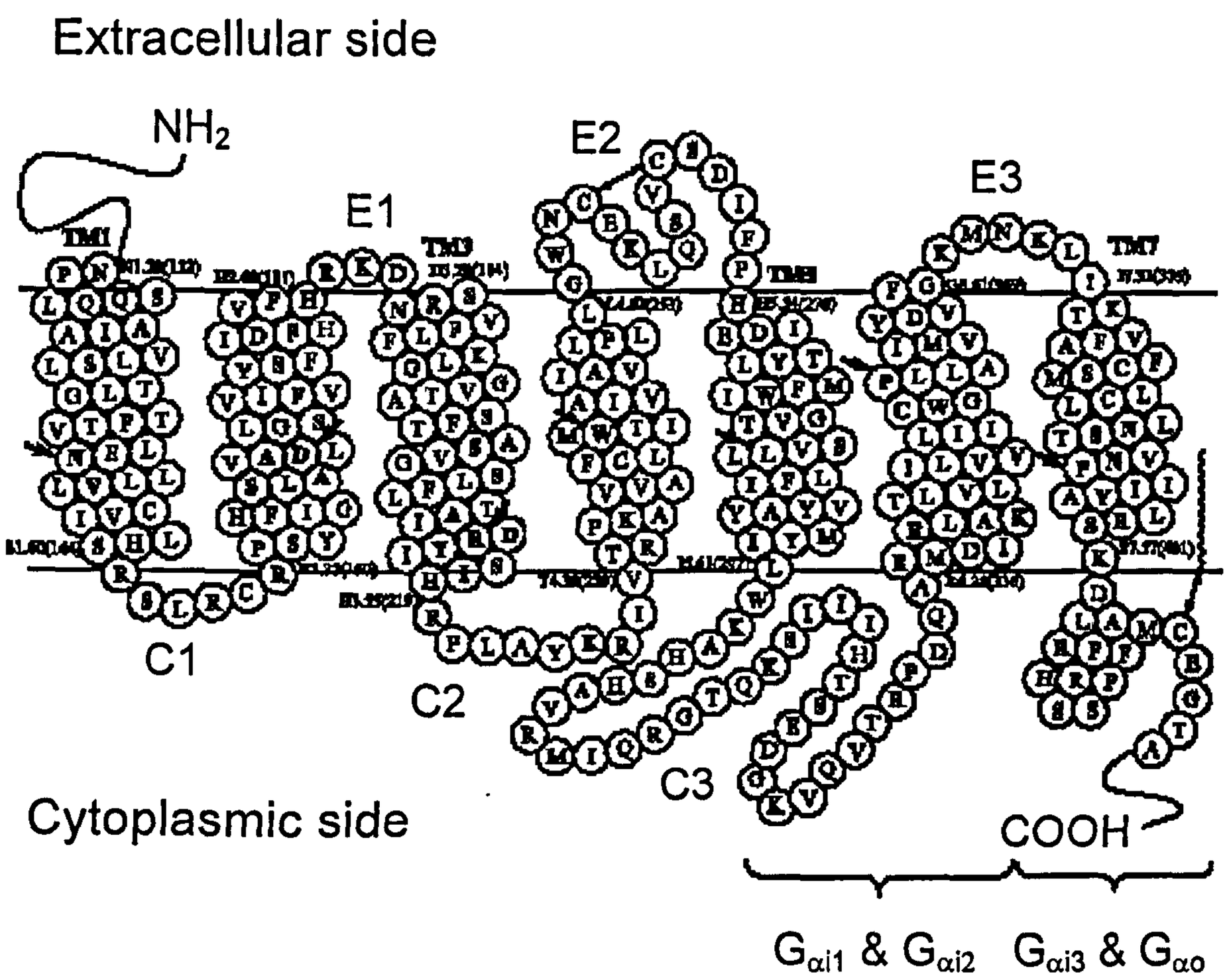


Figure 1.3 Two-dimensional structure of the human CB_1 receptor.
Three extracellular (E1, E2 and E3) and intracellular (C1, C2 and C3) regions are represented. Amino acid residues are denoted by the single letter abbreviation. The characterised sequences of the C3 intracellular residues are important for $G_{\alpha i1}$ and $G_{\alpha i2}$ interactions and the C-terminal residues are important for $G_{\alpha i3}$ and $G_{\alpha o}$ interactions (shaded circles). Figure adapted from Mukhopadhyay *et al.* (2002b).

able to activate enzymes and/ or ion channels, which leads to the induction of certain cellular responses. α-subunits possess intrinsic GTPase activity and α-GTP is eventually hydrolysed to α-GDP, which is able to bind free βγ complexes with high affinity. This reaction returns the system to the resting state.

On the basis of amino acid similarity the G-protein α-subunits can be grouped into four major families: G_s, $G_{i/o}$, G_q and $G_{12/13}$ (Wess, 1998). Presently, 6 different G-protein β- and 12 γ-subunits have also been described (Clapham and Neer, 1997). Activation of α_s- and α_i-subunits is known to activate and inhibit distinct forms of adenylyl cyclase respectively (Simonds, 1999). G-proteins can also modulate the activity of ion channels through the direct binding of the βγ complex (N-type Ca^{2+} channels and inwardly rectifying K^+ (K_{ir}) channels) or indirectly by altering second messenger systems that modulate ion channel activation (A-type K^+ channels) (Jan and Jan, 1997).

Methods have been employed to analyse the selectivity of receptor/ G-protein interactions. Pharmacologically, G-protein-mediated responses have been classified into PTX-sensitive and PTX-insensitive because of the ability of the bacterial toxin to selectively inactivate G-proteins of the $\alpha_{i/o}$ family (Wess, 1998). Biochemically, receptor activation of G-proteins can be measured by receptor stimulated [^{35}S]GTPγS binding. This is a direct assay of receptor activation of G-proteins since it measures the exchange of bound GDP for GTP (or [^{35}S]GTPγS) (Childers and Deadwyler, 1996).

Cannabinoid receptor stimulation of [^{35}S]GTPγS binding has been quantified in rat membranes in the presence of excess GDP (Selley *et al.*, 1996). In addition, most cannabinoid effects are sensitive to PTX implicating a CB_1 receptor coupling to a $G_{i/o}$ protein.

1.5.2 G_s-protein coupling

Evidence exists to suggest that CB_1 receptors can interact with G_s under conditions of PTX treatment that prevents the receptor's interaction with $G_{i/o}$ proteins. Stimulation of cAMP was observed in rat cultured striatal neurons and in CB_1 receptor-transfected CHO cells in response to the CB_1 receptor agonist HU-210 (Glass and Felder, 1997; Felder *et al.*, 1998). This effect was SR 141716A-sensitive but did require the presence of forskolin. HU-210 and the dopamine D_2 receptor agonist quinpirole inhibit cAMP production in striatal cultures when added separately (Glass and Felder, 1997). Interestingly, when these agonists were added together they augmented cAMP accumulation (Glass and Felder, 1997). Furthermore, Maneuf and Brotchie (1997) showed that high concentrations of WIN 55,212-2 could stimulate basal cAMP accumulation in a slice preparation of rat globus pallidus in the absence of forskolin and PTX. This effect was inhibited by SR 141716A.

Another study using human embryonic kidney (HEK) 293 cells transfected with D_2 and CB_1 receptors indicated that expression of D_2 receptors was sufficient to convert the inhibition of forskolin-stimulated cAMP production by CP 55,940 to a stimulation of cAMP production (Jarrahian *et al.*, 2004). This would be consistent with an unmasking of the ability of the CB_1 receptor to couple to G_s in addition to G_i. Evidence supporting this included the observation that, within this experimental model, pretreatment with PTX eliminated cAMP inhibition but did not affect the stimulation of cAMP. Interestingly, the D_2-mediated inhibition of forskolin-stimulated cAMP accumulation was not affected by the expression of CB_1 receptors (Jarrahian *et al.*, 2004). In addition, concurrent activation of the CB_1 and D_2 receptor resulted in increased levels of cAMP compared with the activation of the D_2 receptor alone. The

mechanism for this response could be explained by the ability of D_2 receptors to sequester G_i proteins such that they will no longer be available to couple to CB_1 receptors, leaving the CB_1 receptors to couple to G_s. In support of this mechanism, over expression of $G_{\alpha i}$ allowed the inhibition of cAMP accumulation by CP 55,940 to prevail (Jarrahian *et al.*, 2004). In addition, when the D_2 receptor coupling to G_i was compromised by persistent agonist stimulation (18 hour treatment of the cells with quinpirole), the CB_1 receptor-G_i inhibition was the prevalent response (Jarrahian *et al.*, 2004). It was also shown that a 10 fold increase in the concentration of CP 55,940 was required to stimulate cAMP production than inhibit its accumulation (Jarrahian *et al*, 2004) suggesting that the concentration of cannabinoids used may also determine the activation of either G_i or G_s.

A CB_1 receptor interaction with G_s has also been demonstrated in CHO cells expressing human CB_1 receptors (Bonhaus *et al.*, 1998). Pretreatment with PTX was used to observe receptor coupling with G_s. It was found that cannabinoid agonists (HU-210, CP 55,940, Δ^9-THC, anandamide; order of potency for $G_{i/o}$-coupled effects) were markedly less efficacious when stimulating forskolin-stimulated cAMP production (G_s) than in inhibiting its formation ($G_{i/o}$). The CB_1 receptor antagonist SR 141716A inhibited equally both cannabinoid accumulation and inhibition of cAMP (Bonhaus *et al.*, 1998). Thus, these findings indicate that there is specificity among CB_1 receptor agonists in their relative abilities to activate G_s- and $G_{i/o}$-coupled transduction pathways.

Collectively the data strongly suggests that CB_1 receptors may be dually coupled to both G_s and $G_{i/o}$ proteins but the physiological significance of this needs further investigation.

1.5.3 Regulation of adenylyl cyclase

The first characterised CB_1 receptor signal transduction response was the inhibition of adenylyl cyclase by micromolar concentrations of Δ^9-THC in N18TG2 neuroblastoma cells (Howlett and Fleming, 1984). This response was blocked by PTX suggesting the involvement of $G_{i/o}$ proteins (Howlett *et al.*, 1986). Since then the functional inhibition of adenylyl cyclase (and thus cAMP production) by CB_1 receptors has been identified in many other preparations. Cannabinoids have exhibited PTX- and SR 141716A-sensitive attenuation of cAMP accumulation in CHO cells expressing exogenous CB_1 receptors (Matsuda *et al.*, 1990; Felder *et al.*, 1993; Hillard *et al.*, 1999). Brain regions in which cannabinoids are effective inhibitors of adenylyl cyclase are those most densely populated with cannabinoid binding sites. Thus, cannabinoid-mediated inhibition of cAMP has been demonstrated in slices of rat hippocampus, striatum, cerebral cortex and cerebellum (Bidaut-Russell *et al.*, 1990). In addition, WIN 55,212-2, CP 55,940 and anandamide were shown to inhibit adenylyl cyclase activity in rat cerebellar membranes (Childers *et al.*, 1994). *In vivo*, WIN 55,212-2 reduced forskolin-stimulated cAMP accumulation in the rat striatum, in an SR 141716A-sensitive manner (Wade *et al.*, 2004).

In conjunction with other reports of a CB_1 receptor-mediated increase in cAMP (Glass and Felder, 1997; Felder *et al.*, 1998; Maneuf and Brotchie, 1997), Busch *et al.* (2004) recently showed that anandamide evoked a concentration-dependent increase in cAMP in rat parotid glands. The CB_1 receptor antagonist AM281 inhibited this effect.

Nine distinct isozymes of adenylyl cyclase have been identified, which can be categorised into six distinct classes based on sequence and functional similarities

(Patel *et al.*, 2001): (a) Adenylyl cyclase type I (AC-I) is found mainly in brain, is stimulated by Ca^{2+}/ calmodulin and is inhibited by $G_{\beta\gamma}$ subunits and by $G_{i\alpha}$; (b) AC-VIII is found only in brain and is only stimulated by Ca^{2+}/ calmodulin; (c) AC-II, AC-IV and AC-VII are activated by $G_{\beta\gamma}$, providing that $G_{s\alpha}$ is present; (d) AC-V and AC-VI are highly expressed in brain and heart, and they are inhibited by $G_{i\alpha}$ and low levels of Ca^{2+}; (e) AC-III is stimulated by a high concentration of Ca^{2+}/ calmodulin in the presence of $G_{\alpha s}$; (f) AC-IX is expressed at high levels in skeletal muscle and brain and, as yet, is found to be affected by $G_{\alpha s}$ only.

The influence of the adenylyl cyclase isoform on the outcome of the response to cannabinoid agonists has been investigated (Rhee *et al.*, 1998). Monkey kidney COS-7 cells, expressing exogenous CB_1 receptors, were transfected with each adenylyl cyclase isoform in turn and stimulated with the cannabinoid agonists HU-210 and WIN 55,212-2. AC-I, V, VI and VIII were shown to be inhibited by, whereas types II, IV and VII were stimulated by, CB_1 receptor activation. The inhibition of AC-III by the cannabinoids was only observed when forskolin was used as a stimulant while AC-IX was inhibited only marginally. These results suggest that, in addition to the dual coupling of cannabinoid receptors to G_s and $G_{i/o}$, the contrasting effects of cannabinoids on adenylyl cyclase activity could also be attributed to the specific isoform present in different cellular preparations. For example the globus pallidus contains mRNA encoding for AC-II (Furuyama *et al.*, 1993) an area of the brain where CB_1 receptor activation has been shown to induce an incresae in cAMP accumulation (Maneuf and Brotchie, 1997).

Modulation of the intracellular cAMP concentration, thereby regulating protein kinase A (PKA) phosphorylation, can result in major changes in cellular activity. For example A-type K^+ channels undergo PKA-regulated phosphorylation/

dephosphorylation in the hippocampus, as one mechanism for cannabinoid-induced neuronal responses (Hampson *et al.*, 1995; Mu *et al.*, 2000). Given that gene regulation can also be modulated as the result of cAMP/ PKA (e.g. inhibition of cAMP response elements on genes), this mechanism may be particularly important for long-term changes in gene expression (Childers and Deadwyler, 1996).

1.5.4 Regulation of mitogen-activated protein (MAP) kinase

The MAP kinase pathway is a key signalling mechanism that regulates many cellular functions such as cell growth, transformation and apoptosis. Its activation is normally associated with the initial activation of a tyrosine kinase-linked receptor. This activates Ras and sets-up a signalling cascade beginning with the activation of the serine/ threonine kinase Raf (MAP kinase kinase kinase). Raf activates MAP kinase kinase (MEK) leading to the phosphorylation and activation of MAP kinase, which can phosphorylate various cytoplasmic and nuclear proteins.

CB_1 receptors have been shown to link positively to MAP kinase. In cultured U373MG human astrocytoma and CHO cells, expressing CB_1 receptors, HU-210 and CP 55,940 activated a p42/44 MAP kinase (Bouaboula *et al.*, 1995b; Galve-Roperh *et al.*, 2002). These effects were PTX- and SR 141716A-sensitive (Bouaboula *et al.*, 1995b). Furthermore, the activation of MAP kinase in CHO cells was linked to the activation of a Na^+/ H^+ exchanger (NHE-1), a transporter involved in multiple cellular functions such as intracellular pH regulation and control of cell volume (Bouaboula *et al.*, 1999). *In vivo*, acute administration of Δ^9-THC induces a progressive and transient activation of p42/44 MAP kinase in rat dorsal striatum and nucleus accumbens (NAc) (Valijent *et al.*, 2001), as well as in murine hippocampus (Derkinderen *et al.*, 2003),

striatum and cerebellum (Rubino *et al.*, 2004). These effects were blocked by SR 141716A suggesting an involvement of the CB_1 receptor. CP 55,940, WIN 55,212-2, anandamide and 2-AG have also been shown to stimulate p38 MAP kinase in rat and murine hippocampus (Derkinderen *et al.*, 2001). These effects were also mediated by the CB_1 receptor as they were blocked by SR 141716A. Furthermore, the cannabinoids exhibited no stimulatory action in $CB_1^{-/-}$ mice (Derkinderen *et al.*, 2001).

Mechanisms for the induction of MAP kinase by CB_1 receptors have not been fully elucidated. Given the structure of cannabinoid receptors, and the sensitivity of responses to PTX, they are not believed to act as tyrosine kinase-coupled receptors (*trk*). Studies in primary rat astrocyte cultures showed that Δ^9-THC and HU-210 increased glucose metabolism, phospholipid metabolism and glycogen synthesis through the activation of MAP kinase (Sanchez *et al.*, 1998b). The responses were PTX- and SR 141716A-sensitive. Two signal transduction pathways were proposed. The first involved the activation of phosphatidylinositol-3-kinase (PI3K), which in turn mediated tyrosine phosphorylation and activation of Raf. PI3K may signal via protein kinase B (PKB) as Δ^9-THC and CP 55,940 were able to simulate PKB in U373MG astrocytoma cells, in an SR 141716A-sensitive manner (Gomez del Pulgar *et al.*, 2000; Galve-Roperh *et al.*, 2002). The second pathway was initiated by sphingomyelin hydrolysis, release of the lipid second messenger ceramide and the subsequent activation of the Raf MAP kinase cascade (Sanchez *et al.*, 1998b). A CB_1 receptor-mediated production of ceramide by Δ^9-THC has also been demonstrated in rat C6 glioma cells (Sanchez *et al.*, 1998a; Galve-Roperh *et al.*, 2000). Furthermore, the induction of a PI3K/ PKB pathway, in response to cannabinoid receptor stimulation, was demonstrated in human prostate epithelial PC-3 cells (Sanchez *et al.*,

2003). This in turn evoked the phosphorylation of p42/44 MAP kinase. Interestingly, antagonists at both the CB_1 and CB_2 receptor inhibited the cannabinoid-mediated stimulation of PKB.

CB_1 receptor stimulation may also regulate MAP kinase activity indirectly through its effects on cAMP accumulation. In MCF-7 cancer cells, anandamide induced the activation of MAP kinase in a CB_1 receptor-dependent manner (Melck *et al.*, 1999). Forskolin and the cAMP analogue 8-Br-cAMP inhibited basal MAP kinase activity and significantly reduced the stimulatory effect of anandamide on MAP kinase activity compared to the endocannabinoid alone. In addition, anandamide affected cAMP levels at doses slightly lower than those required to stimulate MAP kinase (Melck *et al.*, 1999). In rat hippocampal slices pretreatment with 8-Br-cAMP completely prevented the activation of MAP kinase by anandamide and 2-AG (Derkinderen *et al.*, 2003). Finally, a study in N1E-115 neuroblastoma cells found that p42/44 MAP kinase activation by WIN 55,212-2 was inhibited by forskolin while the PKA inhibitor H-89 enhanced MAP kinase phosphorylation (Davis *et al.*, 2003). Collectively the data suggests that a decrease in cAMP levels, and consequently in PKA activity, may participate in the stimulatory effects of CB_1 receptor activation on the MAP kinase pathway.

MAP kinase activation can be linked to expression of immediate early genes, as has been demonstrated by a CB_1 receptor-mediated expression of Krox-24 in human astrocytoma cells (Bouaboula *et al.*, 1995a). I.c.v. injection of anandamide evoked an increase in c-Fos protein in rat brain with a generally similar distribution to that of functioning CB_1 receptors (Patel *et al.*, 1998). In MCF-7 cancer cells the anandamide-induced stimulation of MAP kinase was shown to exert a subsequent down-regulation on prolactin receptors and *trk* nerve growth factor receptors (Melck

et al., 1999). This regulation of gene expression was thought to underlie the anti-poliferative effects of anandamide in these cells. In mouse hippocampus Δ^9-THC induced the expression of immediate-early gene products including Krox-24, brain-derived neutrophic factor (BDNF) and c-Fos protein, which was prevented by the inhibition of MAP kinase (Derkinderen *et al.*, 2003). BDNF and Krox-24, in particular, are known to be important for synaptic plasticity (Derkinderen *et al.*, 2003) suggesting that gene regulation, through the activation of MAP kinase, is an important physiological mechanism by which cannabinoids can modulate synaptic plasticity.

1.5.5 Modulation of ion channels

The modulation of voltage-dependent ion channels (primarily N- and P/Q-type Ca^{2+} channels and K_{ir} and A-type K^+ channels) is thought to underlie the cannabinoid-induced inhibition of neurotransmitter release at presynaptic sites (see section 1.9.7). The majority of these effects are mediated through the CB_1 receptor, although there is evidence to suggest that cannabinoids modulate ion channel function directly. Evidence for both mechanisms (CB_1 receptor-dependent and independent) are discussed in this section.

1.5.5.1 Ca^{2+} channels

Anandamide, WIN 55,212-2 and CP 55,940 act via CB_1 receptors to inhibit N-type voltage-operated Ca^{2+} channels (VOCCs) leading to a decrease in Ca^{2+} influx in NG108-15 cells (Mackie and Hille, 1992; Felder *et al.*, 1993; Mackie *et al.*, 1993). The response was blocked by prior treatment of the cells with PTX, demonstrating its

mediation by $G_{i/o}$ proteins, and was independent of the cAMP pathway, as the response was not reversed by the addition of 8-Br-cAMP (Mackie *et al.*, 1993). Sugiura *et al.* (1997) utilised fura-2 imaging to examine depolarisation-induced Ca^{2+} influx in high K^+ depolarised NG108-15 cells. 2-AG and anandamide both attenuated the response. The N-type Ca^{2+} channel was presumed to mediate the depolarisation-evoked increases in Ca^{2+} current because the N-type channel antagonist ω-conotoxin could block these responses (Caulfield and Brown, 1992; Mackie *et al.*, 1993). In rat superior cervical ganglion neurons, transfected with CB_1 receptors, WIN 55,212-2 and CP 55,940 inhibited Ca^{2+} currents (Pan *et al.*, 1996). These effects were both PTX- and ω-conotoxin-sensitive suggesting an inhibitory effect on N-type Ca^{2+} channels. In rat striatal neurons WIN 55,212-2 inhibited corticostriatal glutamatergic synaptic transmission in an SR 141716A- and PTX-sensitive manner (Huang *et al.*, 2001). The inhibition of N-type Ca^{2+} channels was thought to mediate this effect as ω-conotoxin abolished the WIN 55,212-2-mediated synaptic inhibition.

Anandamide inhibited Q-type Ca^{2+} currents in AtT-20 pituitary tumour cells expressing exogenous CB_1 receptors, which was inhibited by PTX (Mackie *et al.*, 1995). Fura-2 studies in rat cortical and cerebellar brain slices showed that anandamide inhibited P/Q-type Ca^{2+} fluxes (Hampson *et al.*, 1998). This response was SR 141716A- and PTX-sensitive, confirming its mediation by $G_{i/o}$ protein-coupled CB_1 receptors.

In cultured rat hippocampal neurons WIN 55,212-2, anandamide and CP 55,940 inhibited N- and P/Q-type Ca^{2+} currents in an SR 141716A- and PTX-sensitive manner (Twitchell *et al.*, 1997; Shen and Thayer, 1998). Activation of CB_1 receptors by WIN 55,212-2 (at nM concentrations) inhibited only a fraction (17%) of the whole-cell Ca^{2+} current, even though more than half of this current is carried by

N- and P/Q-type Ca^{2+} channels (Shen and Thayer, 1998). Interestingly, the same study revealed that further inhibition of Ca^{2+} currents could be obtained using micromolar concentrations of WIN 55,212-2. In addition, the inactive stereoisomer WIN 55,212-3 (μM concentrations) also inhibited Ca^{2+} currents in an SR 141716A-insensitive manner. This clearly indicates that at micromolar concentrations the effects of WIN 55,212-2 are not mediated by CB_1 receptors, which may suggest a direct effect of cannabinoids on Ca^{2+} channels. The inhibitory effect of cannabinoids on N-type Ca^{2+} channels in the hippocampus is in accordance with the observations of Lenz *et al.* (1998). These authors showed that depolarisation-evoked DSI, in rat hippocampal slices, was completely blocked by ω-conotoxin. This suggests that an endocannabinoid-mediated inhibition of N-type Ca^{2+} channels is required for a presynaptic reduction of GABA release.

L-type Ca^{2+} channels can also be regulated via CB_1 receptor stimulation. WIN 55,212-2 inhibited L-type Ca^{2+} currents in cat cerebral arterial smooth muscle cells, in a PTX- and SR 141716A-sensitive manner (Gebremedhin *et al.*, 1999). In retinal slices from larval tiger salamander, activation of CB_1 receptors by WIN 55,212-2 led to the inhibition of L-type Ca^{2+} channels in bipolar cells (Straiker *et al.*, 1999).

T-type Ca^{2+} currents, transfected in HEK293 and CHO cells and endogenously expressed in NG108-15 cells, were inhibited by anandamide (Chemin *et al.*, 2001). This inhibitory effect was not mimicked by synthetic cannabinoids including WIN 55,212-2, CP 55,940 and HU-210 and was not blocked by SR 141716A. PL (PLA_2, PLC and PLD) and PK (PKA and PKC) pathways were also not involved (Chemin *et al.*, 2001). From these observations it was suggested that anandamide directly inhibits T-type Ca^{2+} channels.

1.5.5.2 K^+ channels

Exogenously expressed CB_1 receptors couple positively to endogenous G-protein inwardly rectifying K^+ (GIRK) channels in AtT-20 pituitary tumour cells (Mackie *et al.*, 1995). WIN 55,212-2 activated an inward current, which showed inward rectification and was sensitive to low concentrations of barium (Ba^{2+}) (Mackie *et al.*, 1995). In *Xenopus* oocytes, expressing CB_1 receptors and either GIRK1 or GIRK4 channels, WIN 55,212-2 was able to enhance currents carried by these channels, in a Ba^{2+}-sensitive manner (McAllister *et al.*, 1999). WIN 55,212-2 and CP 55,940 inhibited glutamatergic signalling in the mouse NAc in an SR 141716A-sensitive manner (Robbe *et al.*, 2001). Ba^{2+} blocked this inhibition suggesting a cannabinoid-mediated activation of GIRK channels as a mechanism for the inhibition of neurotransmitter release. Forskolin did not alter presynaptic CB_1 receptor actions suggesting that cannabinoids inhibited glutamate release independently from the cAMP/ PKA pathway (Robbe *et al.*, 2001). Endogenously expressed K_{ir} channels in HEK293 cells, transfected with the CB_1 receptor, were activated by WIN 55,212-2 and anandamide (Vasquez *et al.*, 2003). These effects were antagonised by AM251 implicating a CB_1 receptor-dependent mechanism.

Importantly, Garcia *et al.* (1998) demonstrated that the WIN 55,212-2 evoked activation of GIRK channels, in CB_1 receptor-transfected AtT-20 cells, could be inhibited by stimulation of PKC. The finding that a mutation in the CB_1 receptor prevented the ability of PKC to disrupt ion channel activation suggested that phosphorylation of the G-protein coupled receptor could inhibit its activity (Garcia *et al.*, 1998). Hence, the stimulation of PKC may provide a mechanism to restore neuronal excitability and synaptic strength when endocannabinoid levels are high.

In cultured hippocampal neurons WIN 55,212-2 increased voltage-dependent A-type outward K^+ currents (I_A) (Deadwyler *et al.*, 1995) and decreased voltage-independent D-type outward K^+ currents (I_D or delay current) (Mu *et al.*, 1999) in a concentration-dependent, SR 141716A- and PTX-sensitive manner. A cannabinoid-mediated reduction in cAMP/ PKA was the mechanism shown to activate I_A (Hampson *et al.*, 1995) and inhibit I_D (Mu *et al.*, 1999). It was proposed that phosphorylation of the K^+ channel inactivated I_A and therefore a decrease in PKA would act to reverse this process (Hampason *et al.*, 1995; Mu *et al.*, 2000). As such, PKA inhibitors including IP-20, H7 and H8 mimicked the effects of WIN 55,212-2 on I_A (Hampson *et al.*, 1995; Mu *et al.*, 2000) while activators of PKA including 8-Br-cAMP and forskolin produced opposite effects to WIN 55,212-2 (Hampson *et al.*, 1995). In addition, the phosphatase inhibitor okadaic acid blocked the stimulatory effects of WIN 55,212-2 on I_A (Mu *et al.*, 2000). IP-20 inhibited I_D whereas 8-Br-cAMP potentiated I_D (Mu *et al.*, 1999). This suggests that PKA-mediated phosphorylation activates D-type channels, which may be the reason why WIN 55,212-2 inhibits their activation.

Interestingly, WIN 55,212-2 acting through postsynaptic CB_1 receptors was shown to decrease M-type K^+ currents (I_M) in hippocampal CA1 neurons (Schweitzer, 2000). The authors suggested that a CB_1 receptor-mediated increase in intracellular Ca^{2+} could be one of the mechanisms behind I_M inhibition (see section 1.5.6). By reducing I_M, cannabinoids diminish the ability of neurons to counteract depolarisations, favouring increased firing of action potentials, and thus induce hyperexcitability in the hippocampus (Schweitzer, 2000). Further studies are required to assess the physiological significance of this effect.

In cerebellar granule and COS-7 cells anandamide inhibited the acid-sensitive background K^+ channel TASK-1 (Maingret *et al.*, 2001). This effect was also observed with WIN 55,212-2 and CP 55,940 but not HU-210, 2-AG and Δ^9-THC and was not reversed with SR 141716A. This suggests a direct effect of anandamide on TASK-1 channels. TASK-1 is a member of a family of leak or background K^+ channels that sets resting membrane potential (Maingret *et al.*, 2001). Thus, inhibition of the channel would induce depolarisation and enhance excitability. Taking into account the localisation of TASK-1 in areas of motor control, such as motor neurons and cerebellar granular cells, Maingret *et al.* (2001) suggested that anandamide might influence motor behaviour through an interaction with TASK-1 but further studies are needed to support this hypothesis.

1.5.5.3 Na^+ channels

A study by Nicholson *et al.* (2003) demonstrated the ability of anandamide and WIN 55,212-2 to inhibit voltage-dependent Na^+ channels (activated by veratridine) in mice synaptosomes. The cannabinoids also blocked the veratridine-induced release of neurotransmitters from synaptosomes including GABA and glutamate. The CB_1 receptor antagonist AM251 did not attenuate Na^+ channel inhibition. In addition, anandamide and WIN 55,212-2 were able to displace the binding of [^{3}H]BTX-B to voltage-dependent Na^+ channels (Nicholson *et al.*, 2003). Together the data suggests that cannabinoids can directly modulate the activity of voltage-dependent Na^+ channels, depressing synaptic transmission in the brain and, in turn, reduce both excitatory and inhibitory transmitter release.

1.5.6 Regulation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)

$[Ca^{2+}]_i$ was increased by 2-AG and WIN 55,212-2 in NG108-15 cells (Sugiura *et al.*, 1996a). This effect was both PTX- and SR 141716A-sensitive. The PLC inhibitor U73122 was able to block the response suggesting an inositol triphosphate ($InsP_3$)-mediated release of Ca^{2+} from internal stores (Sugiura *et al.*, 1997). The metabolically stable analogue of anandamide, methanandamide, was shown to deplete $InsP_3$-sensitive Ca^{2+} stores in primary cultures of striatal astrocytes, in a PTX-sensitive manner (Venance *et al.*, 1997). In cultured cerebellar granule cells methanandamide, WIN 55,212-2 and HU-210 augmented the Ca^{2+} signal in response to depolarisation induced by high K^+ (Netzeband *et al.*, 1999). This response was mediated by postsynaptic CB_1 receptors as the effect was antagonised by SR 141716A and PTX. U73122 also blocked the augmented Ca^{2+} release suggesting a CB_1 receptor-mediated release of Ca^{2+} from $InsP_3$-sensitive stores (Netzeband *et al.*, 1999). Collectively the data suggests that CB_1 receptor stimulation is coupled to PLC activation, through $G_{i/o}$ proteins, in turn increasing levels of $InsP_3$ for the induction of Ca^{2+} release from internal stores.

In cultured human arterial endothelial cells anandamide evoked an increase in $[Ca^{2+}]_i$ in an SR 141716A-sensitive manner (Fimiani *et al.*, 1999). This increase in Ca^{2+} was coupled to the production of NO. Anandamide also induced a rise in $[Ca^{2+}]_i$ in human umbilical endothelial cells (Mombouli *et al.*, 1999). This increase was only marginally blocked by SR 141716A, insensitive to PTX and blocked by caffeine suggesting a release of Ca^{2+} from caffeine-sensitive intracellular stores. Anandamide also significantly increased NO synthase (NOS) activity as determined by enhanced conversion of L-[^{3}H]arginine to L-[^{3}H]citruline (Mombouli *et al.*, 1999). These results

suggest that CB_1 receptor-dependent and independent increases in $[Ca^{2+}]_i$ and subsequent NO production may account for some of the vasodilator actions of anandamide.

In Madin-Darby canine kidney (MDCK) cells CP 55,940 increased $[Ca^{2+}]_i$ in a concentration-dependent manner (Chou *et al.*, 2001). CP 55,940 was shown to release Ca^{2+} from thapsigargin (a sarcoplasmic/ endoplasmic reticulum Ca^{2+} adenosine triphosphate (ATP)ase (SERCA) inhibitor)-sensitive stores in an $InsP_3$-independent manner, as the response was not altered by U73122. Filipeanu *et al.* (1997) described an increase in $[Ca^{2+}]_i$ in a hamster vas deferens smooth muscle cell line, DDT_1 MF-2 cells, following CB_1 receptor stimulation by Δ^9-THC. A cannabinoid-mediated release of Ca^{2+} from thapsigargin-sensitive stores was also established. DDT_1 MF-2 smooth muscle cells are employed in the present study to examine the signal transduction pathways induced by CB_1 receptor stimulation, which lead to increases in $[Ca^{2+}]_i$. The cell line is described in more detail in section 1.8.

1.6 Signal transduction mechanisms of CB_2 receptors

The preparations employed in the current study (smooth muscle cells and cultured neurons) do not express CB_2 receptors and therefore the signal transduction pathways of these receptors will not be described in detail. Similar to CB_1, CB_2 receptors can modulate adenylyl cyclase and MAP kinase activity, through their ability to couple to $G_{i/o}$ proteins (Felder *et al.*, 1995; Kobayashi *et al.*, 2001). However, in contrast to CB_1, CB_2 receptor stimulation is believed not to modulate ion channel function, as seen in AtT-20 cells transfected with CB_2 receptors (Felder *et al.*,

1995) and *Xenopus* oocytes transfected with CB_2 and GIRK1/ 4 (Mcallister *et al.*, 1999).

1.6.1 Regulation of adenylyl cyclase

Cannabinoids were shown to inhibit adenylyl cyclase activity in a concentration-dependent manner in CHO cells transfected with the CB_2 receptor (Bayewitch *et al.*, 1995; Slipetz *et al.*, 1995). This effect was PTX-sensitive suggesting signalling through $G_{i/o}$ proteins. The same effect of cannabinoids was also observed in COS cells expressing CB_2 receptors (Slipetz *et al.*, 1995). Again pretreatment with PTX blocked the cannabinoid-mediated inhibition. The inhibitory effect on cAMP production, induced by CB_2 stimulation, is thought to underlie, in part, the regulation of immune function by cannabinoids (Kaminski, 1996).

In contrast to CB_1, CB_2 receptors do not couple to G_s. Stimulation of cAMP accumulation by HU-210 and CP 55,940 was not observed after PTX treatment of CHO cells expressing the human CB_2 receptor suggesting that this novel signalling pathway is unique to the CB_1 receptor (Glass and Felder, 1997; Calandra *et al.*, 1999).

1.6.2 Regulation of MAP kinase

Cannabinoids activate p42/44 MAP kinase in CHO cells (Bouaboula *et al.*, 1996) and HL-60 cells (Kobayashi *et al.*, 2001) expressing the CB_2 receptor. In both studies the effects could be blocked with PTX and the CB_2 antagonist SR 144528. Cannabinoids were also shown to induce the expression of Krox-24 through a PKC-dependent activation of MAP kinase (Bouaboula *et al.*, 1996).

Treatment of human prostate epithelial PC-3 cells with either Δ^9-THC or methanandamide activated the PI3K/ PKB pathway, which in turn induced translocation of Raf-1 to the membrane and phosphorylation of p42/44 MAP kinase (Sanchez *et al.*, 2003). SR 144528 was able to inhibit this induction suggesting the involvement of the CB_2 receptor.

Interestingly, Kaplan and Kaminski (2003) recently showed that WIN 55,212-2 concentration-dependently inhibited p42/44 MAP kinase phosphorylation in stimulated mouse splenocytes. In addition, the MEK inhibitor PD 98059 decreased evoked IL-2 production in these splenocytes (Kaplan and Kaminski, 2003). This suggests that a cannabinoid-mediated reduction of MAP kinase may inhibit IL-2 production in these cells and contribute a mechanism for immunosuppression by cannabinoids. Noteworthy, is that these authors did not determine the cannabinoid receptor subtype involved in mediating this response, although it is likely to be CB_2-mediated as this is the most abundantly expressed cannabinoid receptor subtype in the immune system (Parolaro *et al.*, 2002).

1.6.3 Regulation of $[Ca^{2+}]_i$

Anandamide initiated a rise in $[Ca^{2+}]_i$ in calf pulmonary endothelial cells (Zoratti *et al.*, 2003), which was sensitive to inhibition by the CB_2 antagonist SR 144528 but not the CB_1 receptor antagonist SR 141716A. This increase resulted from the activation of PLC and a subsequent release of Ca^{2+} from $InsP_3$-sensitive stores (Zoratti *et al.*, 2003).

1.7 Interactions of cannabinoids with other receptor systems

A number of investigations have demonstrated the ability of cannabinoids to modulate the activity of other receptor types. Therefore some of the behavioural effects of cannabinoids may occur through a synergistic action with other receptors and their signal transduction pathways. This section briefly describes some of these interactions.

1.7.1 Opioid receptors

Opioid compounds induce their pharmacological effects by activating μ-, δ- and κ-opioid receptors (Thompson *et al.*, 1993; Mansour *et al.*, 1995) and share several actions with cannabinoids including hypothermia, hypotension, motor depression and antinociception (Bloom and Dewey, 1978). Pharmacological interactions between the cannabinoid and opioid systems have been suggested, mainly concerning antinociception.

Smith *et al.* (1998) found that subcutaneous administration of Δ^9-THC enhanced the antinociceptive potency of the opioid agonist morphine in the mouse tail-flick test. This action was SR 141716A-sensitive suggesting it was mediated through the CB_1 receptor. The κ-opioid receptor antagonist nor-binaltorphimine and the δ-opioid receptor antagonist naltrindole was able to block the antinociceptive effect caused by the combination of Δ^9-THC and morphine (Pugh *et al.*, 1996). These authors suggested that the antinociceptive effects of morphine, which are predominantly mediated by μ-receptors, might be enhanced by Δ^9-THC through

activation of κ- and δ-receptors. This fact could be potentially useful in the treatment of pain.

Although the biochemical mechanisms involved in these interactions remain unclear several hypotheses have been formulated. Cannabinoids and opioids might interact at the level of their signal-transduction mechanisms (Manazanares *et al.*, 1999) since opioid and cannabinoid receptors are coupled to similar intracellular signalling systems i.e. inhibition of adenylyl cyclase and Ca^{2+} channels via the activation of GPCRs (Childers *et al.*, 1992; Reisine *et al.*, 1996; Massi *et al.*, 2003).

Cannabinoids may have a direct effect on the synthesis and release of endogenous opioids such as enkephalins and dynorphins. Five day treatment with Δ^9-THC significantly increased proopiomelanocortin gene expression (38%) in the arcuate nucleus of the rat hypothalamus (Corchero *et al.*, 1997b) and increased prodynorphin (39%) and proenkephalin (34%) gene expression in rat spinal cord (Corchero *et al.*, 1997a). This would suggest a cannabinoid-mediated increase in opioid peptide synthesis. Using microdialysis, Valverde *et al.* (2001) showed that acute administration of Δ^9-THC increased the release of enkephalin-like material in the NAc of awake and freely moving rats suggesting cannabinoids can increase opioid release.

1.7.2 Vanilloid VR_1 receptor

The VR_1 receptor is a protein known to be primarily activated by noxious stimuli including heat, H^+ ions and capsaicin, the ingredient found in chilli peppers (see Szallasi and Blumberg, 1999 for review). The receptor can be found on sensory neurons, where VR_1 channel opening causes Ca^{2+} influx and neurotransmitter release.

Studies have demonstrated that anandamide can activate the VR_1 receptor by binding to sites on the cytosolic side of the receptor (De Petrocellis *et al.*, 2001). Anandamide activated cloned VR_1 ion channels expressed in HEK293, which could be blocked by the VR_1 receptor antagonist capsazepine (Zygmunt *et al.*, 1999). Anandamide could also mimic the effects of capsaicin to evoke vascular relaxation in arteries of the guinea-pig, in a capsazepine-sensitive manner (Zygmunt *et al.*, 1999), indicating that anandamide could activate VR_1 channels in physiological preparations. SR 141716A failed to attenuate the vasodilatory actions of anandamide while WIN 55,212-2 had no effect. Some studies have shown that anandamide acts as a partial agonist at vanilloid receptors present in mouse trigeminal sensory neurons (Roberts *et al.*, 2002) and in cultured DRG cells (Hwang *et al.*, 2000). In contrast, other studies have demonstrated that anandamide acts as a full agonist in HEK293 cells transfected with human VR_1 receptors (Smart *et al.*, 2000) and isolated rat mesenteric arteries expressing endogenous VR_1 receptors (Ralevic *et al.*, 2001).

Due to the dual effects of anandamide on inhibitory CB_1 and excitatory VR_1 receptors, Nemeth *et al.* (2003) investigated the effect of different concentrations of anandamide on neuropeptide release from sensory neurons of the rat tracheae, which express both CB_1 and VR_1 receptors. Low concentrations of anandamide (10 μM) inhibited peptide release in an SR 141716A- and PTX-sensitive manner. High concentrations of anandamide (50-100 μM) increased the release of peptides and this response was inhibited by capsazepine. Moreover, anandamide (10 μM) evoked release of peptides in the presence of SR 141716A, in a capsazepine-sensitive manner. This suggests that low concentrations of anandamide can induce neuropeptide release from peripheral sensory nerve terminals by VR_1 receptor activation if the inhibitory CB_1 receptors are blocked. However, since activation of these receptors was only

observed using high concentrations of anandamide the authors concluded that these potentiating effects are not likely to be relevant under physiological conditions (Nemeth *et al.*, 2003).

1.7.3 5-Hydroxytryptamine $(5\text{-HT})_3$ receptor

The 5-HT_3 receptor is a ligand-gated ion channel that is associated with mood, pain and emesis (Greenshaw, 1993). Early work by Fan (1995) in rat nodose ganglion neurons showed that anandamide, WIN 55,212-2 and CP 55,940 inhibited 5-HT-induced currents in a concentration-dependent manner. The inward current was sensitive to blockade by the specific 5-HT_3 receptor antagonist MDL72222 suggesting a cannabinoid-mediated inhibition of 5-HT_3 currents.

A more recent study looked at the effect of cannabinoids in HEK293 cells transfected with the human 5-HT_{3A} receptor only (i.e. no CB-receptor expression) (Barann *et al.*, 2002). The 5-HT-induced currents were inhibited by Δ^9-THC, WIN 55,212-2 and anandamide in a concentration-dependent manner. The WIN 55,212-2-induced inhibition was not altered by SR 141716A and [^{3}H]CP 55,940 was shown not to bind to HEK293 cells further suggesting that the effects are not mediated through either CB_1 or CB_2 receptors. Additional binding studies showed that WIN 55,212-2, anandamide and SR 141716A did not affect [^{3}H]GR65630 binding to the 5-HT_3 receptor but 5-HT caused a concentration-dependent inhibition (Barann *et al.*, 2002). This suggests that the cannabinoids do not interact directly with the active site of the receptor but may instead act allosterically at a 5-HT_3 modulatory site (Barann *et al.*, 2002).

In vivo experiments were conducted to investigate whether cannabinoids also modulate the activity of rat peripheral 5-HT_3 receptors on the terminals of cardiopulmonary afferent C-fibres (Godlewski *et al.*, 2003). In the presence of SR 141716A, injection of the 5-HT_3 receptor agonist phenylbiguanide or capsaicin caused an immediate decrease in heart rate and mean arterial blood pressure. CP 55,940 and WIN 55,212-2 attenuated the 5-HT_3 receptor-induced bradycardia but failed to affect the capsaicin-evoked bradycardia (Godlewski *et al.*, 2003). This data supports that of Barann *et al.* (2002) who suggested that cannabinoids might be mediating their effects through a direct interaction with the 5-HT_3 receptor.

1.7.4 N-methyl-D-aspartate (NMDA) receptor

Areas rich in CB_1 receptors including the basal ganglia and hippocampus also show a high expression of NMDA receptors (a glutamate-sensitive cationic channel involved in excitatory neurotransmission), which are important in the control of movement and memory formation (Ossowska, 1994; Thorat and Bhargava, 1994). Cannabinoids have been shown to have dual effects on NMDA receptor activity. Hampson *et al.* (1998) initially showed that Δ^9-THC and anandamide inhibited NMDA receptor-induced Ca^{2+} influx in rat cortical and cerebellar slices. This effect was CB_1 receptor-mediated as it could be blocked by SR 141716A and PTX and involved the inhibition of P/Q-type Ca^{2+} channels. Interestingly, when the CB_1 receptor component was blocked, anandamide but not Δ^9-THC produced a stimulatory effect on NMDA-induced Ca^{2+} responses including rat cortical, cerebellar and hippocampal slices. This effect was mimicked in *Xenopus* oocytes transfected with NMDA receptors, where both anandamide and the stable analogue methanandamide

dose-dependently potentiated NMDA-induced currents (Hampson *et al.*, 1998). This latter result suggests a direct effect of anandamide on NMDA receptors.

Methanandamide, WIN 55,212-2 and HU-210 have also been demonstrated to enhance NMDA-evoked Ca^{2+} flux in primary cerebellar cultures (Netzeband *et al.*, 1999). This effect was antagonised by SR 141716A, PTX and U73122 suggesting a CB_1 receptor-mediated release of Ca^{2+} from $InsP_3$-sensitive stores was involved. Importantly, blockade of the PLC pathway unmasked a CB_1 receptor-mediated inhibition of the NMDA-evoked Ca^{2+} response (Netzeband *et al.*, 1999).

1.8 Cannabinoid CB_1 receptor-evoked intracellular signalling in DDT_1 MF-2 smooth muscle cells

1.8.1 Physiology of DDT_1 MF-2 cells

The DDT_1 MF-2 smooth muscle cell line was derived from a Syrian hamster vas deferens carcinoma by Norris and Kohler (1974) and many studies have focused on the signal transduction pathways evoked in these cells. Histamine H_1 receptors, P_{2Y}-purinoceptors (stimulated by ATP), α-adrenoceptors and cannabinoid CB_1 receptors are endogenously expressed in this cell line and when stimulated all result in the mobilisation of Ca^{2+} (Molleman *et al.*, 1990, 1991a; Begg *et al.*, 2001). DDT_1 MF-2 cells are convenient for studying receptor-mediated increases in $[Ca^{2+}]_i$ as they do not express VOCCs (Molleman *et al.*, 1991b). An increase in $[Ca^{2+}]_i$ does not contract the cells, as normally observed with most other smooth muscle cells (Webb, 2003), although α-actin polymerisation can be measured suggesting they retain a certain degree of their natural physiology (Mitsuhashi and Payan, 1988).

The intracellular signalling pathways evoked by CB_1 receptor stimulation in DDT_1 MF-2 cells are further investigated in the present study, and compared with previous observations on intracellular signalling initiated by histamine H_1 receptor stimulation. Therefore the following describes the H_1 and CB_1 receptor-induced signalling pathways already known to evoke an increase in $[Ca^{2+}]_i$.

1.8.2 Ca^{2+} modulation in DDT_1 MF-2 cells by H_1 receptor stimulation

H_1 receptors are coupled to G_q proteins and evoke phosphatidylinositol diphosphate (PIP_2) turnover into $InsP_3$ and DAG, through the activation of PLC (Molleman *et al.*, 1990, 1991a; Begg *et al.*, 2001) (Fig. 1.4). The $InsP_3$-induced release of Ca^{2+} from the sarcoplasmic reticulum activates an outward Ca^{2+}-dependent K^+ current ($I_{K,Ca}$) that can be measured using the whole cell version of the patch clamp technique (Den Hertog *et al.*, 1992). This is seen as a transient outward current. Therefore the size of the $I_{K,Ca}$ can be used to indicate increases in $[Ca^{2+}]_i$. Furthermore, measurement of the membrane potential has demonstrated that histamine induces a hyperpolarisation that is most likely a result of the K^+ efflux (Den Hertog *et al.*, 1992). Inositol tetrakisphosphate ($InsP_4$) production was also observed during H_1 receptor stimulation and may be required to promote Ca^{2+} release from internal stores and activate $I_{K,Ca}$, in addition to $InsP_3$ and Ca^{2+} (Molleman *et al.*, 1991a).

In DDT_1 MF-2 cells histamine evokes an outward current in the absence of extracellular Ca^{2+}, consistent with a release of Ca^{2+} from internal stores (Molleman *et al.*, 1991a). Interestingly, subsequent histamine application, 30 minutes later in Ca^{2+}-free conditions, did not result in the mobilisation of $[Ca^{2+}]_i$ and therefore no outward

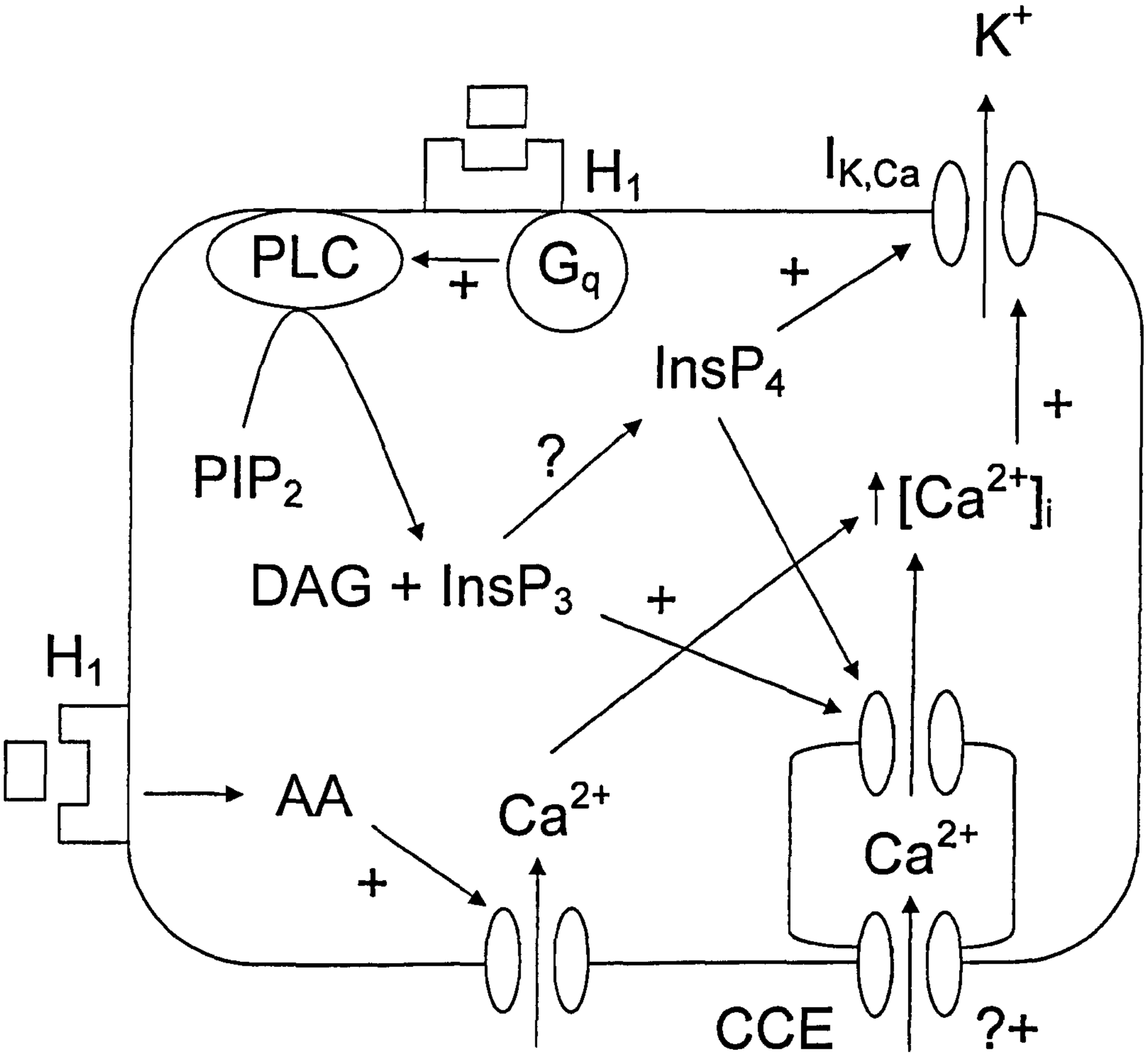


Figure 1.4 The signalling pathways evoked during histamine H_1 stimulation in DDT_1 MF-2 cells.

+ suggests activation, ? suggests a possible mechanism. H_1 receptor stimulation increases $InsP_3$ levels through a G_q protein-coupled activation of PLC. $InsP_3$ induces a release of Ca^{2+} from internal stores, which increases $[Ca^{2+}]_i$ and subsequently activates $I_{K,Ca}$. $InsP_4$ is also produced during H_1 receptor stimulation, possibly from the conversion of $InsP_3$, and modulates Ca^{2+} release from internal stores and $I_{K,Ca}$ activation. The rise in $[Ca^{2+}]_i$ also results from an AA-mediated influx of Ca^{2+} from the extracellular space. Stimulation of CCE may act to replenish depleted intracellular Ca^{2+} stores between histamine responses.

current was evoked (Molleman *et al.*, 1991a). This suggests that extracellular Ca^{2+} is required to refill depleted internal stores between histamine responses and hence, re-addition of Ca^{2+} to the medium re-established the histamine-evoked outward current (Molleman *et al.*, 1991a).

Van der Zee *et al.* (1995) explored the H_1 receptor-evoked intracellular signalling pathways in more detail (Fig. 1.4). In the presence of heparin, to inhibit Ca^{2+} release from internal stores, histamine still evoked an outward current in DDT_1 MF-2 cells that was approximately 65% of control. The remaining current was inhibited by La^{3+}, a non-specific Ca^{2+} channel blocker (Van der Zee *et al.*, 1995). This suggests that the rise in $[Ca^{2+}]_i$ also involves a substantial influx of Ca^{2+} from the extracellular space. Several observations suggest that AA was acting as a second messenger to induce Ca^{2+} influx by activating membrane-bound ion channels (Van der Zee *et al.*, 1995). Firstly, AA evoked a concentration-dependent increase in $[Ca^{2+}]_i$ that was significantly reduced in the absence of extracellular Ca^{2+}. This AA-evoked increase in $[Ca^{2+}]_i$ was also shown to evoke an outward current that was sensitive to La^{3+}. Secondly, histamine stimulated [^{3}H]AA release from DDT_1 MF-2 cells with a similar time course observed to that of the outward current evoked by histamine. Thirdly, inhibitors of the AA breakdown pathway, particularly cyclo-oxygenase and lipoxygenase, did not affect the characteristics of the histamine-induced [^{3}H]AA release. Lastly, pre-treatment of cells with AA reduced the outward current evoked by subsequent histamine application (relating to Ca^{2+} influx) suggesting that AA was involved in the pathways initiated by H_1 receptor stimulation. The fact that the entire outward current was not abolished in the presence of AA and histamine rules out a direct effect of AA on $I_{K,Ca}$.

Van der Zee *et al.* (1995) did not further investigate the signalling pathways leading to the generation of AA. However, H_1 receptor stimulation in DDT_1 MF-2 cells was shown to phosphorylate a p42/44 MAP kinase (Robinson and Dickenson, 2001) whose activation was partly required to induce the histamine-evoked outward current (Begg *et al.*, 2001).

1.8.3 Ca^{2+} mobilisation in DDT_1 MF-2 cells by cannabinoids

In DDT_1 MF-2 cells Filipeanu *et al.* (1997) evoked a concentration-dependent rise in $[Ca^{2+}]_i$ using Δ^9-THC. The rise in Ca^{2+} was directly measured using the fura-2 technique. This mobilisation of Ca^{2+} from internal stores was partially sensitive to inhibition by the CB_1 receptor antagonist SR 141716A, and partially sensitive to thapsigargin. However, there was also a thapsigargin-insensitive component representing Ca^{2+} influx that was neither mediated by the CB_1 or CB_2 receptor.

Begg *et al.* (2001) used the whole cell version of the patch clamp technique to further investigate the signalling pathways evoked by cannabinoid stimulation and in particular the pathways leading to a rise in $[Ca^{2+}]_i$ were explored (Fig. 1.5). CP 55,940 evoked a concentration-dependent transient outward current in DDT_1 MF-2 cells, which was sensitive to inhibition by the CB_1 receptor antagonist SR 141716A but not the CB_2 antagonist SR 144528. Western blot analysis, using antibodies raised against the amino terminus of rat CB_1, confirmed the expression of CB_1 receptors. The rise in $[Ca^{2+}]_i$ was entirely dependent on Ca^{2+} from the extracellular space as extrusion of Ca^{2+} from the extracellular medium abolished the response to CP 55,940. Thapsigargin also significantly reduced the cannabinoid-evoked outward current suggesting a release of Ca^{2+} from thapsigargin-sensitive intracellular stores was

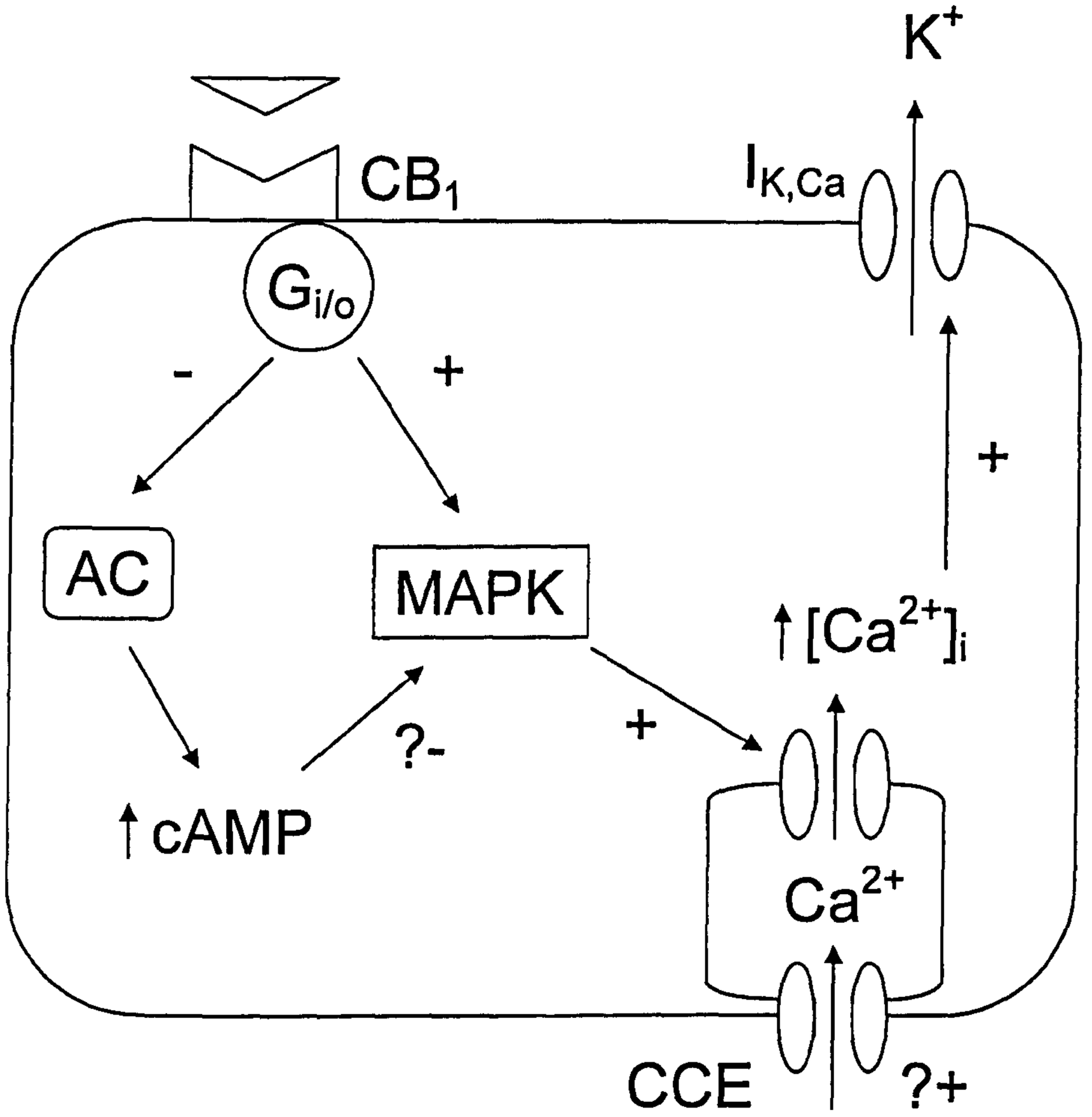


Figure 1.5 The signalling pathways evoked during cannabinoid CB_1 stimulation in DDT_1 MF-2 cells.

+ suggests activation, - suggests inhibition, ? suggests a possible mechanism. CB_1 receptor stimulation activates MAP kinase (MAPK) and inhibits adenylyl cyclase (AC), through $G_{i/o}$ proteins. This induces a rise in $[Ca^{2+}]_i$, which in turn activates $I_{K,Ca}$. An increase in $[Ca^{2+}]_i$ may arise from CCE, via the depletion of thapsigargin-sensitive intracellular Ca^{2+} stores and subsequent Ca^{2+} influx through membrane-bound ion channels. MAPK activation may occur through a CB_1 receptor-mediated reduction in cAMP or a separate pathway associated with $G_{i/o}$ proteins, or both.

required. $InsP_3$ does not mediate the release of Ca^{2+} from internal stores, as the PLC inhibitor U73122 had no significant effect on the CP 55,940-evoked current. Furthermore, both the activation of MAP kinase and the inhibition of adenylyl cyclase are required to induce a rise in $[Ca^{2+}]_i$ suggesting a link between MAP kinase activation and the increase in internal Ca^{2+}. A modulation of cAMP levels may be a means by which MAP kinase activity is regulated in DDT_1 MF-2 cells by cannabinoids (see section 1.5.4).

Stimulation of the CB_1 receptor has been shown to specifically activate the p42/44 MAP kinase in other tissues (Bouaboula *et al.*, 1995b; Sanchez *et al.*, 1998b). This suggests that a p42/44 MAP kinase may also be activated by CB_1 receptor stimulation in DDT_1 MF-2 cells.

1.8.4 Capacitative (CCE) and non-capacitative Ca^{2+} entry (NCCE)

The observation that both Ca^{2+} influx from the extracellular space and a release of Ca^{2+} from thapsigargin-sensitive stores is necessary to evoke a rise in $[Ca^{2+}]_i$ suggests that CB_1 receptor stimulation evokes CCE in DDT_1 MF-2 cells (Putney and McKay, 1999). This is a phenomenon whereby the depletion of intracellular Ca^{2+} stores is coupled to Ca^{2+} influx through store-operated Ca^{2+} channels (SOCCs) at the plasma membrane (Fig. 1.6). CCE can be demonstrated in both non-excitable cells such as epithelial cells (Yang *et al.*, 2003) and excitable cells such as smooth muscle cells (Kwan *et al.*, 1994; Broad *et al.*, 1999). CCE has been proposed to replenish Ca^{2+} stores, as observed in porcine vascular smooth muscle (Weirich *et al.*, 2004). Thus, the observation that extracellular Ca^{2+} is required to produce successive histamine responses in DDT_1 MF-2 cells (Molleman *et al.*, 1991a)

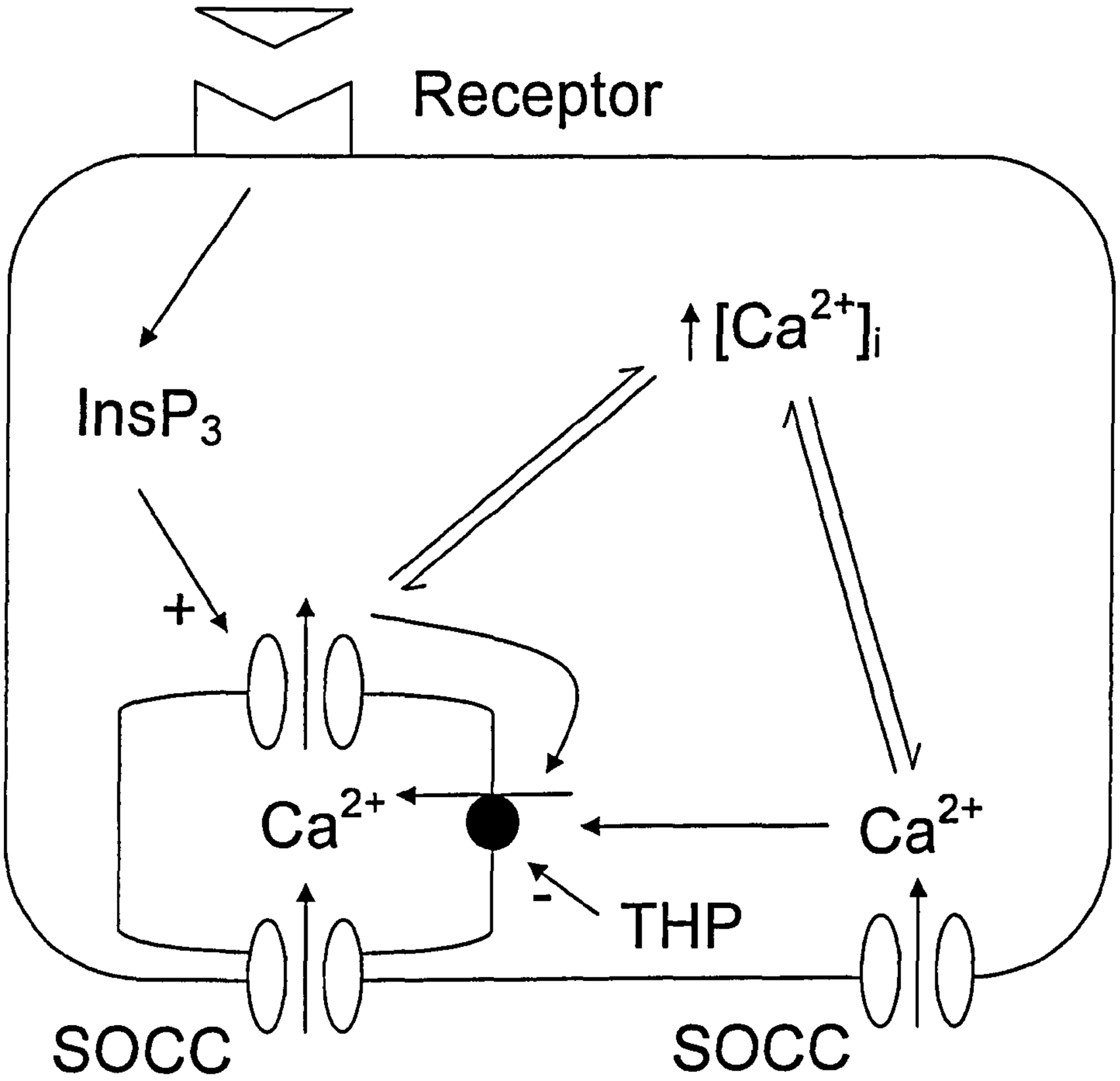


Figure 1.6 Capacitative Ca^{2+} Entry (CCE)
+ suggests activation, - suggests inhibition. The influx of Ca^{2+} through membrane-bound store-operated Ca^{2+} channels (SOCCs), whose activation are dependent on the filling state of the internal stores, has been termed CCE. Hence, a receptor-mediated release of Ca^{2+} from $InsP_3$-senstive stores can activate SOCCs and subsequent Ca^{2+} influx. In addition, agents that indirectly deplete Ca^{2+} stores, such as the Ca^{2+}-ATPase inhibitor thapsigargin (THP), can also evoke CCE. CCE has been shown to replenish intracellular Ca^{2+} stores, as well as provide sustained elevations in $[Ca^{2+}]_i$. The activation of SOCCs may lead to a direct refilling of depleted internal Ca^{2+} stores. Increases in cytosolic Ca^{2+}, mediated by the activation of SOCCs, may provide an alternative mechanism by which intracellular Ca^{2+} stores are replenished, via the action of Ca^{2+}-ATPases. For review see Putney and McKay (1999).

could implicate a role for CCE in intracellular store replenishment. CCE can also provide prolonged, sustained elevation of $[Ca^{2+}]_i$, which may be desirable when tonic responses are needed, for example, in the maintenance of smooth muscle tone (Gibson *et al.*, 1998). In corneal epithelial cells CCE was associated with the initiation of cell proliferation and migration by epidermal growth factor (Yang *et al.*, 2003).

As demonstrated by Van der Zee *et al.* (1995) the increase in $[Ca^{2+}]_i$ in DDT_1 MF-2 cells was partly mediated by an influx of Ca^{2+} attributable to AA production. The same signalling pathway could also potentially mediate the Ca^{2+} entry process observed during CB_1 receptor stimulation. This NCCE pathway, whereby AA mediates Ca^{2+} influx, has also been described in murine Balb-C 3T3 fibroblasts (Munaron *et al.*, 1997), HEK293 cells (Mignen and Shuttleworth, 2000), rat aortic smooth muscle cells (Broad *et al.*, 1999) and bovine aortic endothelial cells (Fiorio Pla and Munaron, 2001) and has been designated I_{ARC} (arachidonate regulated Ca^{2+} current; Mignen and Shuttleworth, 2000). The main property of this process is that Ca^{2+} influx is independent of store depletion and thus is non-capacitative. Thus, exogenous AA application still induced Ca^{2+} influx into cells whose intracellular stores were initially depleted (Broad *et al.*, 1999; Fiorio Pla and Munaron, 2001; Luo *et al.*, 2001b).

1.8.5 Phospholipase A_2 (PLA_2) and the liberation of AA

PLA_2 catalyses the hydrolysis of the *sn*-2 position of membrane glycerophospholipids to liberate AA (Kudo and Murakami, 2002). So far three different types of PLA_2 have been identified including secretory PLA_2 ($sPLA_2$), cytosolic PLA_2 ($cPLA_2$) and Ca^{2+}-independent PLA_2 ($iPLA_2$). The $cPLA_2$ family (also

known as group IV PLA_2) consists of three enzymes including $cPLA_2\alpha$, which is tightly regulated by Ca^{2+} and phosphorylation (Kudo and Murakami, 2002). Ca^{2+} is required for $cPLA_2\alpha$ translocation to golgi, the endoplasmic reticulum and the nuclear envelope, and also stabilises the association of $cPLA_2\alpha$ with the membrane, leading to AA release. Ca^{2+} may also combine with calmodulin (a decoder of Ca^{2+} signals) to form Ca^{2+}/ calmodulin kinase-II, which has been found to bind to $cPLA_2\alpha$ and phosphorylate the Ser^{515} site resulting in an increase in enzyme activity (Kudo and Murakami, 2002).

Maximal activation of $cPLA_2\alpha$ also requires the sustained phosphorylation of Ser^{505} by MAP kinase, which results in up to a 2 to 3-fold increase in the catalytic activity (Kudo and Murakami, 2002).

Therefore, if NCCE mediates Ca^{2+} influx in DDT_1 MF-2 cells, during CB_1 receptor stimulation, the activation of a $cPLA_2$ pathway could provide a link between MAP kinase activation and a resulting rise in $[Ca^{2+}]_i$, via the production of AA. Experiments have yielded results indicating that cannabinoid receptor stimulation can induce AA mobilisation in rat brain cortical astrocytes (Shivachar *et al.*, 1996) and in WI-38 fetal lung fibroblasts (Wartmann *et al.*, 1995). In the latter experiment the rise in AA was associated with an increased phosphorylation and hence activity of both MAP kinase and the $cPLA_2$ subtype. In addition, AA-mediated NCCE in other preparations involved the activation of $cPLA_2$ (Munaron *et al.*, 1997; Osterhout and Shuttleworth, 2000) and MAP kinase (Munaron *et al.*, 1997).

1.9 The myenteric plexus

1.9.1 Structure of the guinea-pig small intestine

The small intestine is composed of two muscle layers, the outer longitudinal and the inner circular muscles (Fig. 1.7). In the outer layer, smooth muscle cells are oriented along the length of the intestinal segments whereas, in the inner layer, smooth muscle cells are oriented transversally to the length of the intestine. A neuronal network, the myenteric plexus, separates these muscle layers and primarily controls contractions and relaxations of the gastrointestinal smooth muscle (Kunze and Furness, 1999). Propulsive motility (peristalsis) is induced by relaxation of intestinal muscle downstream (descending inhibitory reflex) and contraction of muscle upstream (ascending excitatory reflex) of the intestinal bolus. Another neuronal network that resides within the small intestine, the submucosal plexus, controls the secretory/ absorptive functions of the intestinal epithelium, local blood flow and neuro-immune responses (Cooke, 1998).

1.9.2 Types of neurons in the guinea-pig myenteric plexus

The myenteric plexus contains three major types of neurons, motor neurons (excitatory and inhibitory), interneurons and intrinsic primary afferent (sensory) neurons. These can be further subdivided depending on the neurotransmitters they release and the physiological function they evoke (Fig. 1.8). The following describes these subtypes in more detail.

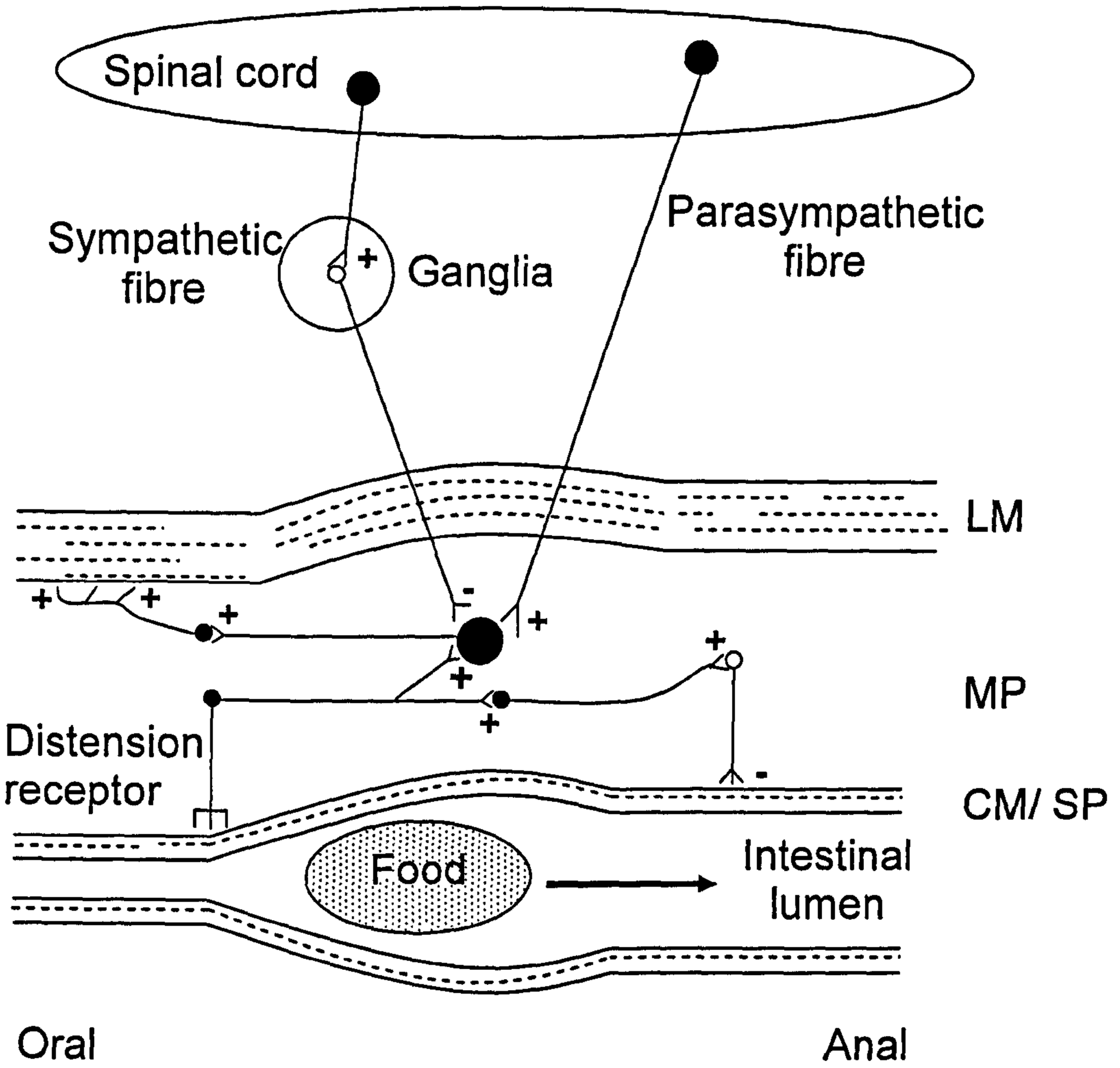


Figure 1.7 Schematic diagram of the guinea pig small intestine.
+ and – are innervations from excitatory (●) and inhibitory (○) neurons respectively. Distension of the circular muscle (CM) at the oral side evokes a relaxation of CM downstream (descending inhibitory reflex) and contraction of the longitudinal muscle (LM) upstream (ascending excitatory reflex) of the intestinal bolus, pushing it along the length of the intestine. Peristalsis is also regulated by centrally-derived sympathetic and parasympathetic nerves. Abbreviations: myenteric plexus, MP, submucosal plexus, SP.

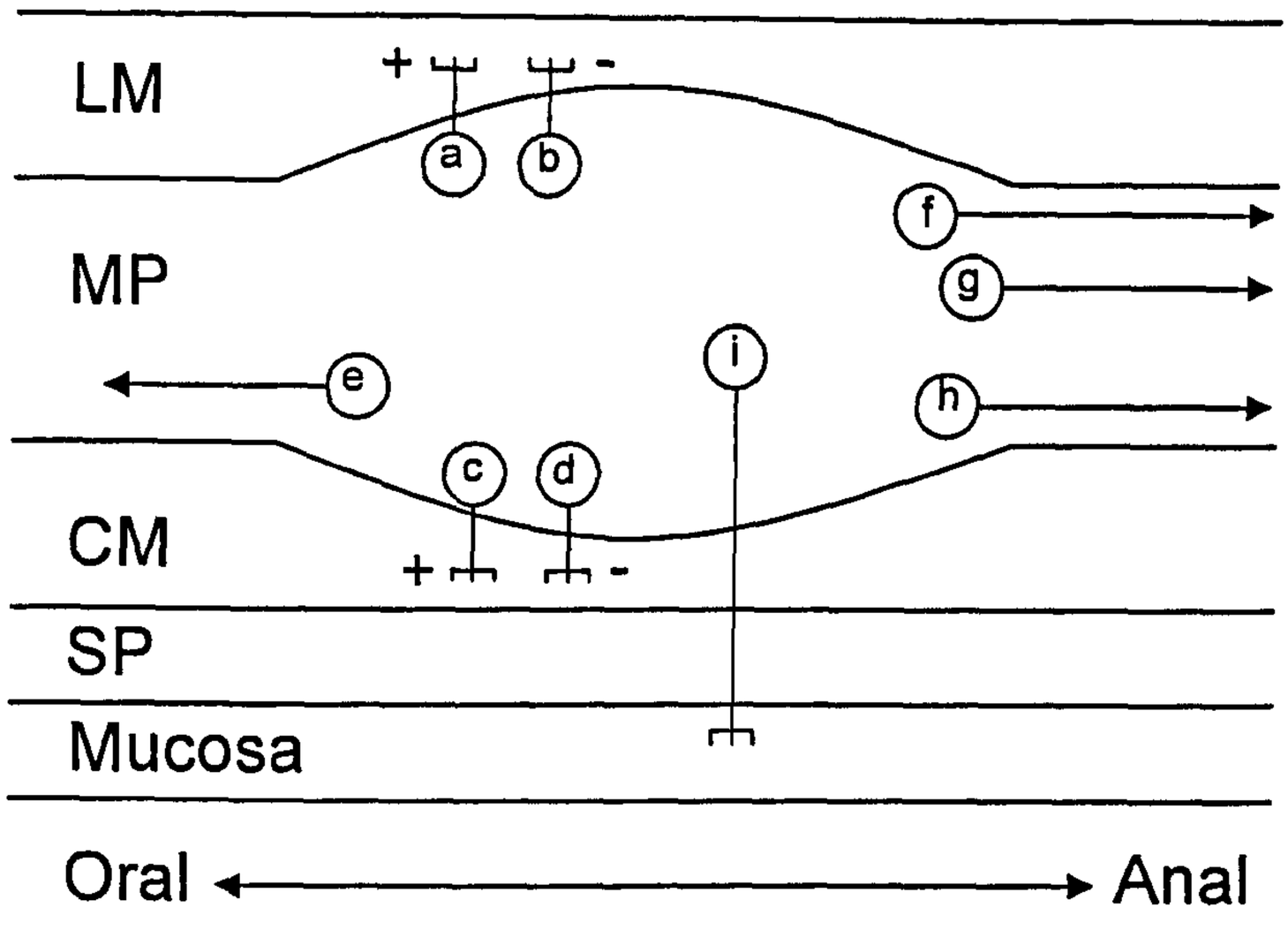


Figure 1.8 Schematic diagram showing the neuronal circuitry involved in regulating motility in the guinea pig ileum.
+ and – are excitatory and inhibitory innervations respectively. The letters correspond to those in the table below, aiding neuronal identification.

	Transmitters	Morphology	Neurons
a	ACh, TK	Dogiel type I/ S	Excitatory motor neuron, LM
b	NO, ATP, VIP, PACAP	Dogiel type I/ S	Inhibitory motor neuron, LM
c	ACh, TK	Dogiel type I/ S	Excitatory motor neuron, CM
d	NO, ATP, VIP, PACAP	Dogiel type I/ S	Inhibitory motor neuron, CM
e	ACh, TK	Dogiel type I/ S	Ascending interneuron
f	ACh, ATP, NO, VIP	Dogiel type I/ S	Descending interneuron
g	ACh, ATP, SOM	Dogiel type I/ S	Descending interneuron
h	ACh, 5-HT	Dogiel type I/ S	Descending interneuron
i	ACh, TK	Dogiel type II/ AH	Primary afferent neuron

Abbreviations: Longitudinal muscle, LM, myenteric plexus, MP, circular muscle, CM, submucosal plexus, SP, acetylcholine, ACh, tachykinins, TK, nitric oxide, NO, adenosine triphosphate, ATP, vasoactive intestinal polypeptide, VIP, pituitary adenylyl cyclase activating peptide, PACAP, somatostatin, SOM, 5-hydroxytryptamine, 5-HT, S-neuron, S, AH-neuron, AH.

1.9.2.1 Motor neurons

Approximately 37% of myenteric neurons in the guinea-pig small intestine are classed as excitatory motor neurons (Furness, 2000). Both muscle layers receive excitatory ascending innervations (12% innervate the circular muscle, 25% innervate the longitudinal muscle), which are predominantly cholinergic and are mediated through muscarinic M_2 and M_3 receptors (Furness and Sanger, 2002). However, there is residual excitation that was resistant to the muscarinic receptor antagonist scopolamine but could be blocked by neurokinin NK_1 receptor antagonists (Galligan, 1999; Schneider *et al.*, 2000). This suggests that this residual excitation is predominantly due to the release of tachykinins (TKs). Consistent with this result, motor neurons are immunoreactive for both the synthesising enzyme for ACh (i.e. choline acetyltransferase (ChAT)) and for TKs (Costa *et al.*, 2000). It is thought that substance P and NKA are the principal TK transmitters (Lippi *et al.*, 1998). In guinea-pigs the simultaneous blockade of NK_1, NK_2 and NK_3 receptors slowed colonic peristalsis, although the effect was much less than that of the muscarinic antagonist atropine (Tonini *et al.*, 2001). This suggests that ACh is the primary transmitter of excitatory motor neurons. Furthermore, low levels of physiological stimulation preferentially activate the cholinergic component of transmission whereas higher levels activate the TK component (Bornstein *et al.*, 2004).

Inhibitory motor neurons, which descend anally, also innervate both muscle layers. Approximately 18% of myenteric neurons are inhibitory (Furness, 2000); 16% innervate the circular muscle while only 2% innervate the longitudinal muscle. The inhibitory neurons contain NO, ATP, vasoactive intestinal peptide (VIP) and pituitary adenylyl cyclase activating peptide (Furness and Sanger, 2002).

Effects on the muscle from both the excitatory and inhibitory motor neurons are relayed at least in part via interstitial cells of Cajal (ICC), which are electrically-coupled to the muscle (Kunze and Furness, 1999). Disruption of ICC by treating mice with antibodies to *kit*, a receptor tyrosine kinase on the ICC, impaired excitatory and inhibitory transmission to the circular muscle of the ileum (Torihashi *et al.*, 1995).

1.9.2.2 Interneurons

In the guinea-pig ileum the ascending myenteric interneurons are immunoreactive for ChAT and substance P (Brookes *et al.*, 1997). Transmission between ascending interneurons and interneurons and ascending excitatory motor neurons is predominantly cholinergic, mediated via nicotinic receptors (see section 1.9.4), although a component is also mediated by NK_3 receptors (Bornstein *et al.*, 2004).

There are at least three distinct classes of descending interneurons (Costa *et al.*, 1996) displaying immunoreactivity for ACh, ATP, NO, 5-HT, VIP and somatostatin. They have outputs to inhibitory motor neurons, other interneurons and ascending interneurons (Mann *et al.*, 1997).

Transmission to and from descending interneurons can be divided into two distinct pathways, descending inhibition and descending excitation. In the descending inhibitory pathway in the guinea-pig ileum, transmission from interneurons to inhibitory motor neurons is largely mediated by ATP acting at P_{2X} receptors (Bian *et al.*, 2000). Descending excitatory pathways in the guinea-pig ileum have been shown to be resistant to the nicotinic antagonist hexamethonium and instead 5-HT_3 and P_{2X} receptors are thought to predominantly mediate neurotransmission (Monro *et al.*,

2002). Descending excitation is distinct from the neural pathways involved in regulating peristalsis as antagonists at these receptors failed to effect evoked peristaltic contractions (Monro *et al.*, 2002).

1.9.2.3 Intrinsic primary afferent neurons (IPANs)

The first neurons in the intrinsic nerve circuits that are activated by appropriate stimuli are the IPANs (sensory neurons). In isolated guinea-pig small intestine, where extrinsic nerves no longer innervated the tissue and time was allowed for their endings to degenerate, reflexes, in response to mucosal distension and distortion, were not diminished relative to intestine that was isolated without denervation (Furness *et al.*, 1995). This indicates that there are IPANs in the intestine, which makes up about 26% of the total number of neurons in the myenteric plexus (Furness, 2000).

Intracellular microelectrodes have been used to record from IPANs in myenteric neurons, which were excited by chemical stimuli. Bertrand *et al.* (1997) demonstrated that acid (pH 3) and acetic acid (pH 7.2), applied to the surface of the guinea-pig ileal mucosa, elicited a burst of action potentials in myenteric IPANs. The neurons continued to respond when the bathing solution was changed to one containing high Mg^{2+} and low Ca^{2+} to block transmission, suggesting that the responses were not due to indirect activation by other neurons.

Responses to tension generated by muscle contraction have also been recorded. Stretch of the intestine by around 40% was enough to excite most neurons (Kunze *et al.*, 1998). However, it was not the stretch that stimulated the neurons but

the contraction of muscle cells in response to the stretch, as neurons were no longer excited in the presence of the muscle relaxant isoprenaline (a β adrenoceptor agonist).

IPANs receive synaptic inputs from other IPANs, which can be blocked by NK_3 receptor antagonists (Alex *et al.*, 2001). IPANs also transmit to interneurons and motor neurons, which may involve both ACh and TKs acting at nicotinic and NK_1 receptors respectively (Furness and Sanger, 2002).

1.9.3 Electrophysiological and morphological classification of myenteric neurons

Hirst *et al.* (1974) introduced a system that differentiated between membrane responses to intracellular derived action potentials. One class was termed an AH-neuron because extremely long after-hyperpolarisations (AHPs) were observed after an action potential. The second class was termed S-neurons because they exhibited prominent cholinergic synaptic inputs but not the slow AHP. The action potential recorded from the soma of S-neurons is completely blocked by the Na^+ channel blocker, tetrodotoxin (TTX) and most S-neurons fire continuously when depolarised with a current pulse applied through the recording microelectrode (Hirst *et al.*, 1974). S-neurons are likely to be interneurons and motor neurons in the myenteric plexus and exhibit fast excitatory postsynaptic potentials (fEPSPs) (Galligan, 2002). These fast synaptic responses are mediated through the activation of ligand-gated ion channels, predominantly nicotinic receptors (see section 1.9.4).

Single electrical stimuli elicit fESPSs in some AH-neurons (Furness *et al.*, 1998) and trains of stimuli elicit slow EPSPs (sEPSPs) in all AH-neurons (Galligan, 2002). The action potential in AH-neurons is partly mediated by an influx of Ca^{2+} and as a result was only partly blocked by TTX (Hirst *et al.*, 1974). The action potential in

AH-neurons is followed by an AHP that lasts from 1-20 seconds. The AHP is mediated by Ca^{2+}-dependent K^+ channels activated by Ca^{2+} entering the neuron during the action potential (North and Tokimasa, 1987; Furness *et al.*, 1998). Under resting conditions AH-neurons fire one or two action potentials as the AHP limits the firing rate. AH-neurons are IPANs in the myenteric plexus (Furness *et al.*, 1998; Galligan, 2000). All sEPSPs have a long latency (often more than 50 ms) between the time of nerve stimulation and the onset of the response, and a long duration (seconds to minutes). The prolonged latency and duration occur because sEPSPs are mediated through intracellular signalling pathways activated by GPCRs, which lead to the inhibition of $I_{K,Ca}$ and neuronal depolarisation. In guinea-pig myenteric AH-neurons sEPSPs were evoked by the NK_3 receptor agonist senktide (Bertrand and Galligan, 1995). This response was augmented by the addition of GTPγS but reduced by PLC and protein kinase inhibitors suggesting the response was mediated by G-protein activation of PLC and protein kinases. 5-HT application also evokes sEPSPs in AH-neurons (Pan *et al.*, 1997). The response to 5-HT was antagonised by PTX and inhibitors of PKC and PKA (Pan *et al.*, 1997). Activation of both PKA and PKC was thought to give rise to the inhibition $I_{K,Ca}$.

Morphologically myenteric neurons were categorised into two distinct groups. Dogiel type I neurons possess a single axon and short dendrites. Dogiel type II cells have multiple long processes arising from a smooth cell body. Within the guinea-pig small intestine Dogiel type II possess the electrical properties of AH-neurons and thus are IPANs (Brookes *et al.*, 1995). Interneurons and motor neurons are classified as Dogiel Type I cells (Brookes *et al.*, 2001).

1.9.4. Nicotinic ACh (nACh) receptors in the myenteric plexus

1.9.4.1 Function of myenteric nACh receptors

ACh acting at nACh receptors is the predominant mechanism of excitatory neurotransmission in the myenteric plexus. All electrically-evoked fEPSPs recorded from S- and AH-neurons are inhibited, in part, by nACh receptor antagonists such as hexamethonium and mecamylamine (Nishi and North, 1973; Hirst *et al.*, 1974). Only about 25% of neurons in the guinea-pig myenteric plexus exhibit fEPSPs that are completely blocked by hexamethonium (Galligan and Bertrand, 1994). In the remaining neurons that exhibit hexamethonium-insensitive fEPSPs, other transmitters contribute to fast synaptic excitation including ATP and 5-HT. Noteworthy is the observation that, although most AH-neurons do not receive fast excitatory synaptic input, they express functional nACh receptors because exogenously applied nicotine was shown to depolarise AH-neurons in the intact guinea-pig myenteric plexus (Schneider and Galligan, 2000).

Microlesion studies in the guinea-pig myenteric plexus showed that nACh receptors are localised on neurons that are in ascending and circumferential pathways (LePard and Galligan, 1999). Stimulation of orally-directed pathways produced fEPSPs that were completely blocked by hexamethonium. Surgical interruption of these pathways reduced the average amplitude but did not change the pharmacological properties of the fEPSP (LePard and Galligan, 1999). This suggests that nACh receptor activation is the predominant mechanism for neurotransmission in ascending pathways in the myenteric plexus. However, neurotransmission in descending excitatory pathways to the muscle layers was largely resistant to

hexamethonium, which is consistent with 5-HT and ATP being the predominant neurotransmitters in this pathway (Monro *et al.*, 2002).

1.9.4.2 Properties of myenteric nACh receptors

ACh and nicotine acting at nACh receptors induces channel opening and an influx of cations into the cell (Paterson and Nordberg, 2000) including Ca^{2+} (Trouslard *et al.*, 1993). The fEPSPs, in response to nACh receptor stimulation, were shown to result from an increase in cation conductance (Galligan and Bertrand, 1994).

Neuronal nACh receptors consist of various complements of α (α2-α7) and β (β2–β4) subunits and assemble according to a general 2α3β stoichiometry, with the possibility of more than one α subunit within a pentamer (Paterson and Nordberg, 2000). Each subunit is composed of four transmembrane segments (TM1-TM4), where TM2 is thought to form the lining of the ion channel (Paterson and Nordberg, 2000).

Recent immunohistochemical studies in cultured guinea-pig myenteric neurons identified the presence of α3, α5 and β2 subunits (Zhou *et al.*, 2002). α7 was also present but only on a few neurons. Zhou *et al.* (2002) also tried to characterise the pharmacological properties of nACh receptors in myenteric neurons. Inward currents evoked by ACh were blocked by nACh receptor antagonists with a rank order potency of mecamylamine > hexamethonium > dihydro-β-erythroidine (DHβE). Mecamylamine and DHβE exhibit high potency at β4 and β2 subunit-containing nACh receptors respectively (Zhou *et al.*, 2002). α-bungarotoxin and α-methylcaconitine, antagonists that block α7 subunit-containing nACh receptors, did not affect nicotine-evoked inward currents in myenteric neurons (Zhou *et al.*, 2002).

Electrophysiological studies performed in the intact myenteric plexus of the guinea-pig showed that cytisine, which is a full agonist at β4 subunit-containing nACh receptors, caused fast depolarisations in 70% of S-neurons but did not depolarise AH-neurons excited by nicotine (Schneider and Galligan, 2000). All responses caused by cytisine were blocked by mecamylamine. Collectively the data indicates that myenteric neurons predominantly express nACh receptors composed of α3, α5, β2 and β4 subunits but do not express functional nACh receptors containing α7 subunits. In addition, most S-neurons express nACh receptors that contain β4 subunits whereas AH-neurons and a subset of S-neurons (~30%) may express nACh receptors that do not contain β4.

1.9.4.3 Presynaptic nACh receptors

From the results described above it seems fairly well established that nACh receptors are localised to the somatodendritic region of neurons where they mediate excitation of these neurons. However, there is evidence to suggest that nACh receptors may also be localised on nerve endings where these receptors mediate the release of neurotransmitters (Fig. 1.9). In whole guinea-pig ileum, contractions evoked by high concentrations of nicotine (> 100 μM) were significantly inhibited (but not completely blocked) by the simultaneous application of TTX (to abolish neurally mediated contractions) and the muscarinic antagonist scopolamine. (Galligan, 1999). The residual contractions evoked by nicotine were significantly reduced by the NK_1 receptor antagonist CP 96,345-1 (Galligan, 1999). These observations would be consistent with the activation of presynaptic nACh receptors on motor neurons at the neuromuscular junction that couple to the release of TKs.

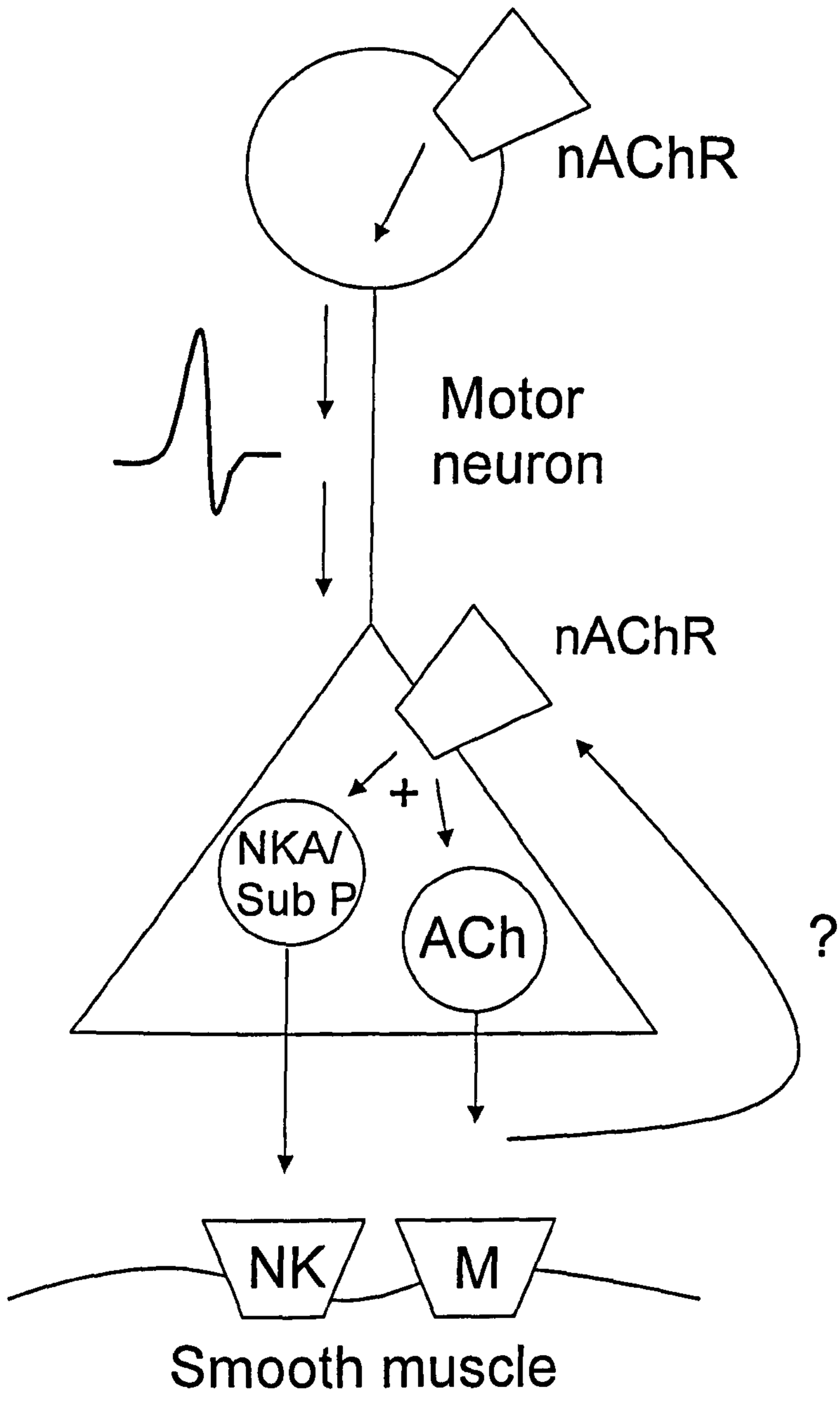


Figure 1.9 Function of presynaptic nACh receptors (nAChRs) on excitatory myenteric motor neurons.

+ suggests activation, ? suggests a possible mechanism. Activation of somatodendritic nAChRs induces neuronal depolarisation and a release of ACh and TKs (NKA and substance P) from the nerve terminal. These act on muscarinic (M) and NK receptors respectively to evoke smooth muscle contraction. Activation of presynaptic nAChRs facilitates neurotransmitter release from the nerve terminal. Presynaptic nAChRs may mediate positive feedback, stimulated by ACh, which is released from the same nerve terminal the receptors are expressed on.

Another study demonstrated that presynaptic nACh receptors are located on myenteric excitatory motor neurons that innervate circular smooth muscle of the guinea-pig ileum (Schneider *et al.*, 2000). The study used a preparation that consisted of the circular smooth muscle and submucosal layers; referred to as the circular muscle-axon (CM-axon). In this preparation the motor neurons are physically separated from their cell bodies by the removal of the myenteric plexus. The nACh receptor agonist DMPP (> 30 μM) evoked contractions of the CM-axon, which were inhibited by the nACh receptor antagonist mecamylamine. Contractions were also inhibited by the addition of scopolamine and completely blocked in the presence of scopolamine and CP-96,345-1. This suggests the presence of presynaptic nACh receptors on motor neurons, which facilitate both ACh and TK release to evoke circular smooth muscle contraction. A recent study by Mandl *et al.* (2003) used the guinea-pig myenteric plexus-longitudinal muscle (MPLM) preparation to confirm the presence of presynaptic nACh receptors. This preparation consists of merely the longitudinal muscle and myenteric plexus and can therefore be used to investigate myenteric neurotransmission involved in ACh-mediated contractions of the longitudinal muscle. The study used a more potent, selective nACh receptor agonist, epibatidine, as high concentrations of nicotine and DMPP, as used in the previous studies, may have had additional non-specific effects (Mandl *et al.*, 2003). In the presence of TTX, epibatidine (30 nM)-evoked contractions of the MPLM were completely inhibited. However, higher concentrations of epibatidine (300 nM) evoked contractions that were insensitive to TTX but were completely blocked by mecamylamine. The data provides further evidence for the existence of presynaptic nACh receptors on the terminals of myenteric motor neurons, although these receptors are operative only if the concentration of agonist is sufficient for their activation.

In the central nervous system the activation of presynaptic nACh receptors positively modulates transmitter release (Wonnacott, 1997). For instance, the activation of presynaptic nACh receptors by nicotine evokes the release of dopamine from striatal synaptosomes (Soliakov and Wonnacott, 1996) and ACh and noradrenaline from hippocampal synaptosomes (Kulak *et al.*, 2001). Hence, presynaptic nACh receptors, on motor neurons in the myenteric plexus, may be a mechanism by which ACh and TK release could be enhanced during periods of high frequency nerve activity, when concentrations of ACh near the neuroeffector junction are high. Indeed, ChAT, substance P and NKA are all found in myenteric motor neurons (Costa *et al.*, 2000) suggesting that these transmitters are released from the same nerve terminals.

As nACh receptors are non-selective cationic channels they may facilitate neurotransmitter release either directly or indirectly (Wonnacott, 1997). nACh receptor activation may result in neuronal depolarisation, which would be sufficient to activate VOCCs and evoke neurotransmitter release. Alternatively, the high Ca^{2+} permeability of nACh receptors may lead to sufficient Ca^{2+} influx to induce the release of neurotransmitter.

1.9.5 The role of cannabinoids in the myenteric plexus

CB_1 but not CB_2 receptor mRNA is present in the guinea-pig myenteric plexus (Griffin *et al.*, 1997). This is consistent with immunohistochemical data, in the guinea-pig, that showed that myenteric primary afferent, interneuronal and motor neuronal cell bodies and nerve fibres express CB_1 receptors (Coutts *et al.*, 2002). Furthermore, approximately 99% of CB_1 receptor-positive cells are cholinergic

(Coutts *et al.*, 2002). This distribution supports the established inhibitory effects of cannabinoids on gastrointestinal motility, propulsion and transit, which is likely to occur through an inhibition of presynaptic ACh release.

CP 55,940 and WIN 55,212-2 inhibited electrically-evoked contractions (via a release of ACh at the neuromuscular junction) of the MPLM, which were reversed by SR 141716A (Pertwee *et al.*, 1996a). ACh-induced contractions were not affected by CP 55,940 suggesting cannabinoids activate CB_1 receptors located at presynaptic sites. This was later supported by the observation that CP 55,940 inhibits [^{3}H]ACh release in the guinea-pig myenteric plexus, in an SR 141716A-sensitive manner (Coutts and Pertwee, 1997). Similar inhibitory effects of cannabinoids, mediated through the CB_1 receptor, have also been described in the guinea-pig ileum circular muscle (Izzo *et al.*, 1998) and the human ileum longitudinal muscle preparation (Croci *et al.*, 1998). Cannabinoids have also been shown to modulate GABA (Begg *et al.*, 2002b) and adenosine (Begg *et al.*, 2002a) transmission in the guinea-pig MPLM. Both ethylenediamine (a GABA releasing agent) and adenosine were able to inhibit electrically-evoked contractions of the MPLM, mediated through the activation of $GABA_B$ and A_1 receptors respectively. This inhibition was reduced in the presence of CP 55,940, which suggests that cannabinoids may also increase excitatory neurotransmission in the myenteric plexus through a reduction in inhibitory tone.

WIN 55,212-2, CP 55,940 and methanandamide inhibited evoked peristalsis in isolated guinea-pig ileum, in an SR 141716A-sensitive manner (Heinemann *et al.*, 1999; Izzo *et al.*, 2000a). Methanandamide was shown to significantly inhibit the distension-induced ascending excitatory motor reflex contraction of the circular muscle (Heinemann *et al.*, 1999). The methanandamide-induced inhibition of peristalsis was attenuated by the NO synthase inhibitor L-NAME and the Ca^{2+}-

dependent K^+ channel inhibitor apamin (Heinemann *et al.*, 1999). Thus, cannabinoids may depress peristalsis through a blockade of ascending excitatory motor pathways and the facilitation of inhibitory pathways operating via apamin-sensitive K^+ channels and NO.

Some effects of cannabinoids in the myenteric plexus are not mediated by the CB_1 receptor. Lopez-Redondo *et al.* (1997) showed that cannabinoids could block excitatory synaptic transmission within the myenteric plexus. Exposure of S-type myenteric neurons to CP 55,940 and WIN 55,212-2 significantly depressed the amplitude of electrically-evoked fEPSPs. Interestingly, the inhibitory effect of WIN 55,212-2 was reversed in only 38% of the neurons with SR 141716A. Furthermore, SR 141716A alone caused a significant reduction in the amplitude of fEPSPs. This suggests a subset of S-neurons express CB_1 receptors that modulate fast excitatory synaptic transmission. However, it would also seem that a subpopulation of myenteric S-neurons do not express CB_1 receptors and therefore WIN 55,212-2 and SR 141716A may act at a novel site(s) to inhibit fESPSs. Although anandamide has been shown to produce a dose-dependent inhibition of electrically-evoked contractions of MPLM (Pertwee *et al.*, 1995) it might be a non-CB_1 receptor-mediated process as SR 141716A was much less effective at blocking these inhibitory effects when compared to synthetic cannabinoid agonists such as CP 55,940 (Mang *et al.*, 2001). Interestingly, anandamide increased both [^{3}H]ACh release and muscle tone in naive MPLM tissue, which could be antagonised by the VR_1 receptor antagonist capsazepine but not SR 141716A. NK_1 and NK_3 receptor antagonists were also able to inhibit the effect suggesting that anandamide may induce a release of TKs via VR_1 receptor stimulation, which in turn would release ACh through the stimulation of NK_1 and NK_3 receptors.

Consistent with the observations *in vitro*, *in vivo* studies have also confirmed the inhibitory actions of cannabinoids on gut motility. WIN 55,212-2 and Δ^9-THC have been shown to reduce gastric motility in mice and rats (Colombo *et al.*, 1998; Izzo *et al.*, 1999; Krowicki *et al.*, 1999). These effects could be reversed with SR 141716A, which alone stimulated gastric propulsion. The inhibition of gastric propulsion may involve CB_1 receptors located in central sites as Δ^9-THC-evoked inhibition of gastric motility, given by i.v. injection, could be mimicked by application of Δ^9-THC to the dorsal surface of the medulla (Krowicki *et al.*, 1999). A later study in rats found that SR 141716A given either orally or centrally (i.c.v. injection) inhibited the WIN 55,212-2-evoked (given i.c.v.) inhibition of gastric motility, whereas, when SR 141716A was administered centrally, it did not affect the gastrointestinal action of WIN 55,212-2, administered by intraperitoneal (i.p.) injection (Landi *et al.*, 2002). Izzo *et al.* (2000b) indicated an exclusively locally elicited intestinal effect of i.p. WIN 55,212-2 in mice, since this persisted in animals in the presence of the ganglionic blocker hexamethonium, which rendered i.c.v WIN 55,212-2 ineffective. Collectively the data suggests that cannabinoids primarily act at peripheral sites to inhibit gastric motility.

Intestinal motility may be tonically inhibited by the endogenous cannabinoid system. Indeed, 2-AG has been isolated from canine gut (Mechoulam *et al.*, 1995) while the rat small intestine contains high amounts of the metabolising enzyme FAAH (Katayama *et al.*, 1997). SR 141716A alone has been shown to potentiate electrically-evoked contractions of (Pertwee *et al.*, 1996a) and ACh release from (Coutts and Pertwee, 1997) the MPLM, but the inverse agonist properties of SR 141716A may detract from a constitutive activity of CB_1 receptors in this system. Compelling evidence for an endocannabinoid tone controlling propulsion comes from *in vivo*

studies in the mouse where anandamide and WIN 55,212-2 were shown to inhibit colonic motility in an SR 141716A-sensitive manner (Pinto *et al.*, 2002). This included the observations that high amounts of anandamide and 2-AG were present in the mouse colon, SR 141716A alone stimulated colonic motility and colonic propulsion could be inhibited by VDM11, an AMT inhibitor. However, addition of the FAAH inhibitor phenylmethylsulfonyl fluoride has no inhibitory effect on electrically-evoked contractions of the MPLM (Pertwee *et al.*, 1995), which suggests a lack of endocannabinoid tone in this preparation.

1.9.6 Neurotransmitter release in the myenteric plexus

The sequence of events underlying the process of synaptic transmission has long been established. Briefly, depolarisation of a neuron, arising from an increase in Na^+ permeability (action potential), initiates the opening of VOCCs. The subsequent influx of Ca^{2+} into the neuron triggers the fusion of neurotransmitter-containing vesicles with the synaptic membrane (at the active zone). VOCC's become inactivated by neuronal hyperpolarisation, arising principally from an efflux of K^+ ions. This rectification reinstates a 'resting' ionic balance across the neuronal membrane allowing the process to occur again.

An early study by Takahashi *et al.* (1992) examined the Ca^{2+} channels necessary for ACh release from isolated ganglia from the guinea-pig myenteric plexus. The nACh receptor agonist DMPP stimulated the release of [^{3}H]ACh, which was blocked by hexamethonium or significantly reduced by the N-type Ca^{2+} channel blocker ω-conotoxin. Later studies using electrically-evoked contractions of the guinea-pig ileum confirmed that activation of N-type Ca^{2+} channels was required to

mediate cholinergic neurotransmission, although only during low frequency field stimulation (Tran and Boot, 1997). This was a presynaptic event as ω-conotoxin did not affect responses to exogenously applied ACh. At higher frequencies a ω-conotoxin-resistant component remained that could not be blocked by atropine or adrenoceptor antagonists propranalol and prazosin. This non-adrenergic non-cholinergic (NANC) component could be inhibited by the P/Q-type Ca^{2+} channel blocker ω-agatoxin (Tran and Boot, 1997). These observations are quite interesting in the light of studies that have shown that higher levels of stimulation are required to evoke TK release from myenteric motor neurons (Bornstein *et al.*, 2004). Together these results may suggest that at lower stimulus frequencies N-type Ca^{2+} channels modulate synaptic release of ACh whereas at higher stimulus intensities TK release involves Ca^{2+} entry through P/Q-type channels. The release of other transmitters in the guinea-pig myenteric plexus has also been shown to rely on N- and P/Q-type Ca^{2+} channels including GABA (Reis *et al.*, 2002) and glutamate (Reis *et al.*, 2000).

Myenteric neurons have also been shown to express A-type K^+ (Ren *et al.*, 2001; Starodub and Wood, 2000) and K_{ir} (Zholos *et al.*, 1999; Ren *et al.*, 2001) channels, which are thought to regulate neuronal excitability in the myenteric plexus (Zholos *et al.*, 1999; Starodub and Wood, 2000).

1.9.7 Cannabinoid-mediated inhibition of neurotransmission in the myenteric plexus

As described in section 1.5.5, CB_1 receptor stimulation can inhibit VOCCs (including N-, and P/Q-type), as well as activate K^+ channels (including K_{ir} and A-type), which could be the mechanism by which cannabinoids inhibit neurotransmitter release in the myenteric plexus (Fig. 1.10). Indeed, the inhibitory actions of WIN

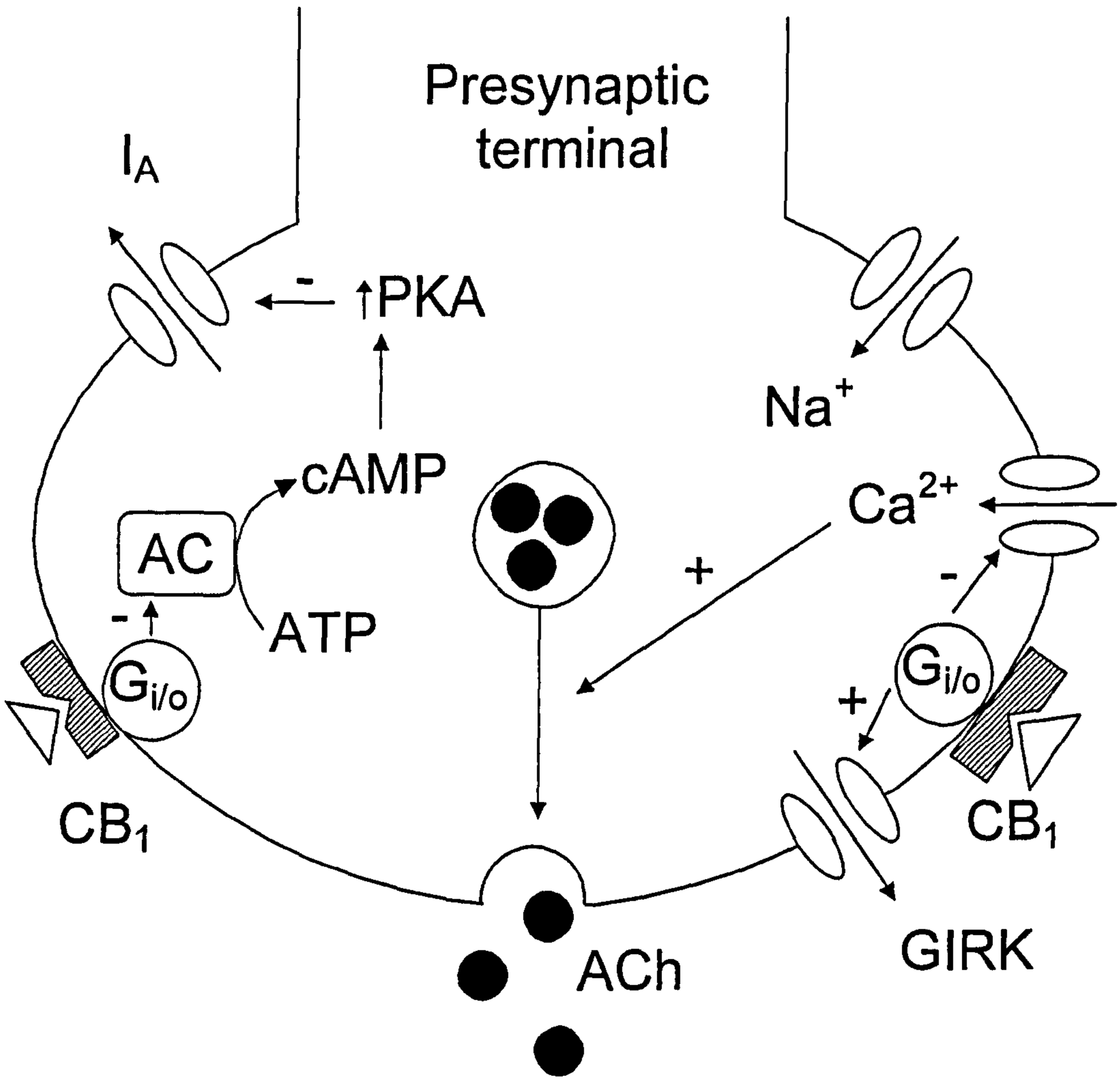


Figure 1.10 Possible mechanisms of cannabinoid-mediated inhibition of neurotransmitter release in myenteric neurons.
+ suggests positive coupling, - suggests an inhibitory action. CB_1 receptor stimulation may decrease neurotransmitter release by a direct inhibitory action of $G_{i/o}$ proteins on Ca^{2+} channels and/ or a direct activation of GIRK channels. The activation of I_A, through the inhibition of AC and subsequent PKA reduction, may serve as another possible mechanism by which cannabinoids inhibit neurotransmitter release.

55,212-2 on electrically-evoked contractions of the MPLM were attenuated by forskolin and augmented by reducing the extracellular Ca^{2+} (Coutts and Pertwee, 1998). This would be consistent with a cannabinoid-mediated inhibition of Ca^{2+} channels and possibly, through the inhibition of the cAMP/ PKA pathway, activation of I_A, although this has not been confirmed by any electrophysiological studies. An activation of GIRK channels may also contribute to the inhibitory effect of cannabinoids on neurotransmitter release.

Recently, anandamide and CP 55,940 have been shown to inhibit inward currents mediated by the activation of α7 nACh receptors in *Xenopus* oocytes (Oz *et al.*, 2003; Oz *et al.*, 2004). The inhibition involved a direct interaction of cannabinoids with the nACh receptors as the cannabinoid antagonists SR 141716A or SR 144528 and the $G_{i/o}$ inhibitor PTX could not reverse the effects. In addition, cannabinoid receptors were not even expressed in the experimental preparation. Although it has been shown that there are no functional α7 subunit-containing nACh receptors on myenteric neurons (Zhou *et al.*, 2002), the hypothesis that cannabinoids may inhibit excitatory neurotransmission in the myenteric plexus, either through the direct inhibition of nACh receptors or in a CB_1 receptor-dependent manner, is intriguing and definitely warrants further investigation.

1.10 Aims and rationale

It is clear that much work is still needed to elucidate the signalling pathways evoked by the activation of both cannabinoid receptor subtypes. Utilisation of diverse effector systems by CB receptors (especially CB_1) may explain how the response to cannabimetics varies across different types of cells. Understanding which physiological responses are mediated by these intracellular signalling systems is of great significance and may provide new grounds for the design of selective cannabimetic agents.

The initial aim of the study is to further establish the intracellular signalling pathways evoked by cannabinoid CB_1 receptor stimulation in smooth muscle cells, in particular, elucidating the intracellular pathways that evoke an increase in $[Ca^{2+}]_i$. Secondly, the present study aims to identify the signalling events that might mediate a cannabinoid-induced inhibition of neurotransmission in the myenteric plexus. Specifically in:-

a) DDT_1 MF-2 smooth muscle cells

- Explore the possibility that CB_1 receptor activation evokes CCE.
- Establish a signalling link between MAP kinase activation and increasing $[Ca^{2+}]_i$, possibly through the generation of AA and hence NCCE.
- Determine the subtype of MAP kinase phosphorylated and a time-course of its activation.
    - Establish a CB_1 receptor-dependent activation of p42/44 MAP kinase.

b) Cultured myenteric neurons

- Validate guinea-pig myenteric neurons in primary culture as a model for those *in situ*.

- Establish the presence of cannabinoid CB_1 receptors on cultured myenteric neurons.

- Ascertain the predominant neuronal type present in culture (e.g. cholinergic) and its expression of CB_1 receptors.

- Determine the signalling events evoked by CB_1 receptor stimulation that could ultimately lead to an inhibition of neurotransmitter release.

    - Establish the effects of cannabinoids on evoked Ca^{2+} currents in cultured neurons.

    - Ascertain if cannabinoids can activate K^+ channels e.g. K_{ir}.

- Determine if cannabinoids can inhibit excitatory neurotransmission via a modulation of nACh receptor signalling.

    - Investigate the effect of cannabinoids on inward currents evoked by nACh receptor activation.

The data obtained from the smooth muscle cell line will provide a greater understanding of the cellular mechanisms by which cannabinoids evoke an increase in $[Ca^{2+}]_i$. This may help to elucidate the cellular actions of cannabinoids in other preparations, where they have also been shown to evoke an increase in $[Ca^{2+}]_i$ i.e. vascular endothelial cells and kidney cells (Fimiani *et al.*, 1999; Chou *et al.*, 2001). In addition, further insight into the possible postsynaptic actions of cannabinoids may be gained. Results acquired from the neuronal cultures will help support existing ideas on how cannabinoids modulate synaptic transmission in both the central and peripheral nervous system i.e. modulation of Ca^{2+} and K^+ conductance. Evidence for effects of cannabinoids on nACh receptor function would identify a novel mechanism through which cannabinoids modulate excitatory neurotransmission in the myenteric plexus and thus gut motility.

METHODS

2.1 Culture and patch clamping of DDT_1 MF-2 cells

2.1.1 Cell culture

DDT_1 MF-2 smooth muscle cells were cultured in monolayers at 37°C (25 cm^2 culturing surface) in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal-calf serum (FCS), penicillin (50 µg/ ml), streptomycin (50 µg/ ml) and L-glutamine (2 mM). They were then kept in an atmosphere of 5% CO_2/ 95% O_2, where they were grown to confluency. At this point the cells were harvested, by means of a plastic cell scraper, and plated onto glass cover slips, housed in 9.6 cm^2 6-well plates. All culture media and supplements were obtained from Gibco (UK).

2.1.2 Patch clamp technique: voltage clamp and current clamp

The patch clamp technique was first described by Neher and Sakmann (1976) and involves the formation of a very high resistance seal (gigaseal, ~10^9 ohms) between a recording micropipette and the membrane of a cell under investigation. These gigaseals are mechanically very stable, even when the area of enclosed membrane is disrupted and broken. Under this 'whole-cell' patch configuration, voltage clamp and current clamp recordings can be performed (see section 2.1.5).

Voltage clamp is a method of maintaining the voltage inside a cell (the membrane potential) at a constant value, while at the same time measuring the membrane current (generated by ion flux through open channels). The technique employs a negative feedback system where the membrane potential is measured and compared with a potential set by the experimenter (holding potential). Any variation

in the measured potential from that of the holding potential is instantly corrected for by an injection of current. This current is proportional (but opposite in polarity) to the membrane current of the patched cell. The technique allows the activity of ion channels to be studied at a constant membrane potential, enabling their properties to be more easily quantified. In addition, it also permits the study of biochemical processes that are taking place within a cell, which are able to modulate the activity of ion channels (e.g. second messenger modulation).

In current clamp, cells are injected with a fixed amount of current and the membrane potential is recorded. Therefore no negative feedback system is required. The recording of action potentials is one example where current clamp is frequently used.

Both techniques also have the added advantage that the pipette filling solution equilibrates with the cytosol of the cell, allowing the experimenter to have control over both the intracellular and extracellular medium.

2.1.3 Micropipette preparation

Borosilicate glass capillaries (Harvard Apparatus; GC150TF-10), containing an internal filament (to ease filling), were heated and pulled (P80/ PC; Sutter Instrument Co., U.S.A.) to produce the micropipettes. They were then heat-polished (MF-830; Narishige, Japan) and backfilled with intracellular solution (ICS; see section 2.1.7 for composition), to give a typical resistance of 2-6 MΩ.

2.1.4 Set-up

Figure 2.1 depicts the patch clamp set-up used for making electrophysiological recordings. In brief, cells were placed in the bath contained in the centre of a perspex block, which in turn was positioned on the microscope table. An inverted microscope (TE 200; Nikon, UK) was used to visualise the cells under high power. The bath was initially filled with a physiological salt solution (ECS; extracellular salt solution, see section 2.1.7 for composition), by means of a gravity-driven superfusion system. Solutions were siphoned out (gravity-assisted) through a metal syringe needle located towards the rear of the perspex bath.

Filled pipettes were mounted into the pipette holder, which was in turn connected to the pre-amplifier (probe). A chloride coated silver wire connected the pipette filling fluid to the probe input. The probe itself was held tightly in a course manipulator (MC35A; Narishige, Japan), permitting movement in the x, y and z direction, while finer movement was achieved using the hydraulic micromanipulator (MHW-3; Narishige, Japan). A tube, also attached to the pipette holder, enabled the internal pressure of the pipette to be changed. Current output was initially amplified (Axopatch 1D; Axon Instruments, U.S.A.) and recorded using a digital interface (Digidata 1200; Axon Instruments, U.S.A.). Changes in membrane current could then be viewed in real-time on a personal computer incorporating the pCLAMP 6 software (Axon Instruments, U.S.A.).

2.1.5 Gigaseal formation

Before patching commenced a positive pressure (of about 4-5 cm of mercury) was applied to the pipette to blow away possible contaminations that could block the

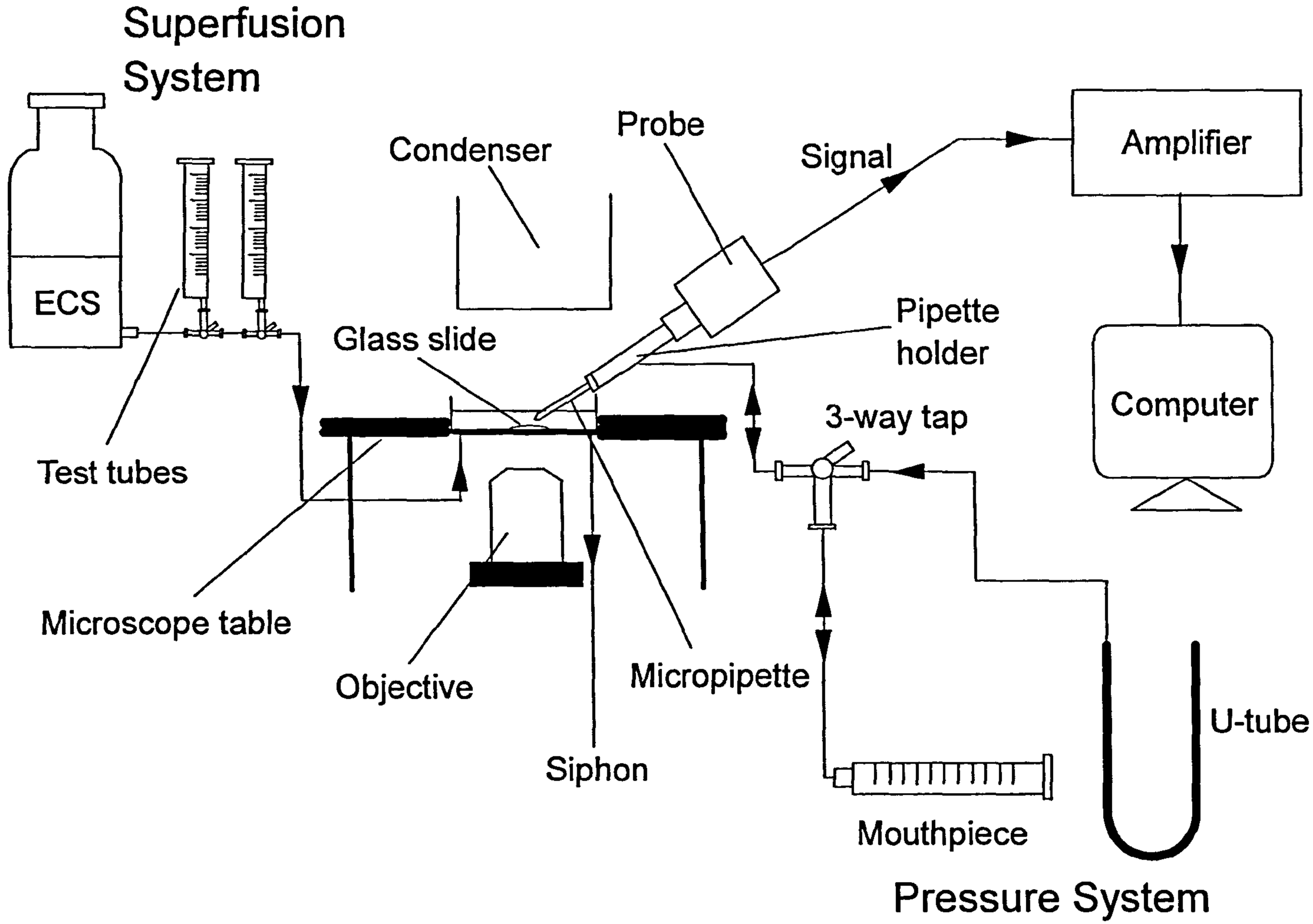


Figure 2.1 Schematic diagram of the patch clamp apparatus.

tip. Monitoring of the pipette approach towards the cell was performed by the application of an electrical pulse (5 mV, 5 ms, 100 Hz) to measure pipette resistance. The approach could also be monitored visually by focusing the microscope above the cells and using the shadow of the pipette as a guide.

The current response to the test pulse indicated the different stages in gigaseal formation (Fig. 2.2). As the pipette entered the bathing fluid a circuit was completed and the pipette resistance decreased. Contact with the cell caused the resistance to increase therefore decreasing the current response. At this point the positive pressure was removed and gentle, continuous suction was applied to form the gigaseal. The next steps were then performed under voltage clamp. Suction pulses were required to disrupt the membrane, under the patched micropipette, enabling whole cell recording. At this stage, both the analysis of resting leak current and compensation (and therefore measurement) of cell capacitance was possible.

2.1.6 Current recording

Experiments were performed at room temperature with cells initially under voltage clamp kept at a holding potential of –60 mV, which is close to the resting membrane potential for these cells (Molleman *et al.*, 1989). A voltage step protocol was used to record currents mediated by the activation of voltage-dependent ion channels present in the preparation. Membrane currents were evoked by stepping the membrane potential from –60 mV to +90 mV, in 10 mV increments.

Membrane currents, in response to drug application, were measured at a holding potential of –30 mV and are expressed as pA per pF membrane capacitance to correct for cell surface area.

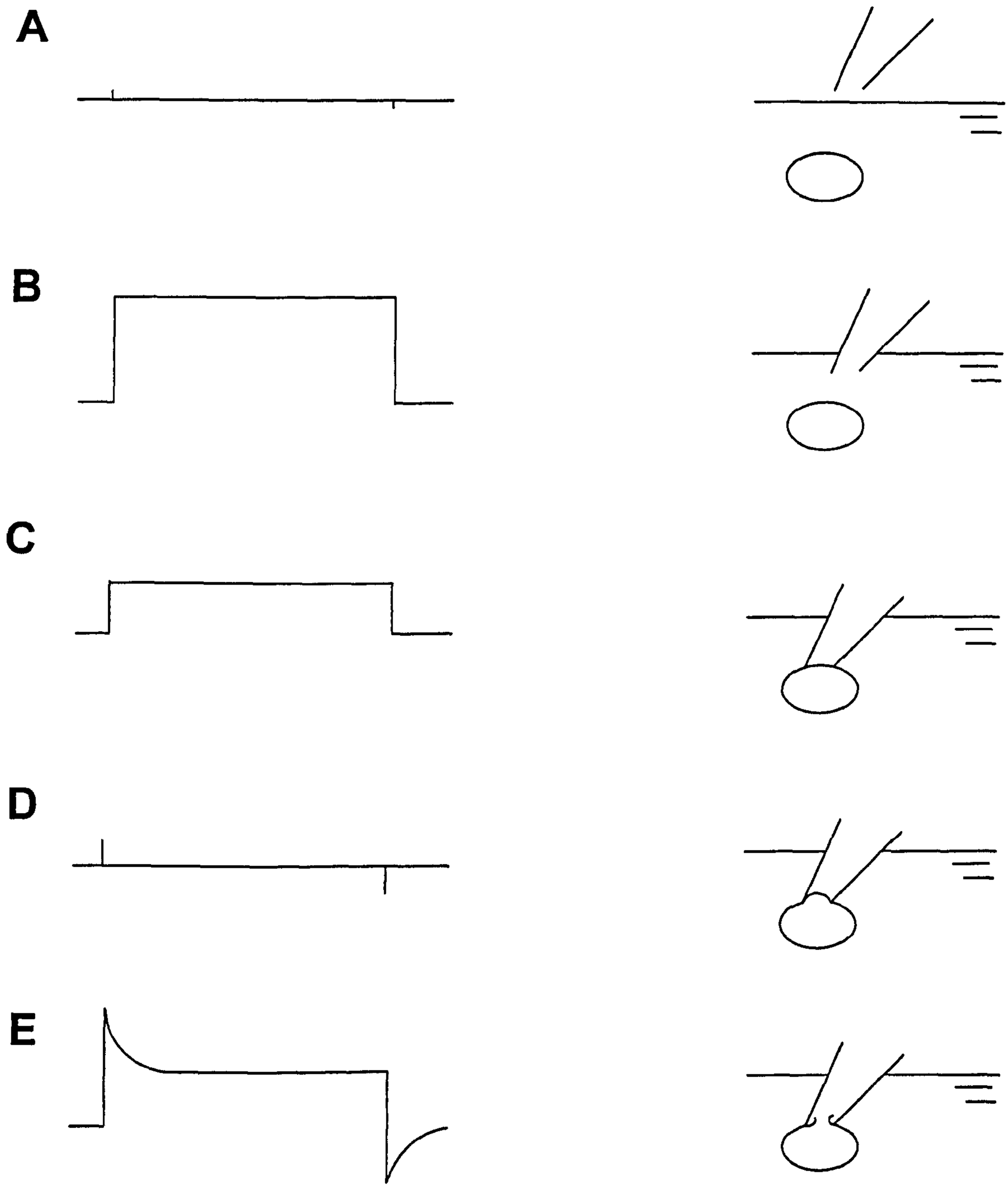


Figure 2.2 Current responses during gigaseal formation.
There is no circuit when the pipette is out of the bathing fluid (**A**). Only when the pipette comes into contact with the bathing fluid, is the circuit completed and current is allowed to flow. The current response is inversely proportional to the pipette resistance (**B**). On contact with the cell, pipette resistance increases, decreasing the current response (**C**). After gigaseal formation, a current response can no longer be visualised (**D**). After successful disruption of the membrane, a large capacitive transient is obtained (RC current) and whole cell recording can begin (**E**).

(Adapted from Molleman, 2003)

2.1.7 Solutions

Electrophysiological measurements were carried out with an ECS containing (mM): NaCl, 125; KCl, 6; $MgCl_2$, 2.5; NaH_2PO_4, 1.2; Hepes, 20; glucose, 11; sucrose, 67; $CaCl_2$, 1.2; pH to 7.4 with NaOH. The ICS contained (mM): NaCl, 5; KCl, 142; $MgCl_2$, 1.2; Hepes, 20; glucose, 11; K-ATP, 5; Na-GTP, 0.1; pH to 7.2 with KOH. All drugs were administered in the ECS, superfused at a rate of 4 ml/ min. Post experimentation the apparatus was washed with dilute hydrochloric acid (0.1 M), absolute ethanol (Fischer, UK) and distilled water to ensure complete washout of residual cannabinoids.

2.2 [^{3}H]Arachidonic acid (AA) release in DDT_1 MF-2 cells

[^{3}H]AA release measurements were carried out in collaboration with the University of Groningen (Netherlands), where all the experimental work was undertaken.

AA release was measured as described previously (Van der Zee *et al.*, 1995). Cells were grown to confluency in 9.6 cm^2 six well plates and labelled with 0.25 μCi of [^{3}H]AA (Sigma, NL) in serum-free DMEM (1 ml), for 3 hours at 37°C. Serum-free DMEM was used as FCS can bind fatty acids, which would significantly reduce the amount of [^{3}H]AA able to diffuse into the cells. Cells were washed once in ECS, twice with ECS containing 1% bovine serum albumin (BSA) (fatty-acid free) and once again with ECS to eliminate any unincorporated radioactivity. They were then allowed to equilibrate for 15 minutes. Inhibitors of the signalling pathway under investigation could then be added at the start of this equilibration period. Following

this the cells were incubated with CP 55,940 for 5 minutes. The experiment ended when the medium was removed and [^{3}H]AA release was determined by liquid-scintillation counting. The effect of the inhibitors alone on AA efflux was also established.

2.3 Intracellular Ca^{2+} measurements in DDT_1 MF-2 cells

$[Ca^{2+}]_i$ measurements were also carried out in collaboration with the University of Groningen (Netherlands), where all experimental work took place.

$[Ca^{2+}]_i$ was measured using fura-2 fluorometry as reported previously (Molleman *et al.*, 1991a). Cells in a monolayer were harvested using a cell scraper and loaded in suspension with 3 μM fura-2 acetoxymethylester at 22°C for 45 minutes in the dark. Fluorescence was measured at 37°C. For measurements made to study CP 55,940 the area under the curve (AUC) was calculated starting at the point of drug application (t=0) to t=150 seconds. A dose-response curve for CP 55,940 was then constructed from the mean of these results.

2.4 Western blot analysis

2.4.1 p42/44 MAP kinase time course

Western blot analysis was used to establish if CP 55,940 induces the phosphorylation of p42/44 MAP kinase in DDT_1 MF-2 cells.

DDT_1 MF-2 cells were grown to confluency in P-60 dishes (Falcon, UK), on DMEM supplemented with 10% FCS, penicillin (50 μg/ ml), streptomycin

(50 μg/ ml) and L-glutamine (2 mM). The cells were then incubated in DMEM (0.1% FCS) for at least 12 hours to ensure minimal activation of MAP kinases in the cells.

Cells were activated with CP 55,940 (10 μM) at time points ranging between 30 seconds and 30 minutes. In experiments involving inhibition with SR 141716A (1 μM), cells were pretreated with the antagonist 15 minutes prior to CP 55,940 activation. Control experiments were performed with vehicle (0.1% ethanol) at the corresponding time points.

After activation, the medium was immediately removed and the cells washed twice with 200 μl of ice-cold phosphate buffered saline (PBS), containing 100 μM sodium orthovanadate to inhibit phosphatases. 300 μl of boiling lysis buffer was then added to the dish, making sure to cover the entire surface. The lysis buffer contained 10% glycerol (Fischer, UK), 2% sodium dodecyl sulphate (SDS), 76.5 mM Tris (Fischer, UK) and 1 mM sodium orthovanadate. The cell lysate was transferred to an Eppendorf tube, heated to 95°C for 5 minutes and then stored at –20°C until required.

2.4.2 Determination of the protein content of cell lysates

Protein content was determined using a bicinchoninic acid (BCA) protein assay. The lysates were defrosted and boiled at 95°C for 5 minutes, before being centrifuged for 3 minutes at 10,000 rpm. Triplicates of each sample (5 μl) were added to the inner wells of a 96-well plate, followed by 10 μl of ultra pure water (BDH, UK) to keep volumes constant. To the outer wells, 10 μl of BSA standards (1-30 μg/ well) were added, again in triplicate. 5 μl of lysis buffer was subsequently added to each standard to ensure all wells were comparable. Addition of 10 μl ultra pure water with 5 μl lysis buffer generated a blank. Finally, 100 μl of BCA reagent (Pierce, UK) was

added to each well, and incubated for 45 minutes at room temperature. Absorbency of the assay solutions was measured at 590 nm (Labsystems Multiskan RC) and the total protein content for each sample calculated using the standard curve. The sample volume required to load 20 μg of protein was then calculated.

2.4.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was used to analyse the sample lysates, using the MINI-Protein II gel apparatus (BioRad). All apparatus was initially cleaned with industrial methylated spirits (IMS, 70%) (Fischer, UK) before being used. A 10% resolving gel was prepared and poured between the glass plates of the gel apparatus, approximately 2/3rds of the way up the glass. Butan-2-ol (Fischer, UK) was applied over the top of the gel to prevent the inhibition of polymerization by air. The gel was left to polymerise at room temperature for 45 minutes. The alcohol was removed and the stacking gel poured over the resolving gel. Insertion of a 0.75 mm comb into the top of the stacking gel created the loading wells. A further 45 minutes was required for the stacking gel to polymerize.

10% Resolving Gel (25 ml)

Ultra pure water	9.90 ml
30% Acrylamide/ bisacrylamide	8.35 ml
1.5 M Tris-HCl (pH 8.8)	6.25 ml
10% SDS	0.25 ml
10% Ammonium persulfate (BDH, UK)	0.25 ml
TEMED (BDH, UK)	15.0 μl

Stacking Gel (20 ml)

Ultra pure water	12.20 ml
30% Acrylamide/ bisacrylamide	2.60 ml
0.5 M Tris-HCl (pH 6.8)	5.00 ml
10% SDS	0.20 ml
10% Ammonium persulfate	0.10 ml
TEMED	20.0 µl

Sample lysates were initially sonicated for 15 seconds to reduce their viscosity. Equal volumes of lysate and loading buffer were added together in a separate Eppendorf and boiled at 95°C for 5 minutes, before being spun at 10,000 rpm for 3 minutes. An SDS molecular weight marker (26 to 180 kDa) was also boiled for 5 minutes at 95°C. The marker (10 µl) was loaded into the first lane followed by the prepared samples in subsequent lanes. Lanes that contained neither marker nor sample were filled with loading buffer (10 µl). The gel apparatus was transferred to an electrophoresis tank, filled with tank buffer (National Diagnostics, UK) and then run at 200 V until the dye was seen to reach the foot of the resolving gel (~45 minutes).

Loading Buffer (1ml)

Ultra pure water	180 µl
0.5 M Tris-HCl (pH 6.8)	180 µl
10% SDS	400 µl
Glycerol	200 µl
2% Bromophenol blue	10 µl
β-Mercaptoethanol (BDH, UK)	20 µl

2.4.4 Immunoblotting of proteins

The gel was removed from the tank and the resolving gel separated from the stacking gel. The resolving gel was incubated for 10 minutes in transfer buffer. Filter

paper was cut to match the size of the gel and placed in transfer buffer. Polyvinylidene diflouride (PVDF) membrane (Amersham, UK) was cut to match the size of the gel, dipped in absolute methanol (Fischer, UK) for 10 seconds, washed with Milli-Q water and also placed in transfer buffer.

Transfer Buffer (1000 ml)

39 mM Glycine	2.93 g
48 mM Tris	5.82 g
20% Methanol	200 ml

The base of a Trans-Blot Semi-Dry electrophoretic cell (BioRad) was initially dampened with transfer buffer. A piece of filter paper was placed in the middle, followed by the PVDF membrane, then the gel, and finally the second piece of filter paper. Protein transfer, from gel to membrane, proceeded at 0.8 Amps/ cm^2 for 90 minutes. The membrane was removed from the transfer cell and immediately placed into blocking buffer (wash buffer supplemented with 3% BSA), where it was agitated at room temperature for 3 hours.

Wash Buffer (1000 ml)

10 mM Tris	1.21 g
100 mM NaCl	5.84 g
0.1% Tween 20	1.00 ml

After blocking the membrane was incubated in anti-phospho p42/ p44 polyclonal antibody (New England Biolabs, UK) at a dilution of 1:1000 in blocking buffer. It was then agitated overnight at room temperature. Following primary antibody incubation, the membrane was washed in wash buffer for 30 minutes, with

replacement of the buffer every 5 minutes. The membrane was then incubated with secondary antibody (goat anti-rabbit-HRP), diluted to 1:10,000 in blocking buffer, for 1 hour at room temperature on a rotating plate. Washing was then repeated (as above).

2.4.5 Film development

The membrane was placed onto cling-film, while equal volumes (3 ml) of enhanced chemo-luminescence (ECL) detection solutions A and B (Amersham, UK) were mixed and added to the protein side of the membrane. It was left for 1 minute, and the excess removed. The cling-film was then folded over, enveloping the membrane, making sure not to trap air bubbles. The membrane was exposed to autoradiography film (Hyperfilm-ECL from Amersham, UK) for 30 seconds to 10 minutes, followed by incubation in Dektol developer (Kodak, UK) until bands were visualized. At this point the film was washed in water (1-2 minutes), then placed in Unifix fixer (Kodak, UK) before being washed again in water (1-2 minutes).

2.4.6 Scanning densitometry

Densitometry was used to quantify changes in band intensity and thus p42/44 MAP kinase phosphorylation. Using the 'Genesnap' programme, western blots were visualised using a transilluminator and the images saved onto a personal computer. Captured images were then transferred to the 'Genetools' programme in order to measure band absorbance. Lanes were determined manually. Absorbance was determined by placing a border around the control band (at t=0) and then reading the

value obtained. Values from subsequent bands were determined as a % change in absorbance, compared to the control band.

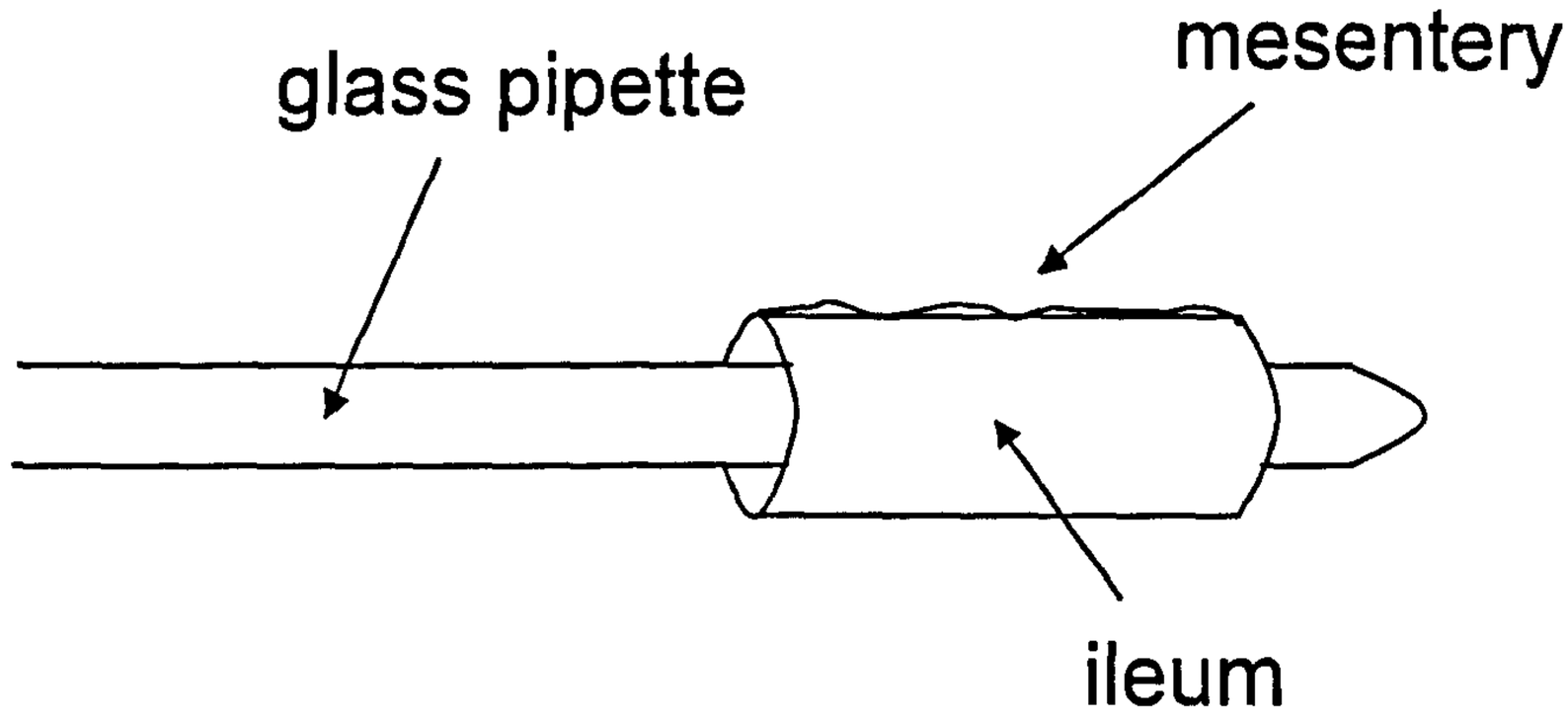
All materials were obtained from Sigma (UK) unless otherwise stated.

2.5 Culture and patch clamping of myenteric plexus neurons

2.5.1 Preparation of the myenteric plexus longitudinal muscle (MPLM)

Dunkin Hartley guinea-pigs (450-550 g), of either sex, were sacrificed by cervical dislocation. The abdomen was cut open and the ileum removed by gently pulling away from the mesentery. Care was taken not to stretch the tissue. The tissue was immediately placed in Krebs solution, continuously gassed with 95% O_2/ 5% CO_2. Krebs solution contained (mM): NaCl, 118.3; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25; Glucose, 11.1; $CaCl_2$, 2.5. A 4 cm strip of ileum was stretched onto a glass pipette and immediately moistened with Krebs solution. The longitudinal strips were obtained by first lining up the mesentery along the length of the pipette and stroking tangentially away from it with a cotton wool bud, which had been soaked in Krebs solution (Fig. 2.3). This continued until a strip of longitudinal muscle along with the myenteric plexus was separated from the underlying circular muscle and mucosa. During the procedure, MPLM and ileum were kept moist in Krebs solution.

A Side view



B Top view

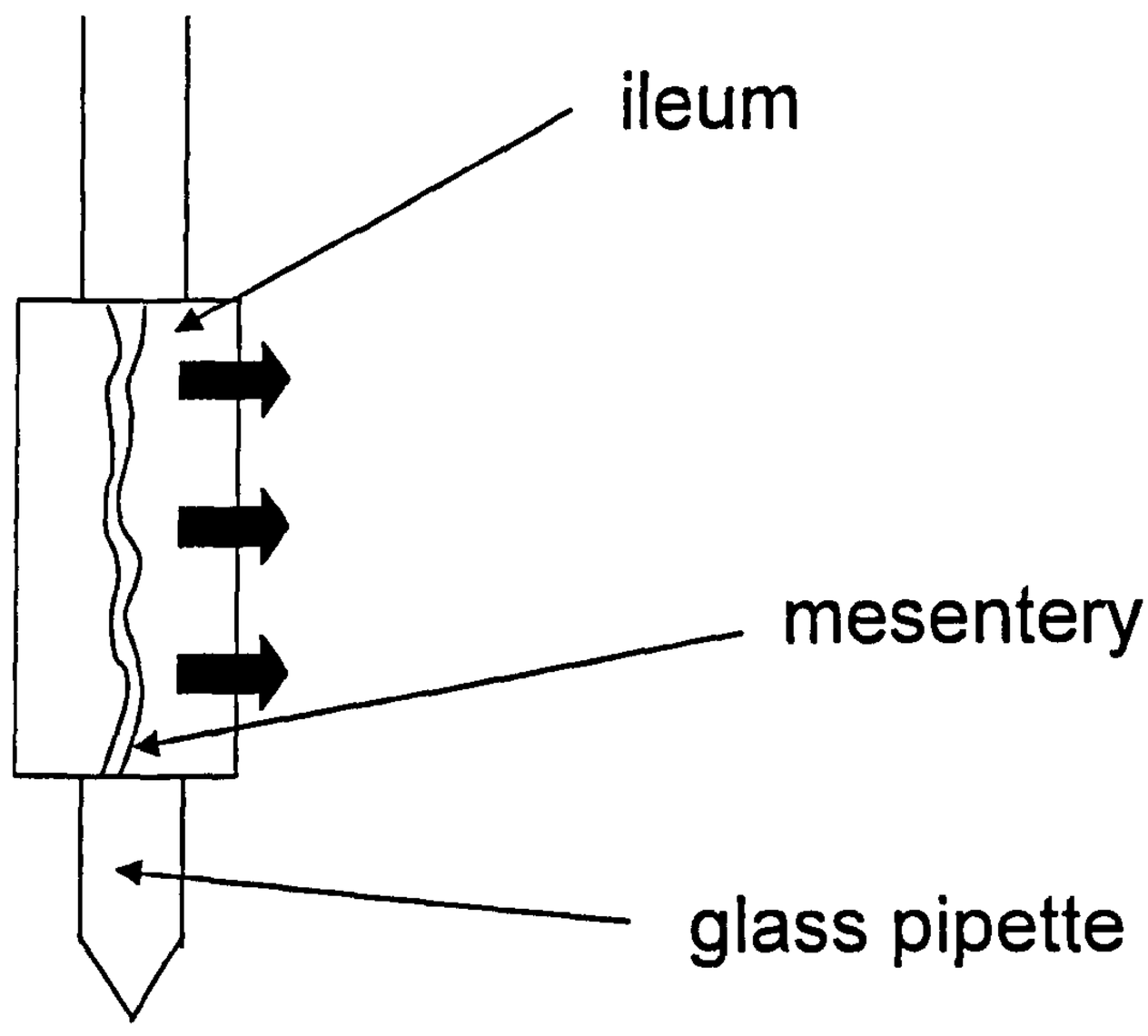


Figure 2.3 Preparation of the MPLM.
Ileum was stretched onto a glass pipette and moistened with Krebs solution (**A**), with the mesentery lined up along the length of the rod (**B**). The longitudinal strips were obtained by stroking tangentially away from the mesentery with a cotton wool bud, in the direction of the arrows (**B**). Eventually a strip of longitudinal muscle, along with the myenteric plexus was separated from the circular muscle and mucosa.

2.5.2 Preparation of cultured myenteric neurons

A 4cm strip of MPLM was cut into 0.5 cm sections and transferred to a sterile 15 cm^3 centrifuge tube, containing 3 ml of papain solution. The tube was placed in a waterbath (37°C) for 10 minutes, removed and the papain solution replaced by 3 ml of collagenase solution. It was then returned to the waterbath for a further 10 minutes. The tissue was triturated with a sterile, flame-polished tip pasteur pipette, and then placed back into the waterbath. The trituration process was repeated for a further two times before the cells were centrifuged for 5 minutes at 600 rpm, the supernatant discarded, and the cells resuspended in feeding medium. Centrifugation was repeated, the medium replaced and the cells resuspended. The cell suspension was transferred to individual P-60 dishes, housing collagen (Sigma, UK)-covered glass slides. Cultured neurons were incubated at 37°C in an atmosphere of 95% O_2/ 5% CO_2. The feeding medium was refreshed every 3 days.

Papain Solution (warmed to 37°C in a waterbath before use)

Hank's Balanced Salt Solution (Sigma, UK)	9 ml
pH balanced (7.4) with 0.15 $NaHCO_3$	
L-cysteine (Sigma, UK)	3.6 mg
Papain (Roche, UK)	0.1 ml

Collagenase Solution

Hank's Balanced Salt Solution	9 ml
Collagenase Type I (Sigma, UK)	9 mg
Dipase (Roche, UK)	28 mg

Feeding Medium

DMEM	189 ml
Antimitotic stock (see below)	3 ml
Antibiotic stock (see below)	3 ml
FCS	5 ml

Antimitotic Stock (3 ml aliquots, stored at –20°C)

Ultra pure water	300 ml
Cytosine β-D-arabinofuranoside hydrochloride (Sigma, UK)	24 mg
(+)-5-fluorodeoxyuridine (Sigma, UK)	246 mg
Uridine (Sigma, UK)	12 mg

Antibiotic stock (3 ml aliquots, stored at –20°C)

Ultra pure water	60 ml
L-glutamine (Sigma, UK)	584 mg
Penicillin	3 mg
Streptomycin	3 mg
Glucose	6 g

2.5.3 Whole-cell recordings from myenteric neurons

Cultures were left to attach for at least 3 days before patching commenced and recordings were taken. Cells were patched and drugs administered in the same manner as described for DDT_1 MF-2 cells, using the same composition of ECS and ICS unless otherwise stated (see section 2.1). Under voltage clamp myenteric neurons were kept at a holding potential of –60 mV, while under current clamp enough current was injected to keep the membrane potential close to –80 mV.

2.5.3.1 Isolation of voltage-dependent Ca^{2+} currents

Voltage-dependent Ca^{2+} currents were recorded using ECS containing (mM): NaCl, 115; KCl, 6; $MgCl_2$, 2.5; NaH_2PO_4, 1.2; TTX, 0.003; TEA, 10; Hepes, 20; glucose, 11; sucrose, 67; $CaCl_2$, 2.5; pH to 7.4 with NaOH. The ICS contained (mM): CsCl, 142; $MgCl_2$, 1.2; Hepes, 20; EGTA, 0.1; glucose, 11; K-ATP, 5; Na-GTP, 0.1; pH to 7.2 with NaOH. Tetrodotoxin (TTX) and tetraethylammonium (TEA) were

used in the ECS to block voltage-dependent Na^+ currents and K^+ currents respectively, unmasking and therefore enabling voltage-dependent Ca^{2+} currents to be recorded. Cs^+, also used to block K^+ channels, replaced K^+ in the ICS. EGTA, a Ca^{2+} chelator, was added to the ICS to 'mop up' any Ca^{2+} ions within the pipette solution attributable to contaminations within the salts and distilled water. This decreases the $[Ca^{2+}]_i$ (compared to using ICS without EGTA), which results in an increased Ca^{2+} concentration gradient between the intracellular and extracellular space. Therefore the driving force of Ca^{2+} into the cell is increased, thus potentiating evoked Ca^{2+} currents. Raising the $CaCl_2$ concentration in the ECS from 1.2 mM to 2.5 mM also increased the driving force of Ca^{2+} into the cell.

Stepping the membrane potential from –60 mV to +50 mV, in 10 mV increments, evoked inward Ca^{2+} currents. Positive/ negative (P/N) leak subtraction was used when recording Ca^{2+} currents. This technique removes the passive components (attributable to capacitive transients and responses relating to leak resistance and membrane resistance) normally included within voltage step data, thus producing a 'cleaner' trace of active currents. 4 sub steps (opposite in polarity and ¼ of each subsequent voltage step) were applied from a holding potential of –60 mV and their cumulative current response added to the current response obtained by the subsequent voltage increment.

2.5.3.2 Peak positive and negative membrane currents

Membrane currents were evoked in myenteric neurons using a voltage step protocol. Cells were stepped from –100 mV to +50 mV, in 10 mV increments and the effect of CP 55,940 on these evoked currents was then established. Membrane

currents were recorded every 30 seconds for a period of 5 minutes, in the presence of CP 55,940. At each 30 second interval the peak negative current was taken as the most negative value during a single step protocol (i.e. –100 mV to +50 mV) and the peak positive current was taken as the current produced during a +50 mV step. Membrane currents recorded in the presence of CP 55,940 were then compared to currents recorded in the absence of cannabinoids. Each cell acted as its own control.

2.5.3.3 Ramp protocol

A ramp protocol was used to see if CP 55,940 activates K_{ir} channels in myenteric neurons. During a ramp recording the membrane potential was stepped to –120 mV for 200 ms and then increased to –50 mV over 800 ms. Ramps were taken at 30 second intervals for a period of 5 minutes, in the presence of CP 55,940. The ramp response under control conditions (no cannabinoid) was subtracted from the ramp response in the presence of CP 55,940. The data was then plotted as a current-voltage relationship.

2.5.3.4 Current clamp

Action potentials were recorded from myenteric neurons under current clamp. Action potentials were evoked by 6 consecutive current steps. To allow comparisons to be made between cells, current was adjusted to produce the same (passive) deflection in membrane voltage in each cell. The amount of current injected to produce this voltage deflection was calculated using Ohms law, due to the variation in membrane resistance between cells:-

$$I \text{ (current)} = V \text{ (voltage)} / R \text{ (resistance)}$$

Ohms law was used to work out the deflection in membrane voltage in the first cell, produced by current injection. This voltage deflection was then used as the standard for subsequent cells:-

$$V \text{ (membrane voltage)} = I \text{ (injected current)} \times R \text{ (membrane resistance)}$$

As the amount of current injected was constant $V \propto R$, where an increase in membrane resistance will produce a greater deflection in membrane voltage. Therefore the amount of current that was injected into subsequent cells (to produce the same voltage deflection as the first cell) was calculated using:-

$$I_{NC} = I_{C1} \times (R_{C1} / R_{NC})$$

I_{NC} = current to be injected into the new cell
I_{C1} = current injected (per step) into the 1st cell
R_{C1} = membrane resistance of the 1st cell
R_{NC} = membrane resistance of the new cell

Before recording cells were kept close to a membrane potential of –80 mV by constant current injection.

2.5.4 Single channel recordings

Single channel recording can be used to study the characteristics of individual ion channels in greater detail. The technique can also be used to investigate the direct

effects of ligands on ion channels, independent of intracellular signaling pathways induced by the activation of other cellular receptors.

Single channel recordings in myenteric neurons were obtained in the 'outside-out' patch configuration, using low tip resistance pipettes (2-6 MΩ). The configuration was obtained by pulling away the patch pipette from the cell membrane in the 'whole-cell' configuration, eventually breaking the membrane. The membrane then folds back on itself to form a gigaseal, with the extracellular surface still in contact with the ECS. The activation of individual ion channels located within this patch of membrane can then be recorded.

Currents were amplified (×100 gain) using the Axopatch-1D amplifier, as described earlier for whole-cell recordings in DDT_1 MF-2 cells (see section 2.1). The composition of the ECS and ICS was also identical. Currents were sampled at 2 kHz and filtered at 1 kHz. Measurements were carried out at a holding potential of –70 mV.

2.6 Immunohistochemical studies of cultured myenteric neurons

Cells were fixed and sent to Dr. Angela Coutts, at the University of Aberdeen, for immunohistochemical study. The following briefly describes the methods used.

2.6.1 Fixation

Cultured cells were fixed in 4% paraformaldehyde (Sigma, UK) for 10 minutes at room temperature then washed with PBS (pH 7.4). Cells were kept in PBS at 4°C until required for labelling.

2.6.2 Permeabilisation

Fixed cells were permeabilised in 0.1% Triton X-100 (Sigma, UK) for 5 minutes at room temperature then washed once with PBS. Non-specific binding sites were blocked by incubation of cultured cells with 25% donkey serum (Scottish Antibody Production Unit, Scotland) in PBS, for 20 minutes at room temperature. A blocking serum is normally used from the species which is the host for the secondary antibody. As donkey anti-rabbit Cy3 was used as the secondary antibody for CB_1 receptor detection, donkey serum was used to block non-specific binding sites.

2.6.3 Cannabinoid CB_1 receptor labelling

Cells were incubated overnight at 4°C with a polyclonal antibody, raised in the rabbit against the C-terminus (401-473) of rat CB_1 receptor. The antibody was obtained from Dr. Ken Mackie (Univ. of Washington, Seattle, Washington, U.S.A.). The stock solution concentration was 1 mg/ml and the dilution was 1:500 in glucose-free PBS. The primary antibody was removed, the cells washed three times with PBS then incubated at room temperature with Cy3-conjugated donkey anti-rabbit (1:250) (Jackson Immunoresearch Laboratories, U.S.A.) for 90 minutes. Finally, cells were washed three times with PBS, mounted on microscope slides in Vectashield and sealed with nail varnish. Cells were either visualised in glucose-free PBS in plastic culture dishes before mounting, or through water immersion on the slide.

2.6.4 Dual labelling with cannabinoid CB_1 receptor antibody and ChAT antibody

ChAT polyclonal antibody, raised in sheep (Chemicon International, U.S.A.), was used to identify cholinergic neurons. Fixed and permeabilised cells were incubated overnight at 4°C with a mixture of ChAT antibody (1:500) and C-terminus CB_1 receptor antibody (1:500). After incubation, the primary antibody was removed, the cells washed three times with PBS, then incubated at room temperature with a mixture of Cy3-conjugated donkey anti-rabbit (1:250) and Alexa 488-conjugated donkey anti-sheep (Molecular Probes, NL) for 90 minutes. Finally, cells were washed and mounted on slides as above.

2.6.5 Dual labelling with cannabinoid CB_1 receptor antibody and neurofilament antibody (NFP-200)

Fixed and permeabilised cells were incubated overnight at 4°C with a mixture of NFP-200 antibody (1:500) (Sigma, UK) and C-terminus CB_1 receptor antibody (1:500). After incubation, the primary antibody was removed, the cells washed three times with PBS then incubated at room temperature with a mixture of Cy3-conjugated donkey anti-rabbit (1:250) and Alexa 488-conjugated goat anti-mouse (Molecular Probes, NL) for 90 minutes. Finally, cells were washed and mounted on slides as above.

2.6.6 Control experiments

2.6.6.1 Non-specific labelling with secondary antibodies

Fixed and permeabilised cells were incubated overnight at 4°C with PBS only, omitting the primary antibody. Labelling with the secondary antibodies was carried out as outlined above. Cells were washed and mounted on slides for visualisation.

2.6.6.2 Blocking of staining with the appropriate blocking peptide

To check for specific staining with the cannabinoid CB_1 receptor antibody, blocking peptide (100 mg/ ml^{-1}) was applied for 60 minutes at room temperature before CB_1 labelling. The blocking peptide is used to prevent specific antibody interactions with the antigen of interest e.g. C-terminus CB_1 receptor protein. Hence, the blocking peptide should significantly reduce (or abolish) the amount of immunostaining normally observed.

2.6.7 Image acquisition and processing

Laser-scanning confocal imaging systems, either BioRad (Hercules, U.S.A.) MicroRadiance attached to an Olympus (Tokyo, Japan) BX50WI microscope, Olympus U-RFL-T mercury lamp and Olympus TH3 light source or BioRad MRC 1024 attached to Nikon Diaphot 200 microscope, Nikon HB-10101AF mercury lamp and light source, were used for image acquisition and processing. With the

MicroRadiance system, Cy3 was excited with a dedicated 543 nm line and emitted light passed through an E570LP filter, whereas Alexa 488 was excited with a 488 nm line and emitted light passed through an HQ515/30 filter. In multiple-labelling experiments images were obtained sequentially and merged off-line. Images were acquired and processed with LaserSharp software (BioRad). Images were edited with PhotoShop software.

2.7 Data analysis

Values are expressed as means ±standard error of the mean (S.E.M.). Comparison of pairs of treatments was determined by Student's *t*-test. As control and experimental data had been obtained from separate cells, an unpaired *t*-test was used to analyse the results. A one way analysis of variance (ANOVA) test was performed, followed by a *post hoc* Dunnett test to assess significant differences between control and multiple test values. $P<0.05$ was considered to be significant.

2.8 Drugs used in the study

NAME	MAIN CELLULAR ACTION	SUPPLIER	SOLVENT
Anandamide (AEA)	Endogenous cannabinoid agonist	Tocris-Cookson (UK)	Ethanol
2-aminoethoxydiphenylborane (2-APB)	$InsP_3$ receptor antagonist/ SOCC inhibitor	Tocris-Cookson (UK)	DMSO
Arachidonic acid (AA)	Cellular 2nd messenger	Sigma (UK)	Ethanol
Arachidonyl trifluoromethyl ketone (ATK) 1,1,1-Trifluoro-6Z, 9Z, 12Z, 15Z-heneicosateraen-2-one	Selective inhibitor of cytosolic PLA_2	Tocris-Cookson (UK)	Ethanol
4-bromophenacyl bromide (4-BPB)	Non-specific inhibitor of PLA_2	Sigma (UK)	DMSO
Cadmium (II) chloride (Cd^{2+})	Non-specific Ca^{2+} channel blocker	BDH (UK)	Distilled water
Caesium chloride (Cs^+)	Non-specific K^+ channel inhibitor	BDH (UK)	ICS
CP 55,940 (-)-*cis*-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-3(3-hydroxypropyl)cyclohexanol	Cannabinoid receptor agonist (CB_1/ CB_2)	Pfizer (UK)	Ethanol
EGTA Ethylene glycol-bis-(2-aminoethyl)-N,N,N',N'-tetraacetic acid	Ca^{2+} chelator	Sigma (UK)	ICS
Gadolinium (III) chloride (Gd^{3+})	Non-specific Ca^{2+} channel blocker	Sigma (UK)	Distilled water
Hexamethonium (C6)	Non-specific nACh receptor antagonist	Sigma (UK)	Distilled water
5-Hydroxytryptamine (5-HT)	5-HT receptor agonist	Sigma (UK)	Distilled water
Indomethacin	Cyclo-oxygenase inhibitor	Sigma (UK)	Ethanol
Lanthanum (III) chloride (La^{3+})	Non-specific Ca^{2+} channel blocker	Sigma (UK)	Distilled water
Methyllycaconitine citrate (MLA)	α7 neuronal nACh receptor antagonist	Tocris-Cookson (UK)	Distilled water
Nickel (II) chloride (Ni^{2+})	SOCC inhibitor	Sigma (UK)	Distilled water
Nicotine	nACh receptor agonist	Sigma (UK)	Distilled water
Palmitoylethanolamide (PEA)	Endogenous cannabinoid ligand (independent of CB_1/ CB_2 receptor activation)	Tocris-Cookson (UK)	Ethanol
Pertussis toxin (PTX)	$G_{i/o}$ inhibitor	Sigma (UK)	50% glycerol, 0.5 M NaCl, 0.05 M Tris-glycine
Phenidone	Lipoyxgenase/ cyclo-oxygenase inhibitor	Sigma (UK)	DMSO
Quinacrine	Non-specific inhibitor of PLA_2	Sigma (UK)	Solubilised in DMSO; diluted in distilled water

NAME	MAIN CELLULAR ACTION	SUPPLIER	SOLVENT
SKF 96365 1-[2-(4-methoxyphenyl)-2-[3-(4-methoxyphenyl)propoxy]ethyl]-1*H*-imidazole hydrochloride	Inhibitor of SOCCs	Tocris-Cookson (UK)	Distilled water
SR 141716A N-Piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide	CB_1 receptor antagonist	Sanofi (France)	Ethanol
Tetraethylammonium (TEA) bromide	K^+ channel inhibitor	Sigma (UK)	Distilled water
Tetrodotoxin (TTX)	Voltage-dependent Na^+ channel inhibitor	Tocris-Cookson (UK)	Solubilised in acidic buffer (pH 4.8); diluted in distilled water
Thapsigargin	SERCA inhibitor	Sigma (UK)	DMSO

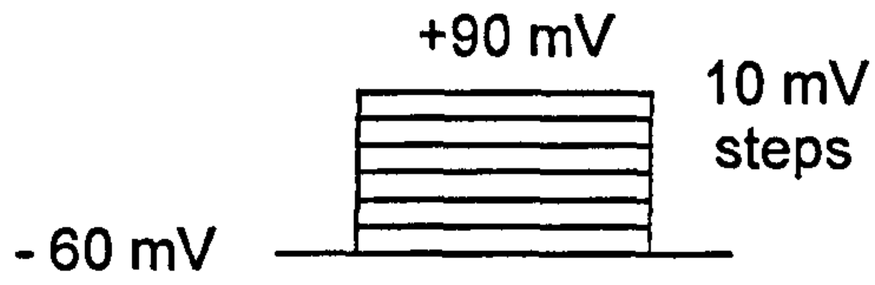
RESULTS

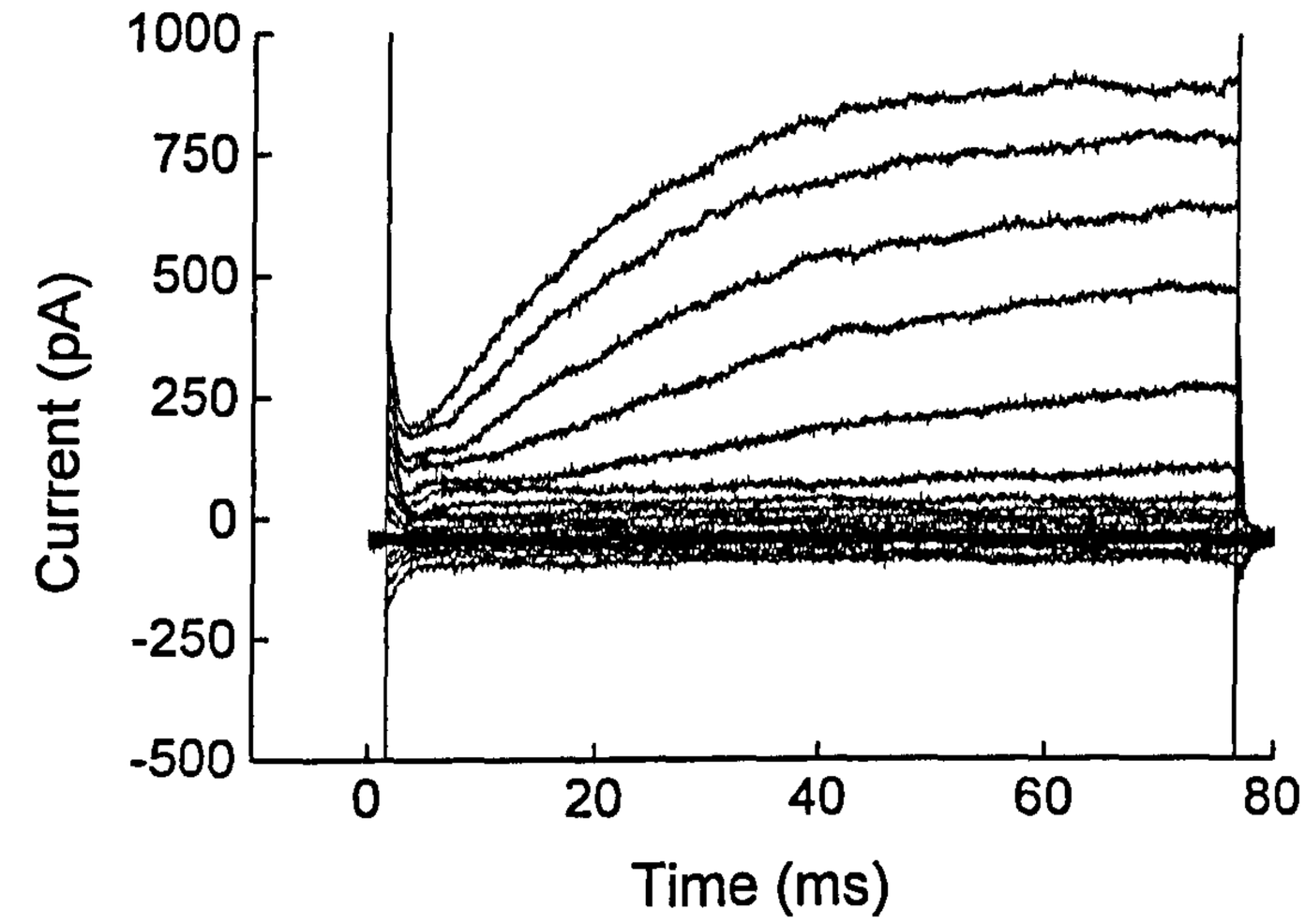
3.1 CB_1 receptor signalling in DDT_1 MF-2 smooth muscle cells

The following drugs, used to investigate the intracellular signalling pathways that lead to a CB_1 receptor-mediated increase in $[Ca^{2+}]_i$, were either dissolved in distilled water, ethanol or dimethyl sulfoxide (DMSO). Therefore the effects of ethanol (0.2%) and DMSO (0.1%) on membrane currents in DDT_1 MF-2 cells were established. Ethanol (n=5) and DMSO (n=6) were found to have no observable effect on membrane currents (controls initially performed consecutively). Subsequent ethanol and DMSO controls were then performed randomly, throughout the investigation.

3.1.1 Electrophysiological properties of DDT_1 MF-2 cells

DDT_1 MF-2 cells exhibited a cell capacitance of 31.1 ±2.6 pF (n=11). To activate voltage-dependent ion channels, the membrane was stepped from –60 mV to +90 mV, in 10 mV increments. Voltage steps evoked outward currents in DDT_1 MF-2 cells, at higher membrane potentials (Fig. 3.1). Previous studies have shown that these outward currents are due to the activation of voltage-dependent K^+ currents as they were abolished in the presence of Cs^{2+} (Begg, unpublished data). In addition, the synthetic cannabinoid agonist CP 55,940 (10 μM) has been shown to have no effect on these K^+ currents (Begg, unpublished data).





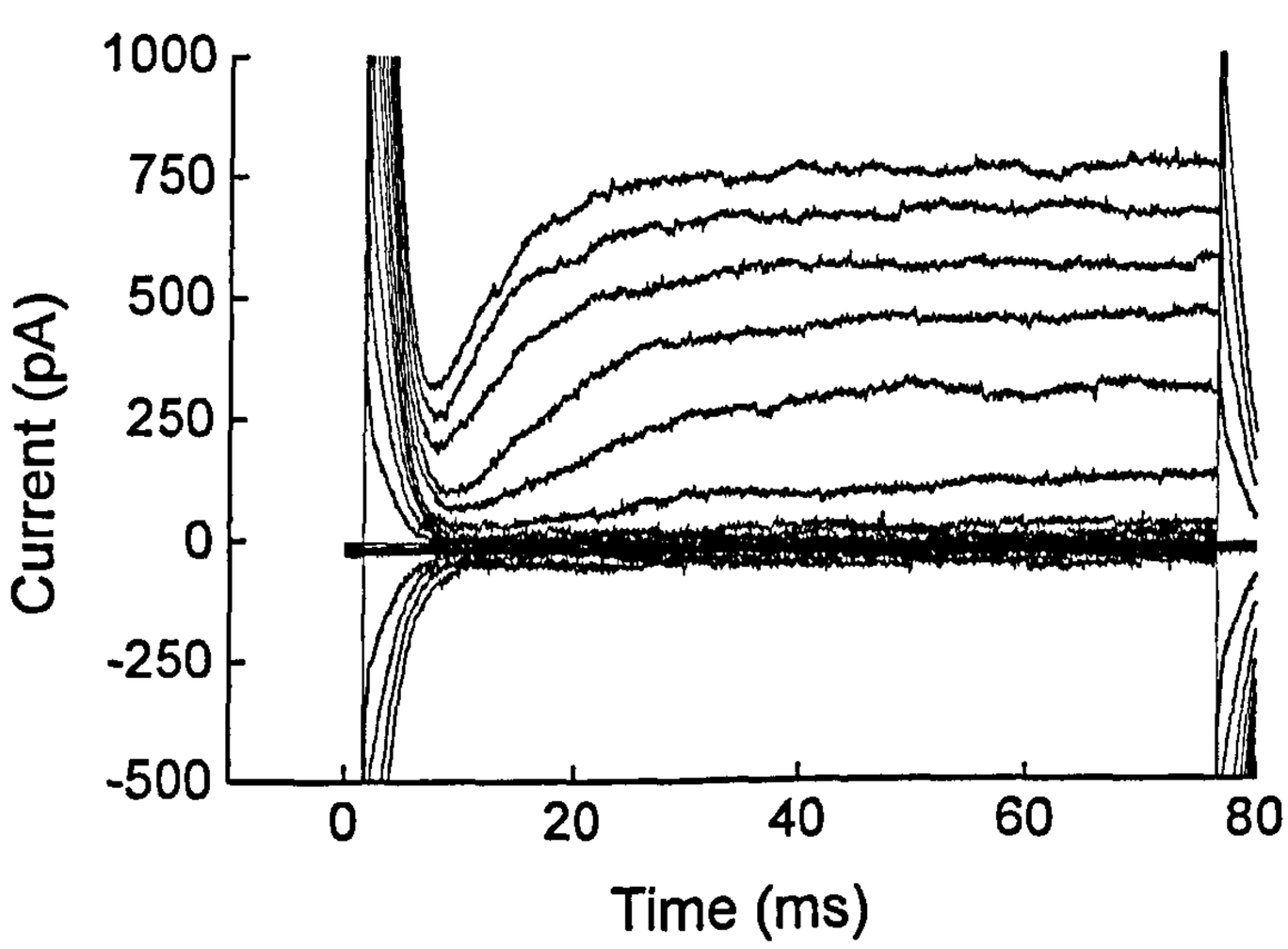


Figure 3.1 The effect of voltage steps on membrane currents in DDT1 MF-2 cells. Voltage-dependent K^+ currents were evoked in DDT_1 MF-2 cells by stepping the membrane potential from –60 mV to +90 mV, in 10 mV increments (n=15). Each trace represents a separate cell.

3.1.2 The effect of CP 55,940 on membrane currents: role of extracellular Ca^{2+}

CP 55,940 was used at a concentration of 10 μM in all electrophysiological experiments, using the DDT_1 MF-2 cell line. This is due to work by Begg *et al.* (2001) who demonstrated that 10 μM CP 55,940 evoked a maximal response, which was sensitive to SR 141716 suggesting a CB_1-dependent mechanism. CP 55,940 (10 μM) evoked a transient outward current, in DDT_1 MF-2 cells, with peak amplitude of 21.3 ±0.9 pA/ pF after 167.7 ±21.1 seconds (Fig. 3.2A, n=11). Histamine (10 μM), which releases Ca^{2+} from $InsP_3$-sensitive stores and induces Ca^{2+} influx (Molleman *et al.*, 1990, 1991b), also produced a transient outward current with a peak amplitude of 31.4 ±5.9 pA/ pF after 46.9 ±6.8 seconds of drug application (Fig. 3.2B, n=8).

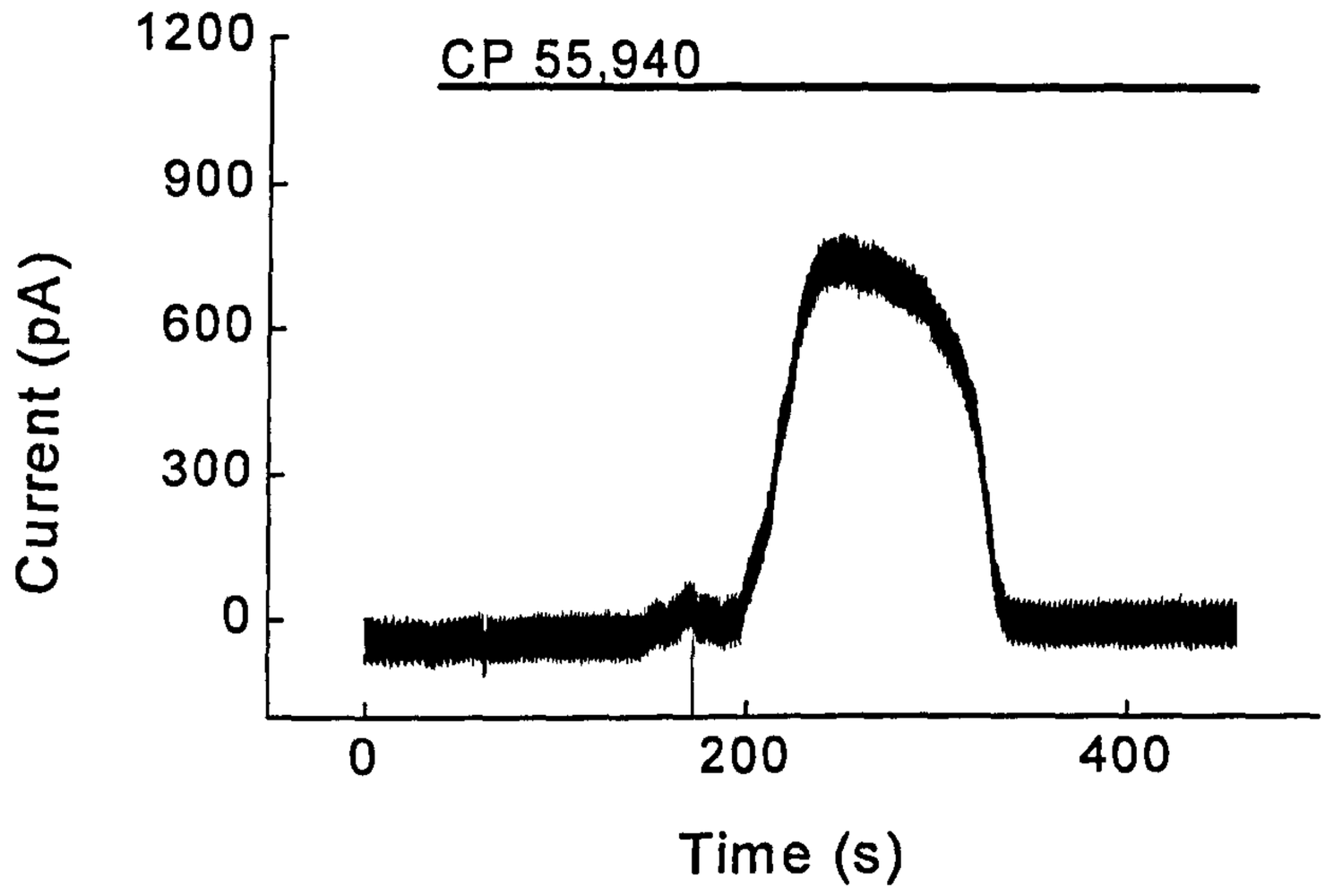
CP 55,940 (0.01-100 μM) induced a concentration-dependent increase in $[Ca^{2+}]_i$, which was completely abolished with the removal of extracellular Ca^{2+} (Fig. 3.3, n=4). However, the increase in $[Ca^{2+}]_i$ was only significant when CP 55,940 concentrations of 10 μM or greater were used. Moreover, in Ca^{2+}-free medium higher concentrations of CP 55,940 decreased $[Ca^{2+}]_i$, which was significant at 100 μM (Fig. 3.3, n=4, $P<0.05$, one way ANOVA).

3.1.3 Capacitative Ca^{2+} entry (CCE)

3.1.3.1 The role of $InsP_3$ receptors

To examine the involvement of $InsP_3$ receptors in the cannabinoid-induced response, the membrane-permeable $InsP_3$ receptor blocker 2-aminoethoxydiphenylborane (2-APB) was used (Maruyama *et al.*, 1997). To

A



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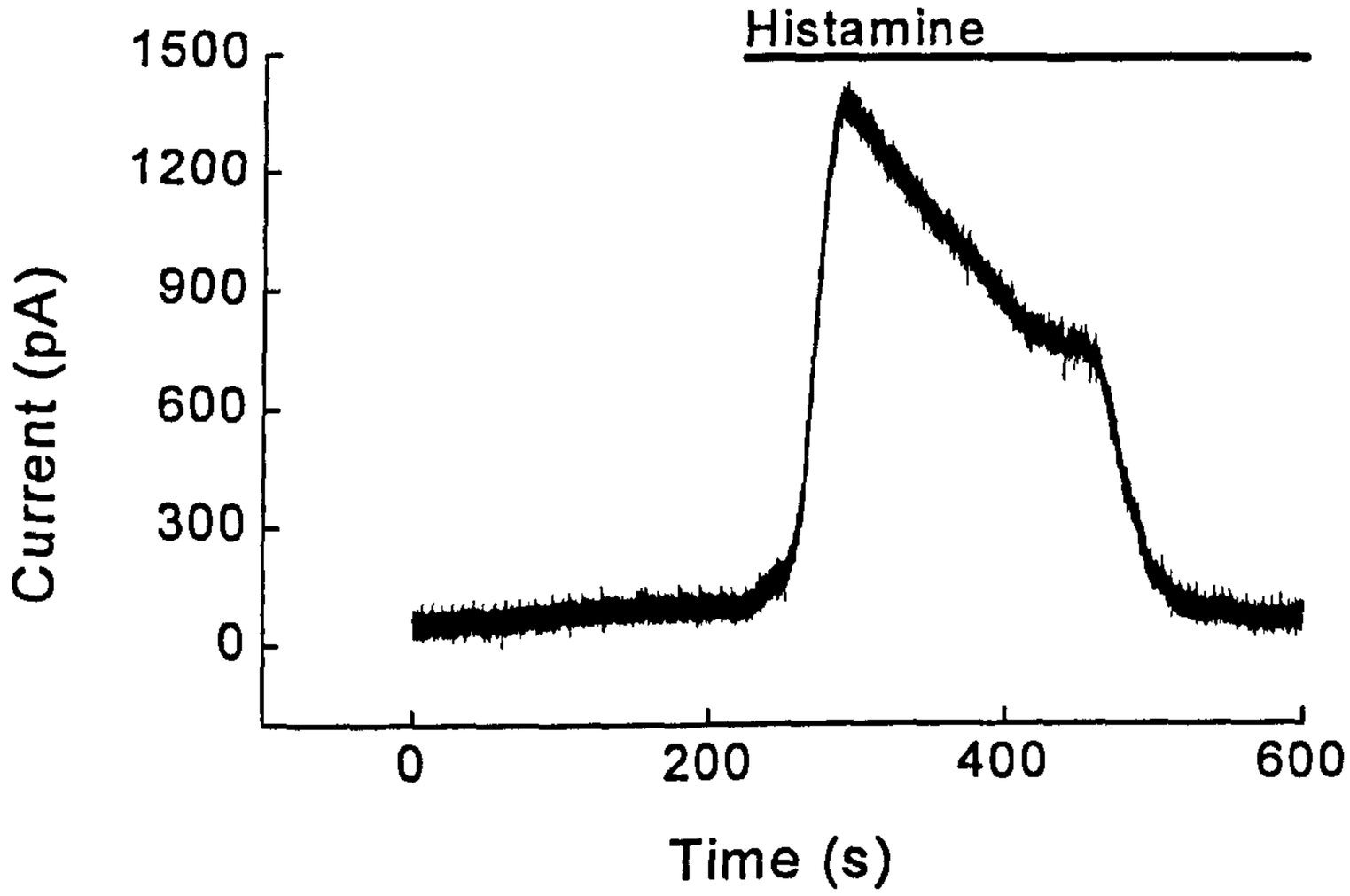


Figure 3.2 The effect of the cannabinoid receptor agonist CP 55,940 and histamine on membrane currents in DDT_1 MF-2 cells.
A, a sample trace of the effect of 10 μM CP 55,940 on resting membrane current (n=11). ***B***, a sample trace of the effect of 10 μM histamine on resting membrane current (n=8). Horizontal bars indicate the presence of ligands.

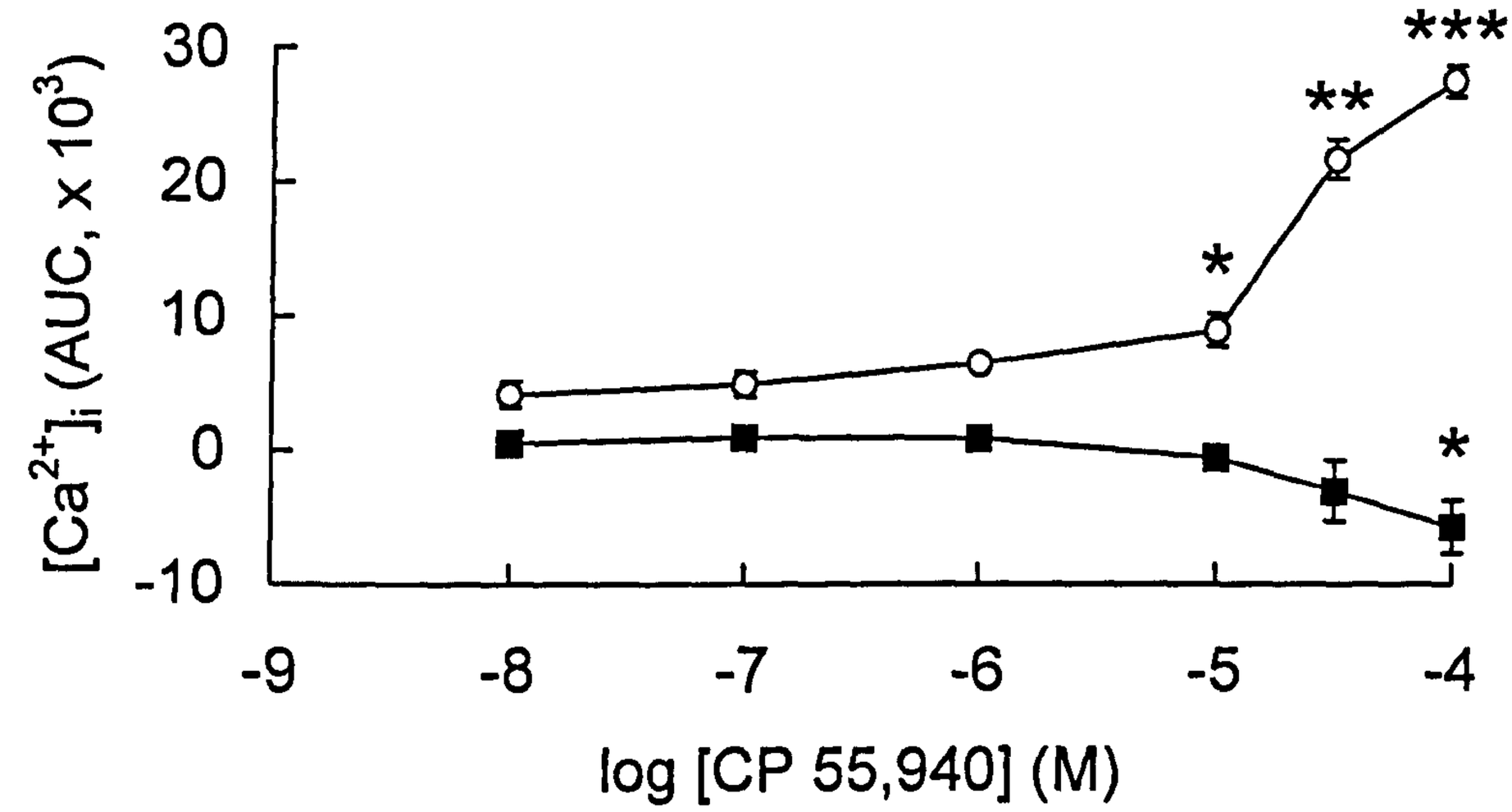


Figure 3.3 The effect of CP 55,940 on $[Ca^{2+}]_i$, in the presence and absence of extracellular Ca^{2+}.

Fura-2 fluorometry was used to measure changes in $[Ca^{2+}]_i$ in DDT_1 MF-2 cells, in response to CP 55,940 application (0.01-100 μM). Measurements were made in the presence (○, n=4) and absence (■, n=4) of extracellular Ca^{2+} (1 mM). Data represented as the mean $[Ca^{2+}]_i$ (±S.E.M.).

Significant difference from CP 55,940 (0.01 μM): *P<0.05, **P<0.01, ***P<0.001 (one way ANOVA).

determine a suitable concentration at which to use the inhibitor its actions on the histamine-evoked outward current were also assessed.

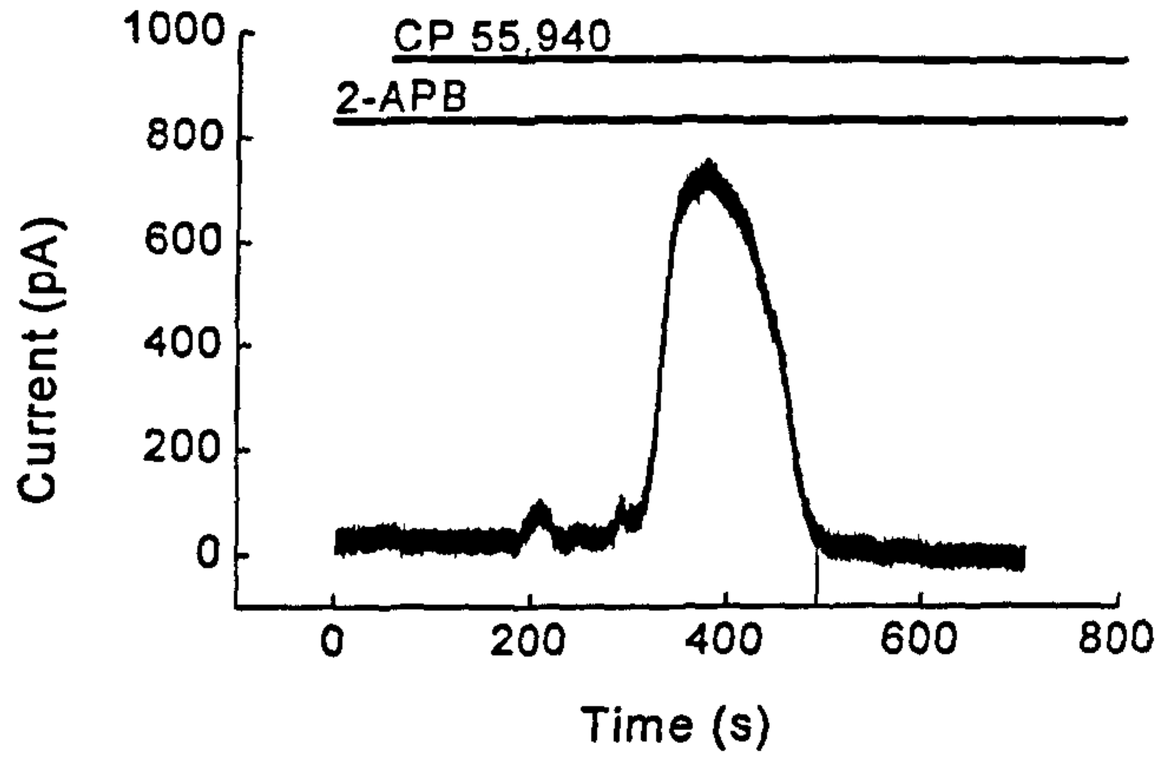
Application of 2-APB (10 μM) had no effect on the outward current evoked by 10 μM CP 55,940 (23.6 ±9.1 pA/ pF, Fig. 3.4A and C, n=6) but at the same concentration 2-APB significantly reduced the histamine response to 2.7 ±2.3 pA/ pF (Fig. 3.4B and C, n=5, $P<0.01$). 2-APB (10 μM) alone had no effect on membrane currents (n=4).

3.1.3.2 The role of store-operated Ca^{2+} channels (SOCCs)

We investigated the possibility that CB_1 receptor stimulation evokes a rise in Ca^{2+} via CCE. The SOCC inhibitor SKF 96365 has been shown to inhibit receptor-mediated Ca^{2+} entry in platelets stimulated with thrombin, with an IC_{50} of approximately 10 μM (Merritt *et al.*, 1990). At a concentration of 10 μM, SKF 96365 had no effect on the cannabinoid-induced outward current (22.6 ±4.0 pA/ pF, Fig. 3.5, n=6).

To determine if SKF 96365-sensitive channels are present in this cell type, the effect of the inhibitor on the response to histamine (10 μM) was examined. Two applications of histamine, 10 minutes apart, evoked reproducible, transient currents, the second response being 97.9 ±19.0% of the first response (Fig. 3.6A, n=6). In the presence of 10 μM SKF 96365 there was no significant change to the initial histamine response (26.7 ±6.0 pA/ pF, n=5) but the second response was significantly reduced to 49.2 ±9.1% of control (Fig. 3.6B and C, n=5, $P<0.05$). When applied alone SKF 96365 (10 μM) produced no observable change in membrane currents (n=4).

A



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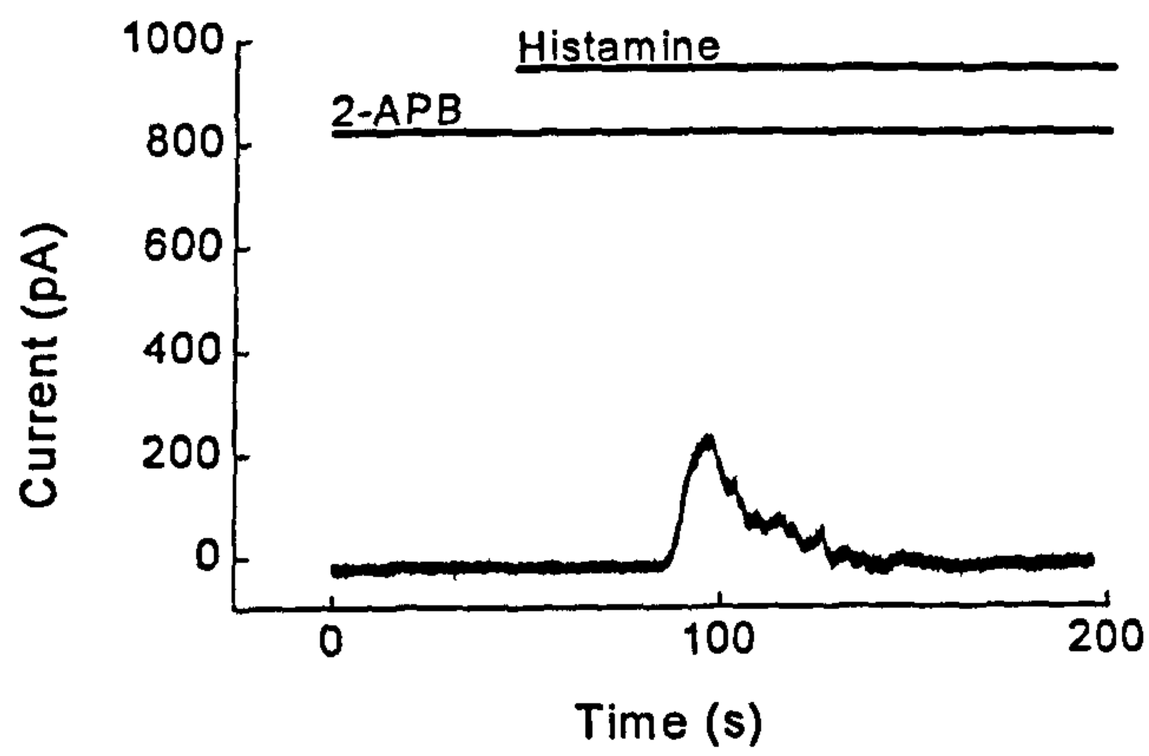

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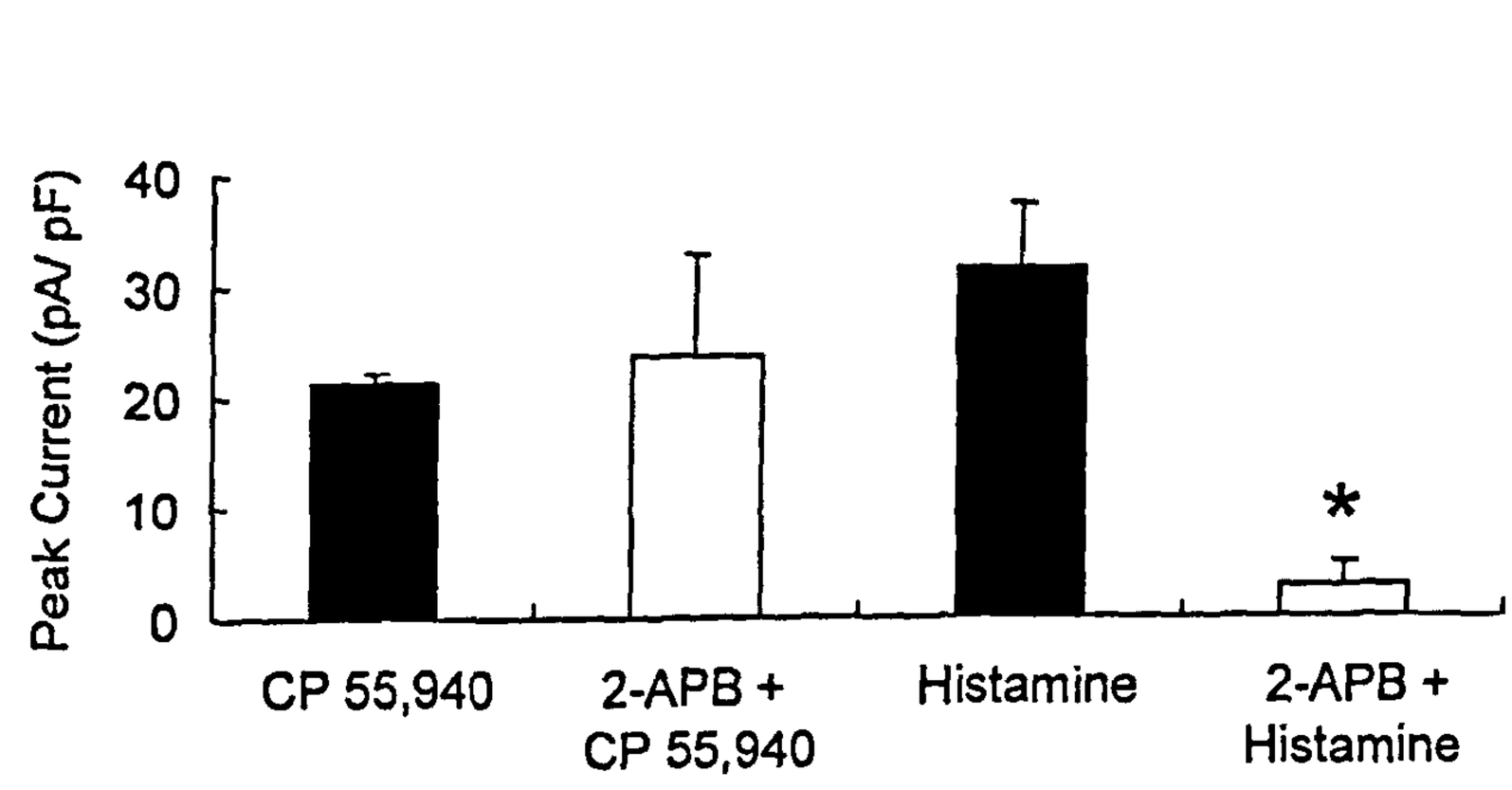
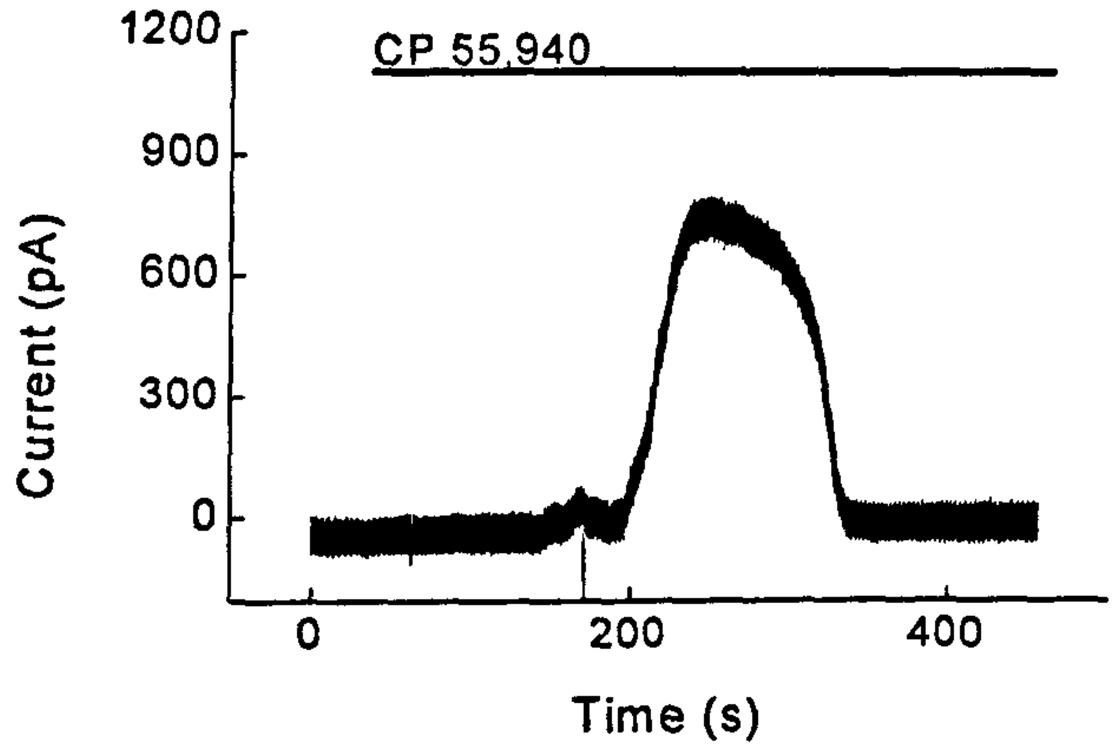


Figure 3.4 The effect of 2-APB on currents evoked by CP 55,940 and histamine.
2-APB (10 μM) was applied to cells for 10 minutes prior to application of either CP 55,940 (10 μM) or histamine (10 μM). ***A***, a sample trace of the effect of 2-APB on the CP 55,940-mediated current. ***B***, a sample trace of the effect of 2-APB on the histamine-evoked current. Horizontal bars indicate the presence of ligands. ***C***, the effect of 2-APB on the mean peak current (±S.E.M.) evoked by CP 55,940 (n=6) and histamine (n=5).
Significant difference from histamine control: **P*<0.01.

A



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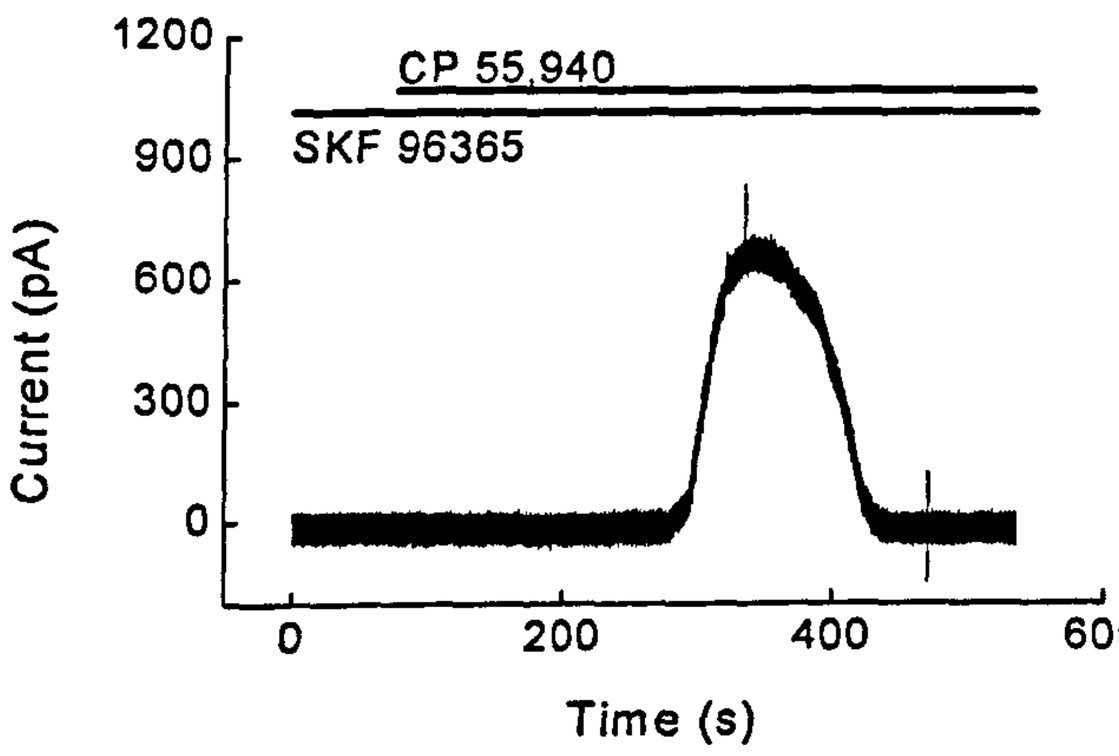

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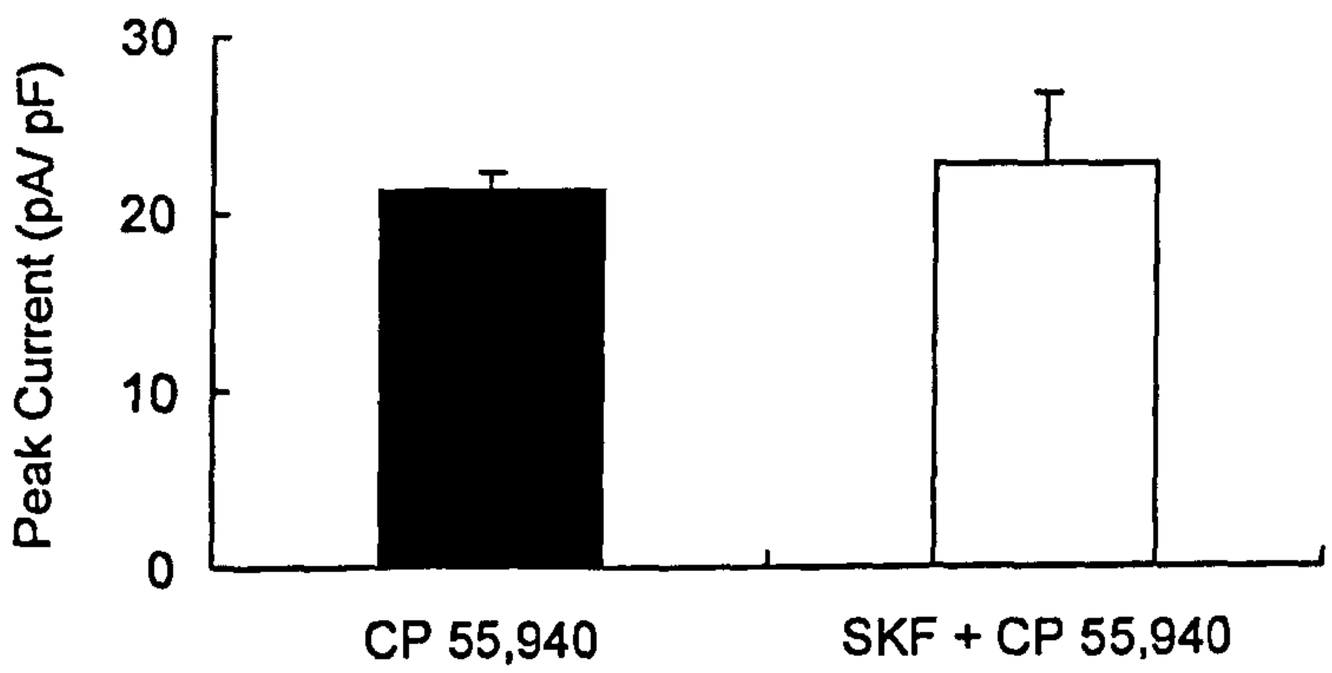
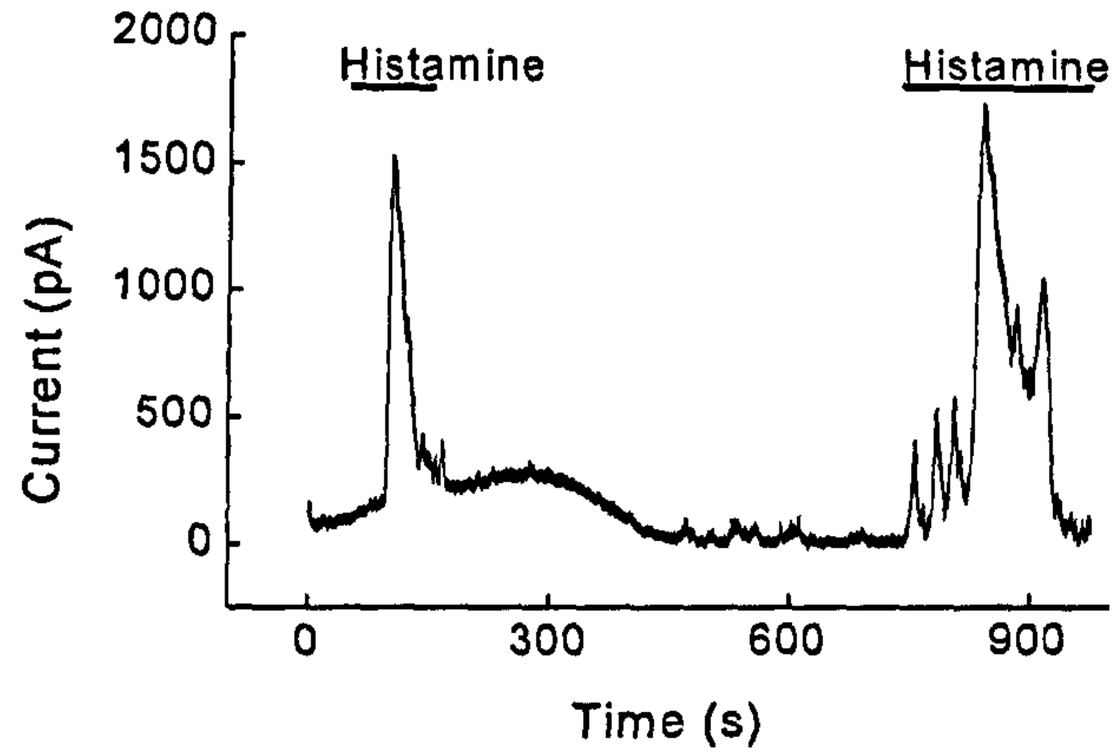


Figure 3.5 The effect of SKF 96365 on the CP 55,940-evoked outward current. SKF 96365 (SKF, 10 μM) was applied for 10 minutes prior to CP 55,940 (10 μM) application. *A*, a sample trace of the effect of CP 55,940 on resting membrane current in DDT_1 MF-2 cells (n=11). *B*, a sample trace of the effect of SKF 96365 on the outward current evoked by CP 55,940. Horizontal bars indicate the presence of ligands. *C*, the effect of SKF 96365 on the mean peak current (±S.E.M.) induced by CP 55,940 (n=6).

A



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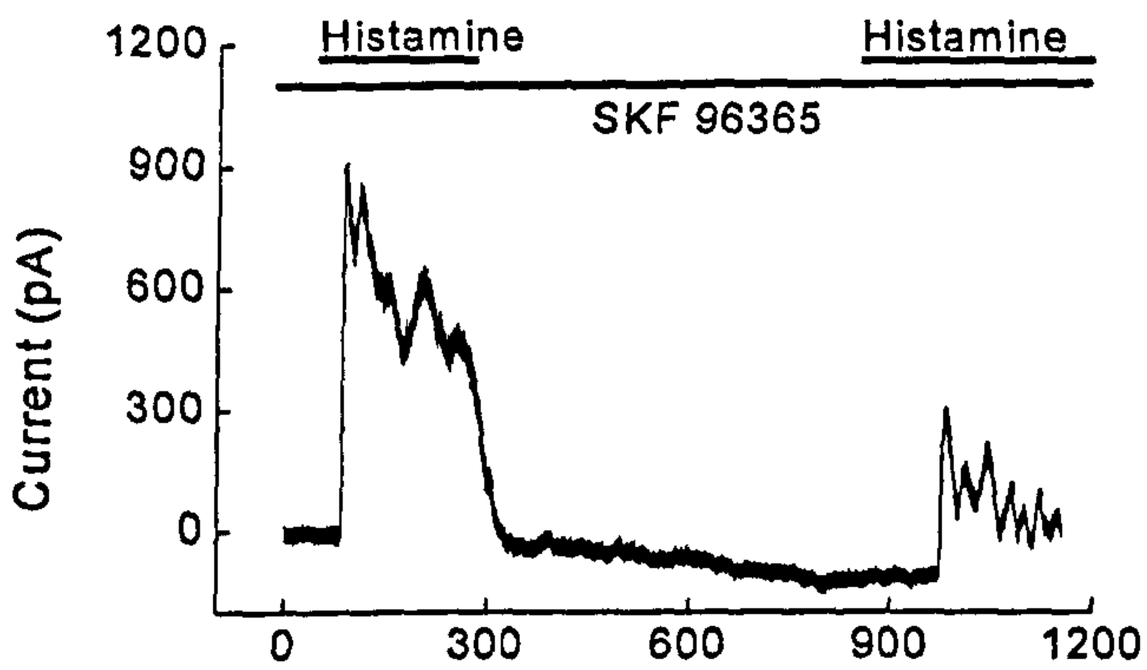

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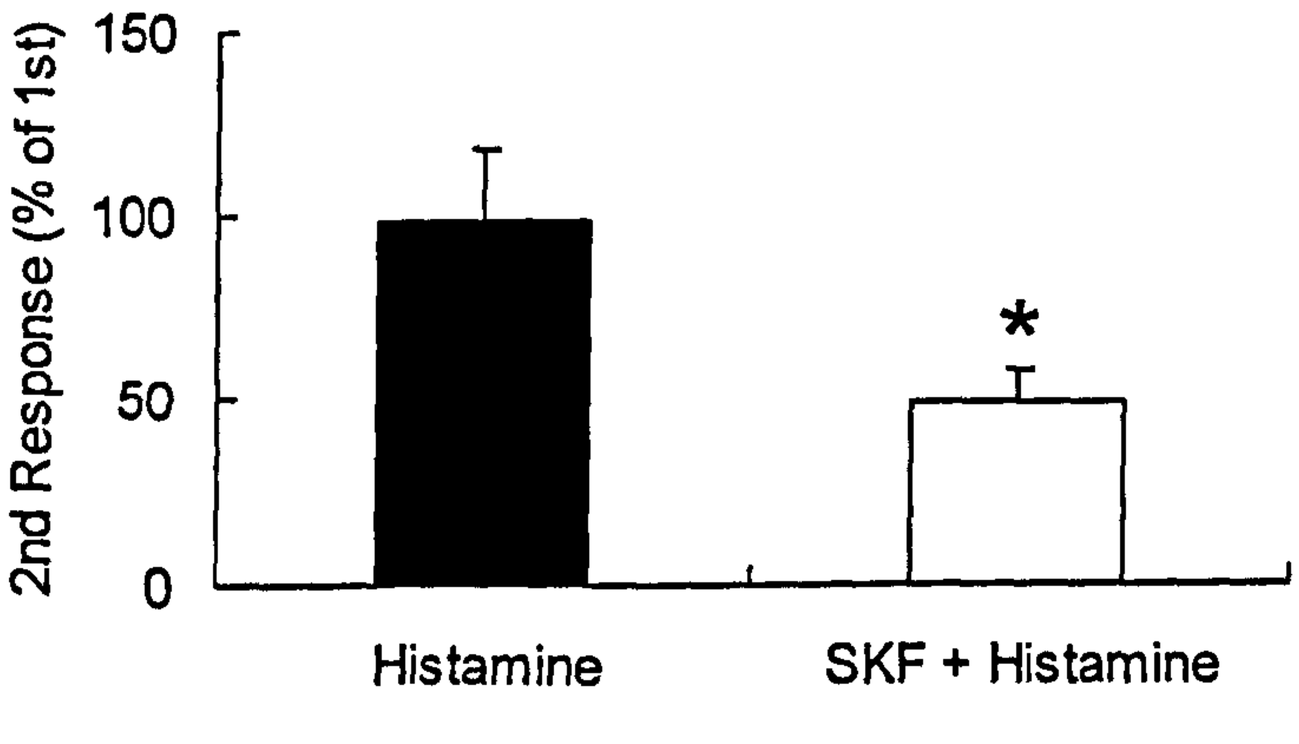


Figure 3.6 The effect of SKF 96365 on reproducible histamine-evoked currents. Histamine (10 μM) was applied twice to cells, separated by a 10 minute washout. SKF 96365 (SKF, 10 μM) was applied for 10 minutes prior to initial histamine application and then continuously throughout the experiment. ***A***, a sample trace of the currents evoked by histamine application (n=6). ***B***, a sample trace of the effect of SKF 96365 on histamine-evoked currents. Horizontal bars indicate the presence of ligands. ***C***, the effect of SKF 96365 on the mean peak current (±S.E.M.) evoked by the 2nd application of histamine, taken as a % of the 1st current response to histamine (n=5). Significant difference from histamine control: * $P<0.05$.

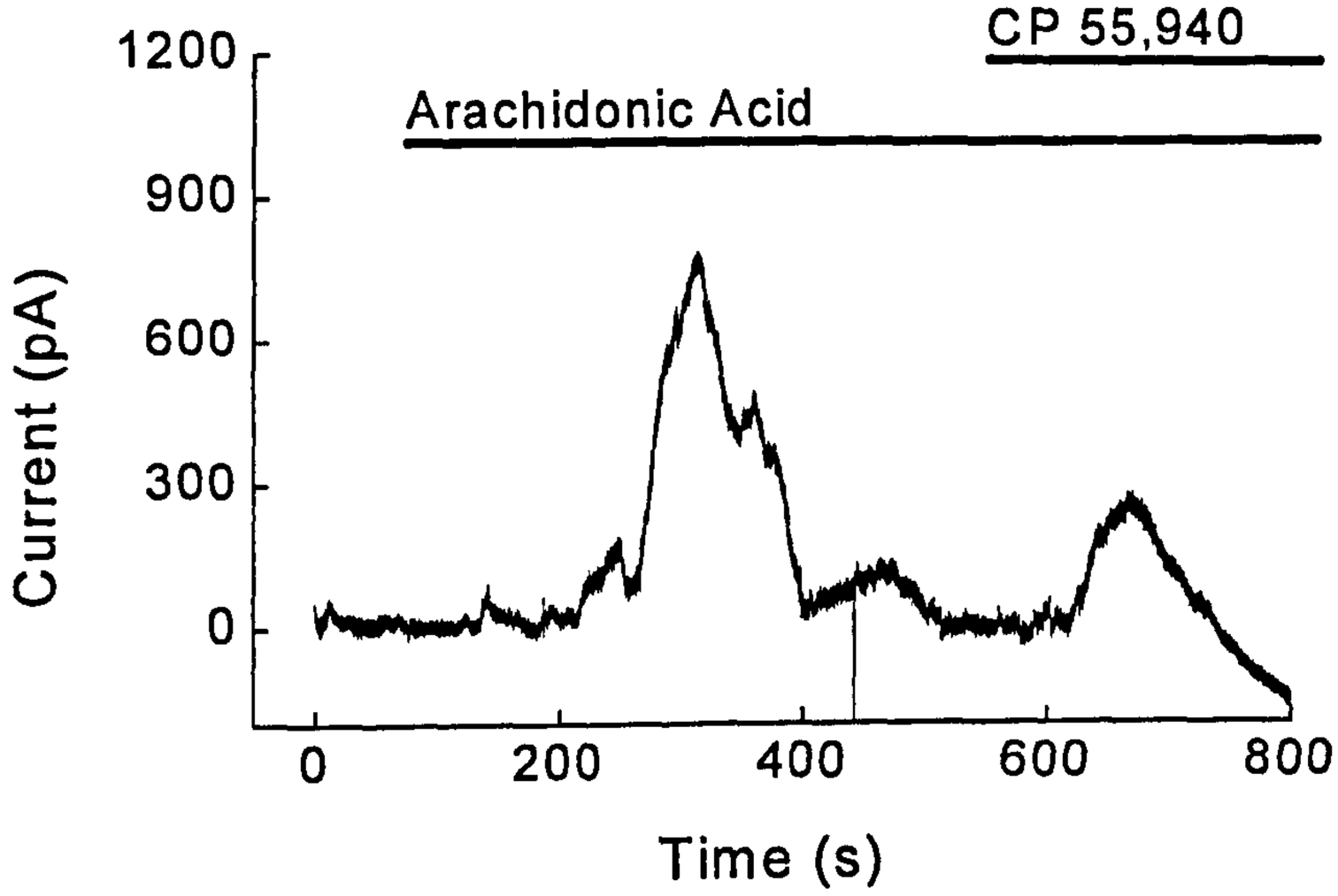
3.1.4 Non-capacitative Ca^{2+} entry (NCCE)

Relocation of the electrophysiology apparatus made it necessary for a new set of CP 55,940 controls to be established. 10 μM CP 55,940 evoked a transient, outward current with a peak amplitude of 32.4 ±1.3 pA/ pF after 223.7 ±54.6 seconds (n=6, data not shown). The following experiments were compared against these new controls, with the exception of those using arachidonyl trifluoromethyl ketone (ATK). These results were compared against the first set of CP 55,940 controls as they were obtained under the original conditions, before the electrophysiology apparatus was relocated. The new CP 55,940 controls produced a significantly greater increase in current compared to the initial set of CP 55,940 controls (n=6, $P<0.0001$), which was also reflected by an increased latency to reach peak current. A shorter superfusion tube, used on the relocated electrophysiology apparatus, may have minimised the surface area available for the cannabinoids to stick to. Hence, a greater concentration of CP 55,940 may have entered the bath, evoking a larger current response in the cells.

3.1.4.1 The role of arachidonic acid (AA) in Ca^{2+} signalling

AA has been shown to produce a concentration-dependent rise in $[Ca^{2+}]_i$ in DDT_1 MF-2 cells, with a maximal release of Ca^{2+} obtained at 1 mM AA (Van der Zee *et al.*, 1995). This increase in Ca^{2+} was therefore expected to evoke an outward K^+ current. In the present study, AA evoked a concentration-dependent, transient outward current (Fig 3.7, n=5).

A



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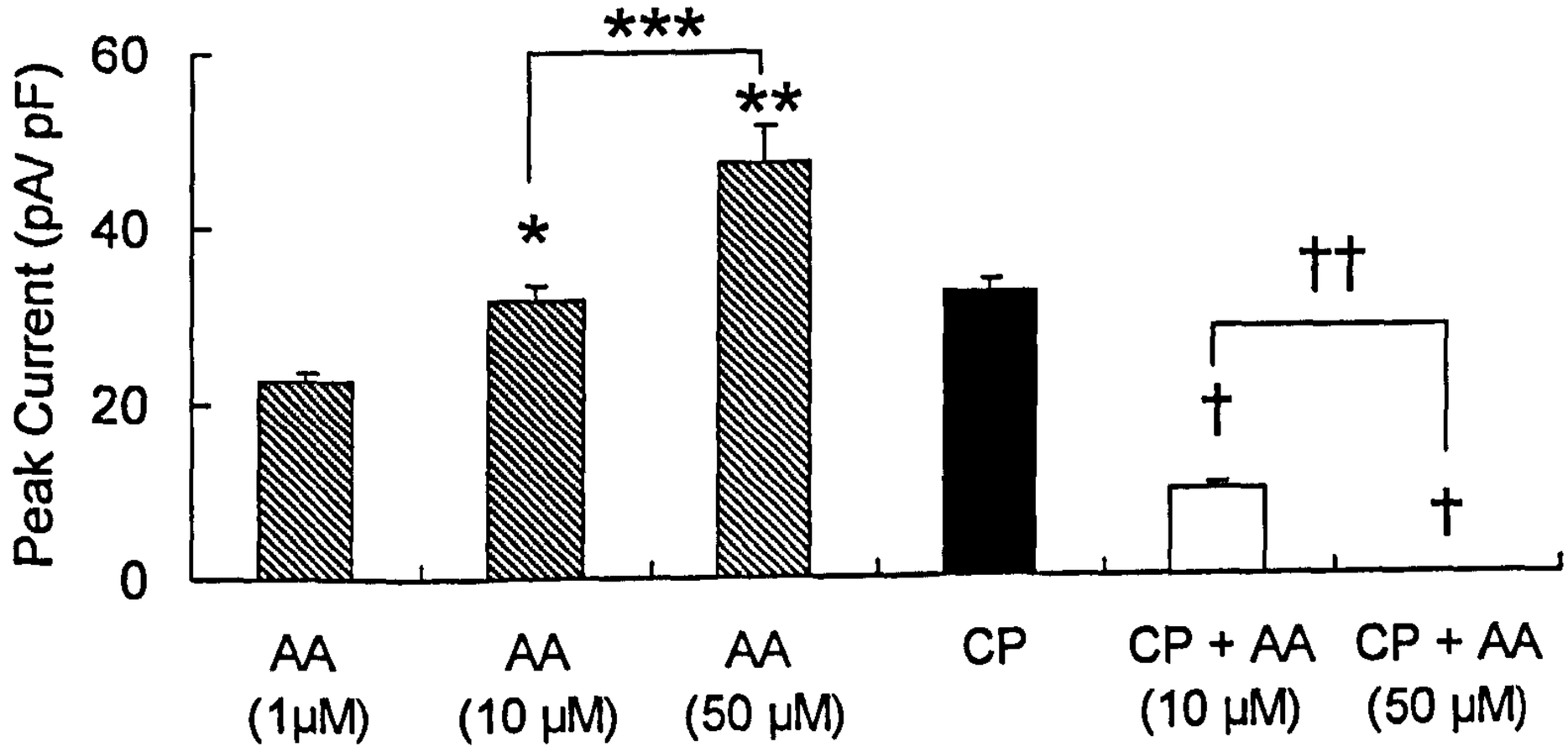

Figure 3.7 The effect of AA on membrane current and CP 55,940-induced currents.

AA (1-50 μM) was applied to DDT_1 MF-2 cells and the resulting change in membrane currents recorded. Once AA (10 and 50 μM)-mediated currents had returned to baseline, CP 55,940 (CP, 10 μM) was applied. ***A***, a sample trace of the transient outward current evoked by 10 μM AA and its effect on the CP 55,940-induced current. Horizontal bars indicate the presence of ligands. ***B***, the effect of AA on membrane currents (n=5) and subsequent currents evoked by 10 μM (n=4) and 50 μM CP 55,940 (n=5). Data represented as the mean peak current (±S.E.M.).
Significant difference from AA (1 μM): $*P<0.01$, $**P<0.001$ (one way ANOVA).
Significant difference from CP 55,940 control: $\dagger P<0.0001$.
Significant difference between test groups: $***P<0.01$, $\dagger\dagger P<0.0001$.

If pre-exposure to AA reduced the outward current seen to subsequent application of 10 μM CP 55,940, this would suggest that AA is involved in the intracellular signalling pathways that are evoked during CB_1 receptor stimulation. However, if AA has no effect on the CP 55,940-evoked outward current this would suggest that the CB_1 receptor signalling pathways do not involve AA production. DDT_1 MF-2 cells were initially exposed to AA to induce a transient response. As soon as membrane currents had returned to baseline CP 55,940 was administered. 10 μM AA significantly inhibited the outward current induced by 10 μM CP 55,940 to 9.9 ±0.7 pA/ pF (Fig. 3.7, n=4, $P<0.0001$, one way ANOVA) and at 50 μM completely abolished the cannabinoid-evoked response (Fig. 3.7B, n=5, $P<0.0001$, one way ANOVA).

To further test the hypothesis that AA is generated in response to CB_1 receptor stimulation, AA release was measured in [^{3}H]AA-pre-labelled DDT_1 MF-2 cells. CP 55,940 produced a concentration-dependent efflux of AA, with maximal release achieved at 0.3 μM (Fig. 3.8A). To determine if a receptor-mediated production of AA was involved, the CB_1 receptor antagonist SR 141716 (Rinaldi-Carmona *et al.*, 1994) was used to attempt to inhibit the [^{3}H]AA efflux observed to 1 μM CP 55,940. SR 141716 (1 μM) alone induced a significant release of AA to 135.6 ±19.3% of basal (Fig. 3.8B, n=7, $P<0.05$), suggesting a partial agonist effect of this compound in this assay. CP 55,940 (1 μM) evoked a significant release of AA to 199.2 ±23.4% of basal (Fig. 3.8B, n=30, $P<0.01$). In the presence of SR 141716 (1 μM) the CP 55,940-mediated release of AA was reduced to 139.8 ±13.8% of basal (Fig. 3.8B, n=14), comparable to the efflux observed with SR 141716 alone.

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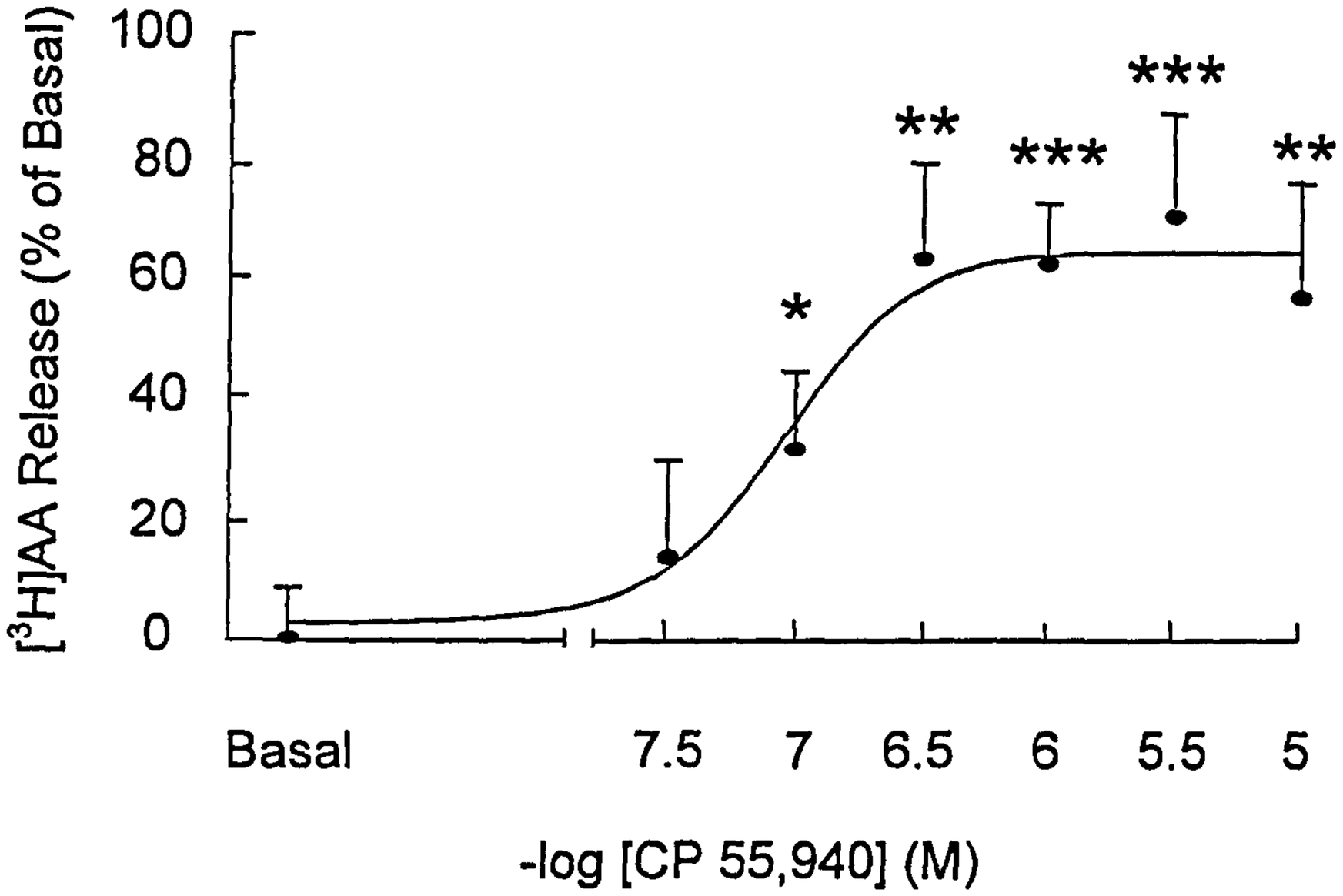

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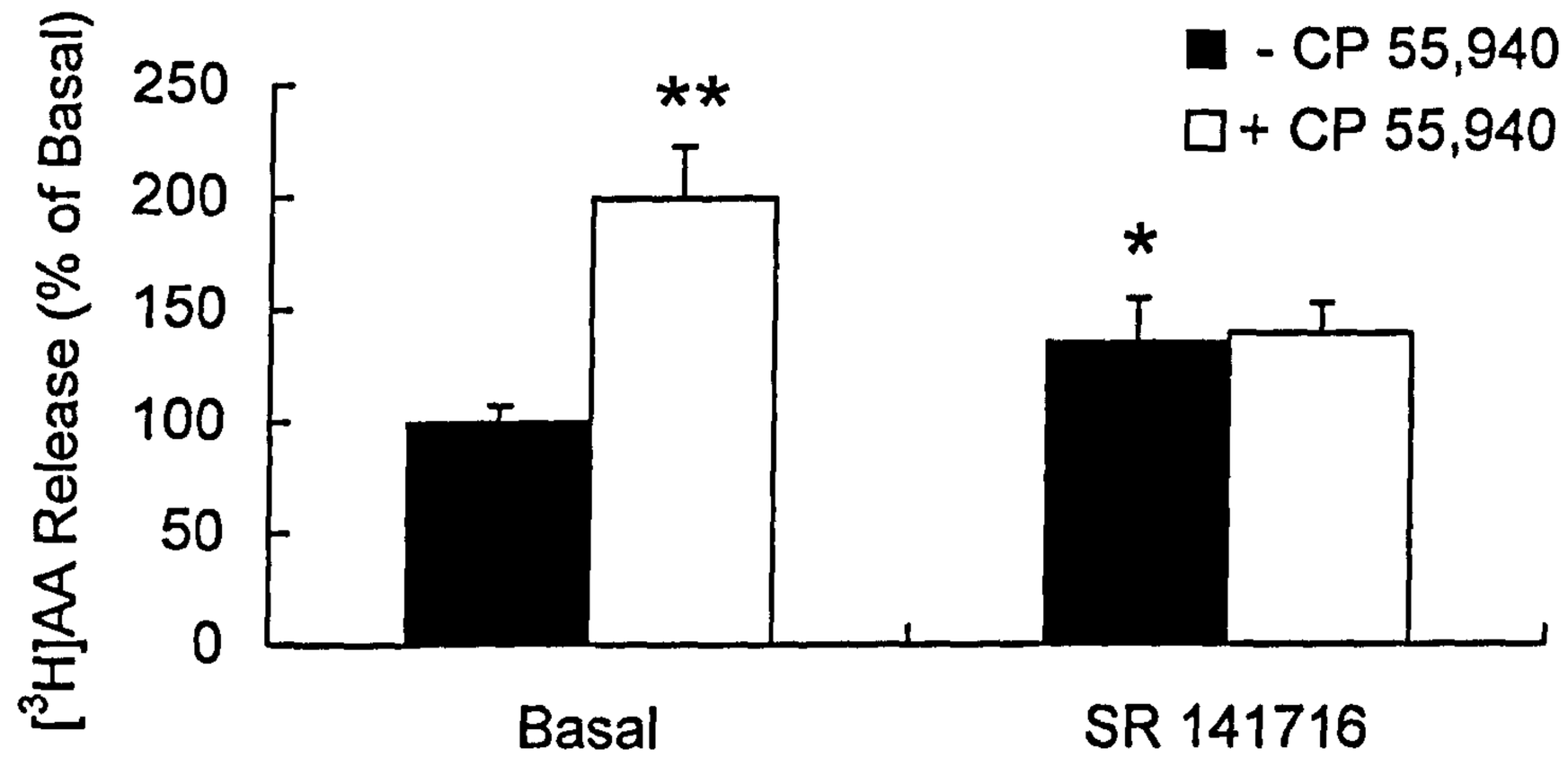


Figure 3.8 The effect of CP 55,940 on [^{3}H]AA release in DDT$_1$ MF-2 cells.
A, [^{3}H]AA-pre-labelled DDT$_1$ MF-2 cells were stimulated with CP 55,940 (0.03-10 μM) and the release of radiation measured (n=8 experiments in triplicate). ***B***, [^{3}H]AA release was measured from cells stimulated with 1 μM CP 55,940 (n=30). These results were compared to [^{3}H]AA measurements taken from cells pre-treated with SR 141716 (1 μM; for 15 minutes), before stimulation with CP 55,940 (n=14). To establish the effects of SR 141716, [^{3}H]AA release was measured from cells treated with SR 141716 alone (n=7).
Basal AA release was taken as the amount of [^{3}H]AA released in response to vehicle (DMSO, 0.1%). Data represented as the mean release of [^{3}H]AA (±S.E.M.), taken as a % of basal.
Significant difference from basal (DMSO control): **P*<0.05, ***P*<0.01, ****P*<0.001.

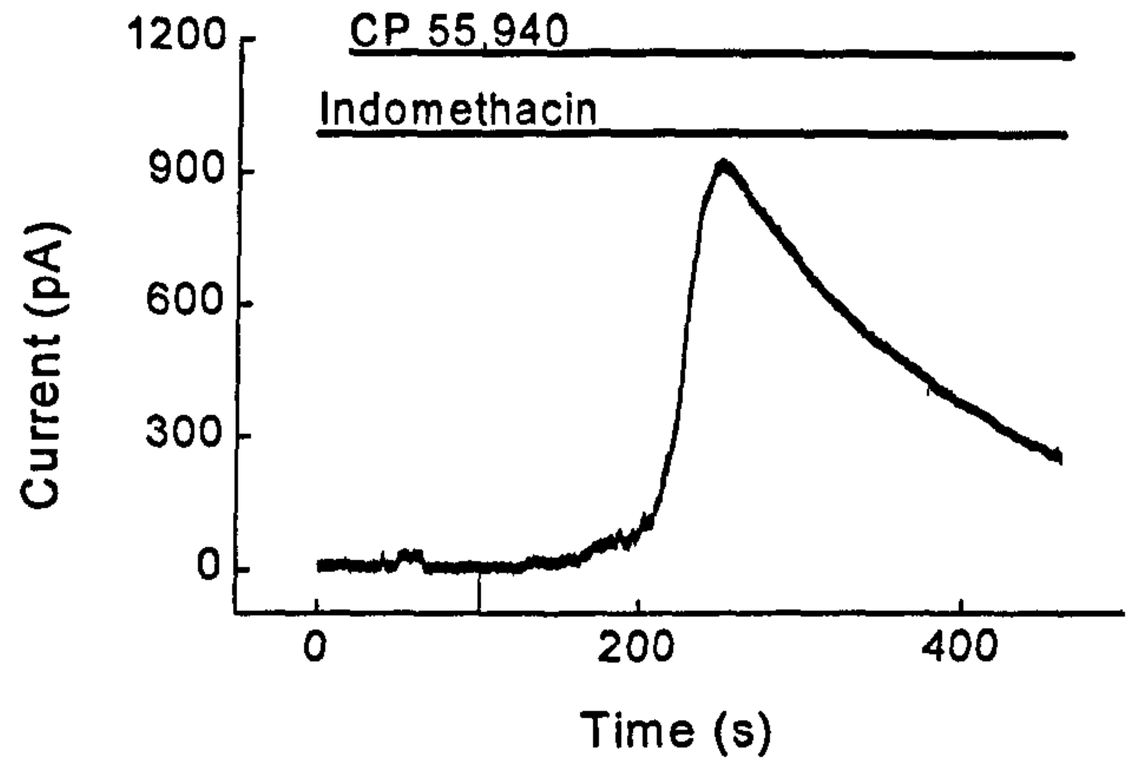
3.1.4.2 The role of AA metabolites

Indomethacin, a non-selective cyclo-oxygenase inhibitor (Bakalova *et al.*, 2002) and phenidone, a dual inhibitor of both cyclo-oxygenase and lipoxygenase (Kim *et al.*, 2000) were used to establish if the response to CB_1 receptor stimulation requires the production of AA metabolites. Pre-treatment of cells with indomethacin (10 μM) had no significant effect on the outward current evoked by 10 μM CP 55,940 (30.1 ±3.2, pA/ pF, Fig. 3.9A and C, n=7). Phenidone (100 μM) also had no effect on the CP 55,940-mediated response (36.9 ±4.2 pA/ pF, Fig. 3.9B and C, n=5). No significant change in membrane currents was observed when indomethacin (10 μM, n=5) or phenidone (100 μM, n=6) were administered alone.

3.1.4.3 Lanthanum (La^{3+})-sensitive Ca^{2+} influx

It has been reported that in the presence of the non-specific Ca^{2+} channel blocker La^{3+} (50 μM), the histamine-evoked Ca^{2+} influx in DDT_1 MF-2 cells was completely abolished, leading to a reduction of the outward current (Van der Zee *et al.*, 1995). This Ca^{2+} influx may represent the same NCCE pathway observed in other cell lines, which is also sensitive to inhibition by La^{3+} (Fiorio Pla and Munaron, 2001; Mignen and Shuttleworth, 2000). To establish if La^{3+}-sensitive Ca^{2+} channels are activated during CB_1 receptor stimulation, its effects on the CP 55,940-evoked outward current were determined. Application of 50 μM La^{3+}, prior to CP 55,940 (10 μM) application, completely abolished the cannabinoid response (Fig. 3.10A and C, n=7, $P<0.0001$). La^{3+} (50 μM) alone evoked no change in membrane currents (n=5).

A



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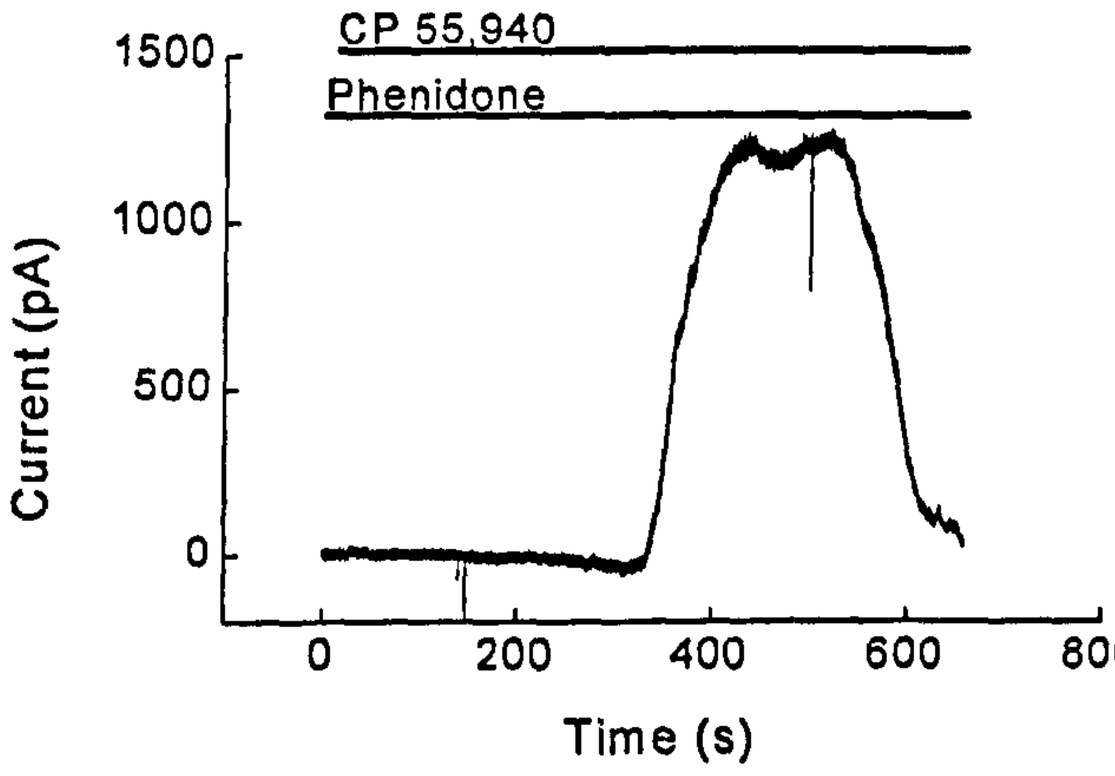

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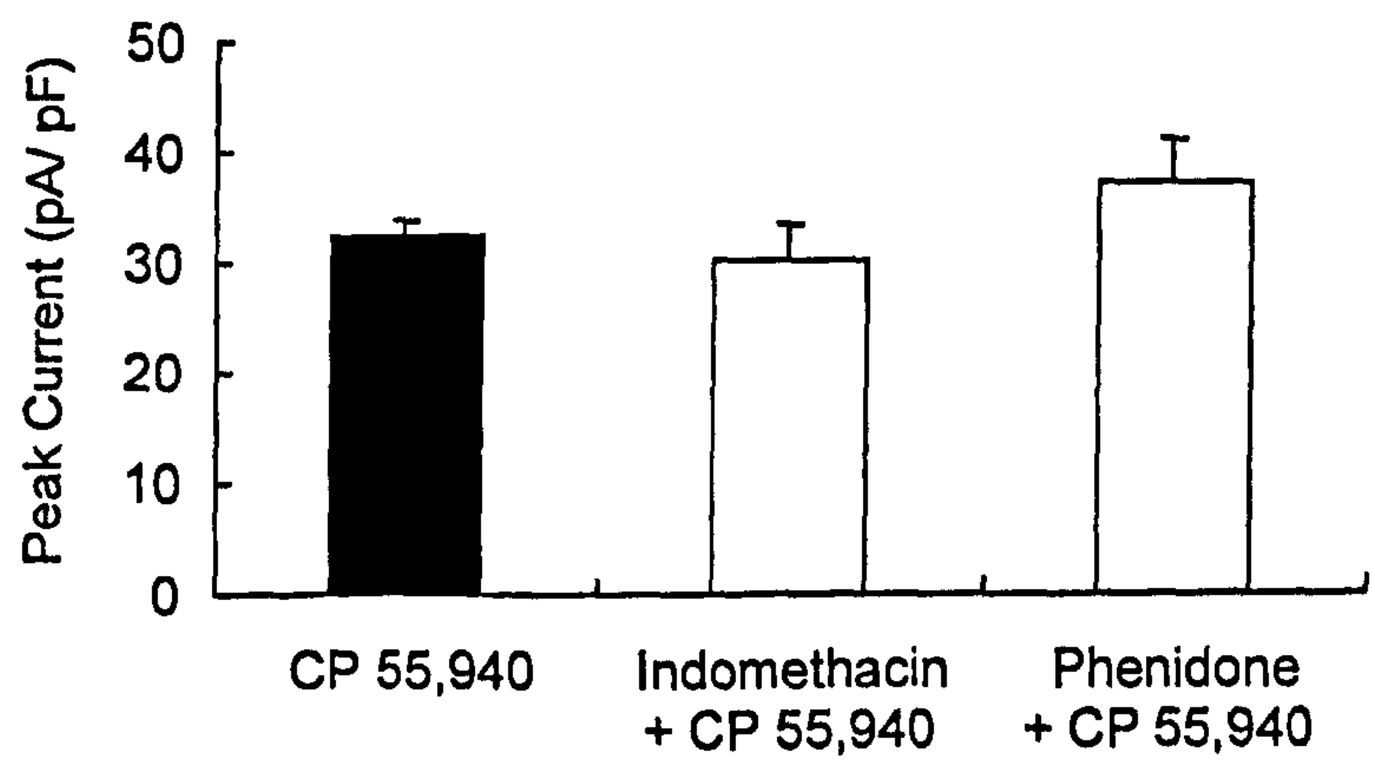
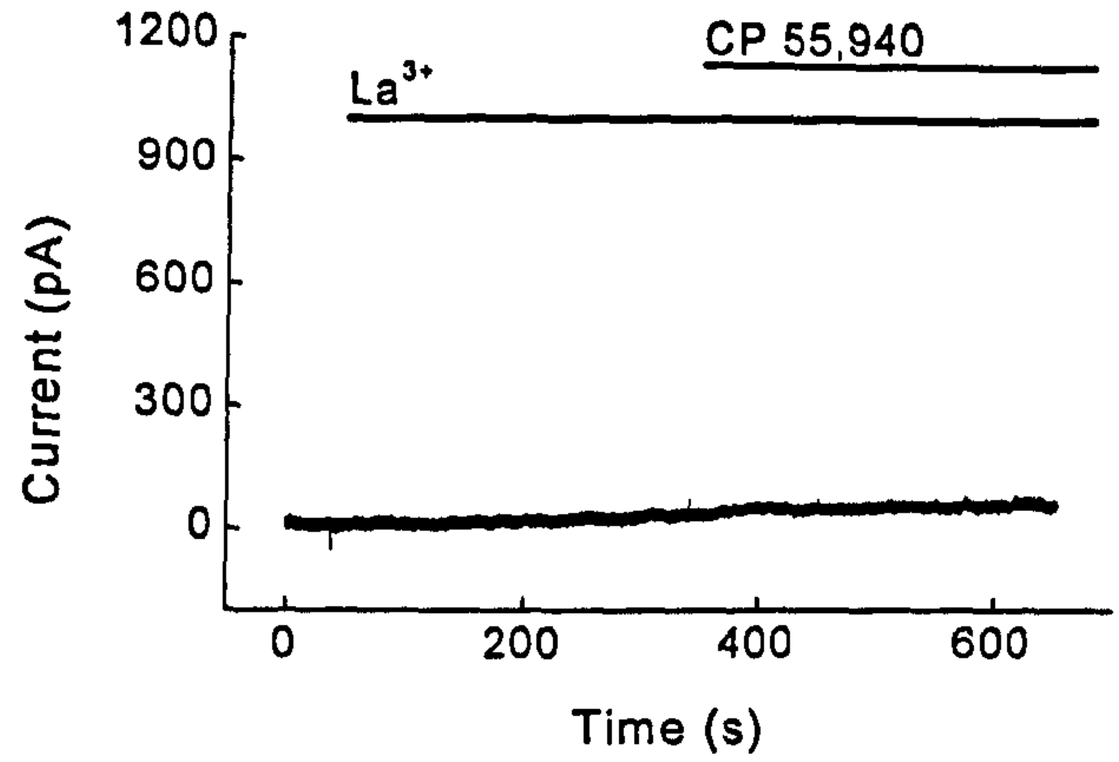


Figure 3.9 The effect of indomethacin and phenidone on CP 55,940-evoked outward currents.

Indomethacin (10 μM) and phenidone (100 μM) were applied to cells for 10 minutes prior to CP 55,940 (10 μM) application. ***A***, a sample trace of the effect of indomethacin on the outward current evoked by CP 55,940. ***B***, a sample trace of the effect of phenidone on the CP 55,940-evoked current. Horizontal bars indicate the presence of ligands. ***C***, the effect of indomethacin (n=7) and phenidone (n=5) on the mean peak current (±S.E.M.) induced by CP 55,940.

A



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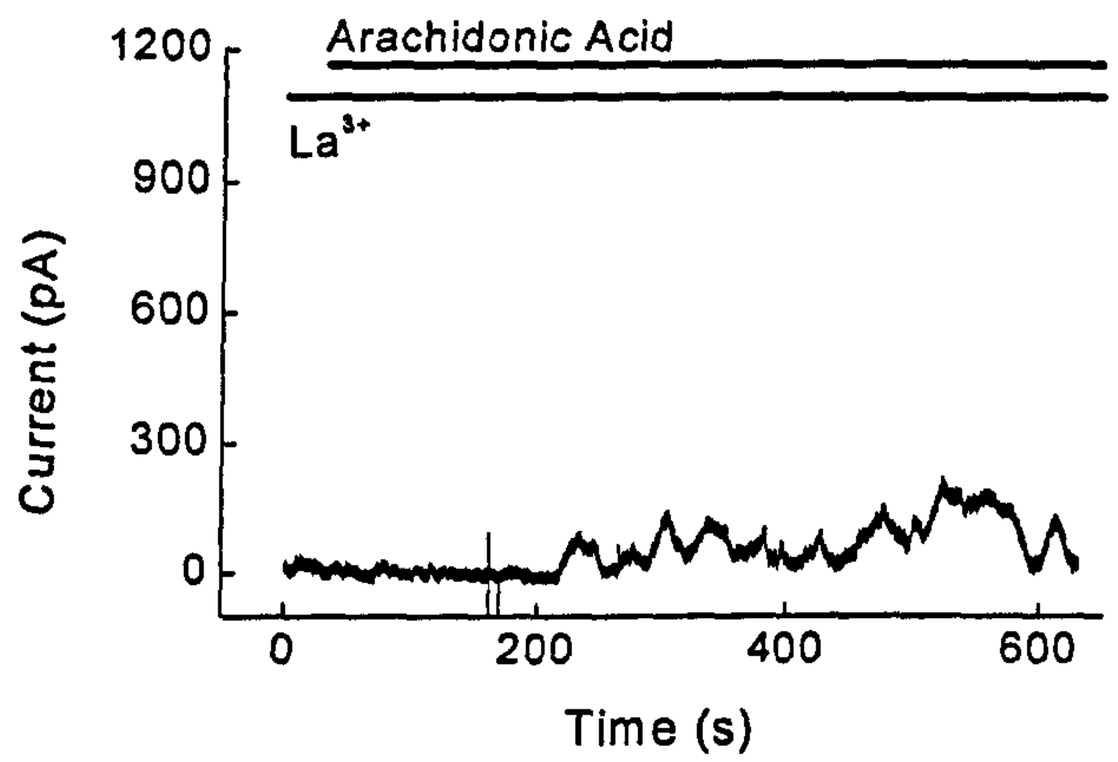

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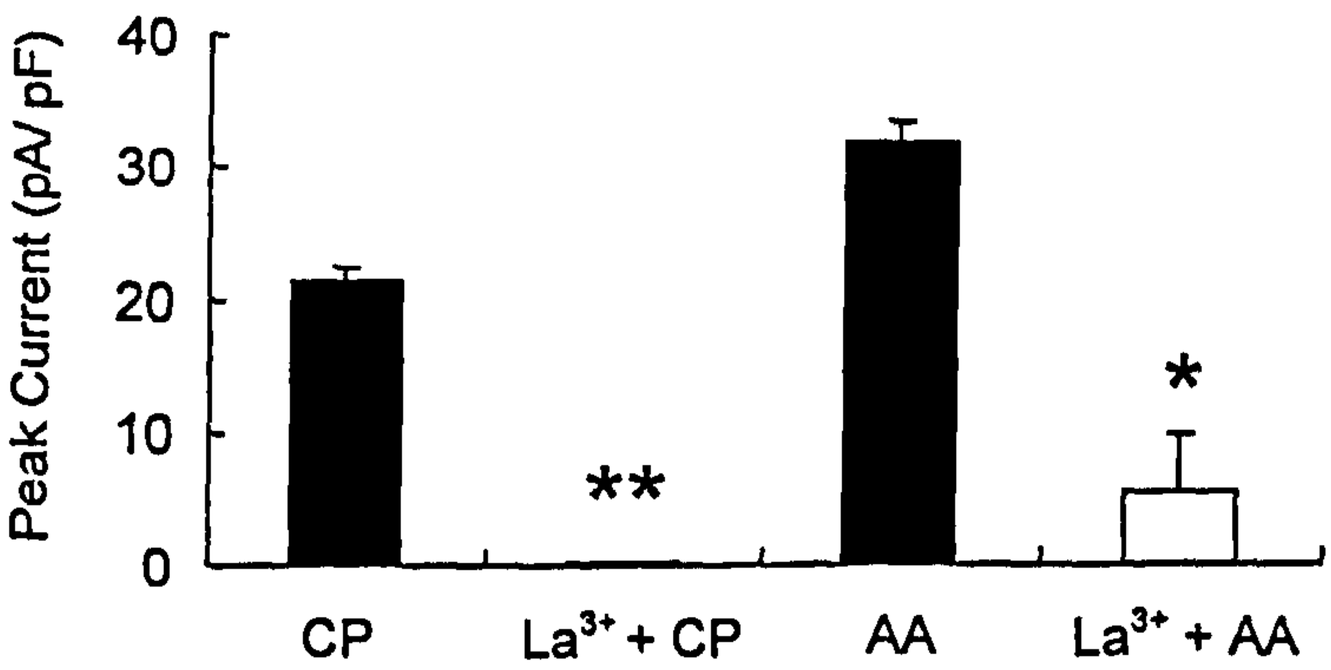


Figure 3.10 The effect of La^{3+} on currents evoked by CP 55,940 and AA.
La^{3+} (50 μM) was applied for 5 minutes prior to CP 55,940 (CP, 10 μM) and AA (10 μM) application. ***A***, a sample trace of the effect of La^{3+} on the CP 55,940-evoked current. ***B***, a sample trace of the effect of La^{3+} on the AA-induced current. Horizontal bars indicate the presence of ligands. ***C***, the effect of La^{3+} on the mean peak current (±S.E.M.) induced by CP 55,940 (n=7) and AA (n=6).
Significant difference from CP 55,940 control: $^{**}P<0.0001$.
Significant difference from AA control: $^{*}P<0.001$.

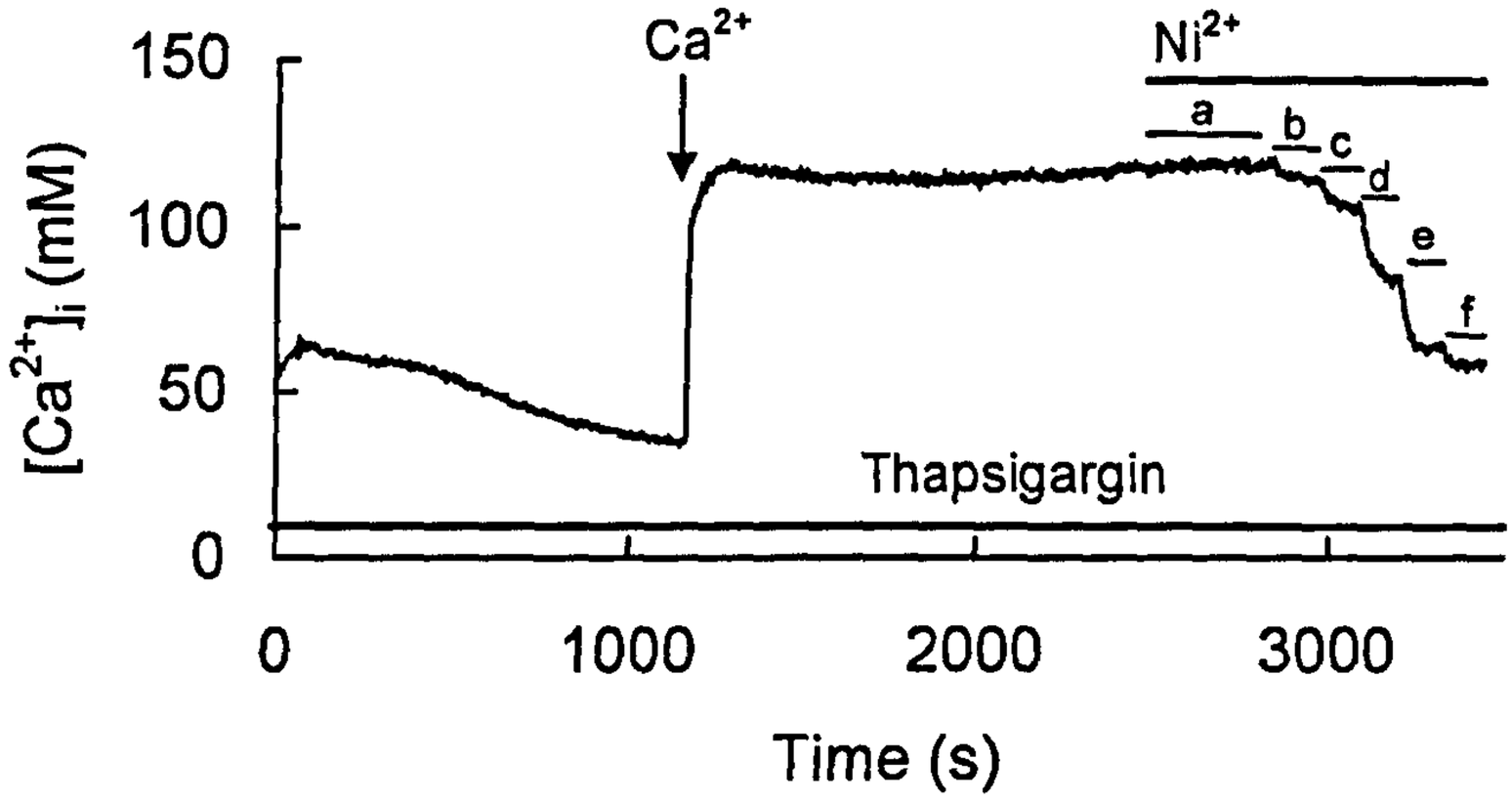
To further explore a cannabinoid-mediated activation of NCCE, the effect of La^{3+} on AA-induced Ca^{2+} entry was established. AA has also been shown to activate $I_{K,Ca}$ (Kirber *et al.*, 1992), and therefore the study would help ascertain if AA production does indeed occur upstream of the Ca^{2+} entry process. La^{3+} (50 μM) significantly reduced the response to 10 μM AA to a peak current of 5.4 ±4.3pA/ pF (Fig. 3.10B and C, n=6, P<0.001).

La^{3+} can also potently inhibit CCE (Putney, 2001), so its effect on CCE induced in DDT_1 MF-2 cells was established. CCE in DDT_1 MF-2 cells was evoked with the SERCA inhibitor thapsigargin. In Ca^{2+}-free medium, 1 μM thapsigargin induced an initial rise in $[Ca^{2+}]_i$ resulting from the depletion of intracellular Ca^{2+} stores (Fig. 3.11A). Re-addition of Ca^{2+} (1 mM) to the medium produced a sharp increase in $[Ca^{2+}]_i$ reflecting the activation of CCE (Fig. 3.11A). Nickel (Ni^{2+}) has been shown to inhibit Ca^{2+} influx mediated by SOCCs in T-lymphocytes (Kerschbaum and Cahalan, 1999). In DDT_1 MF-2 cells, Ni^{2+} inhibited the Ca^{2+} influx component of thapsigargin in a concentration-dependent manner (EC_{50}: 0.8 mM, Fig. 3.11, n=3). At a maximum concentration of 100 μM, La^{3+} had no effect on the thapsigargin-induced elevation in $[Ca^{2+}]_i$ (Fig. 3.12A, n=3), suggesting that separate Ca^{2+} influx pathways are initiated by CP 55,940 (NCCE) and thapsigargin (CCE).

3.1.4.4 Gadolinium (Gd^{3+})-sensitive Ca^{2+} influx

In rat aortic smooth muscle cells low concentrations (1 μM) of Gd^{3+} have been reported to inhibit CCE, while higher concentrations (100 μM) were shown to inhibit both CCE and NCCE (Broad *et al.*, 1999). The effect of Gd^{3+} on CP 55,950-evoked membrane currents was investigated. Gd^{3+} (1 μM) inhibited the outward current

A



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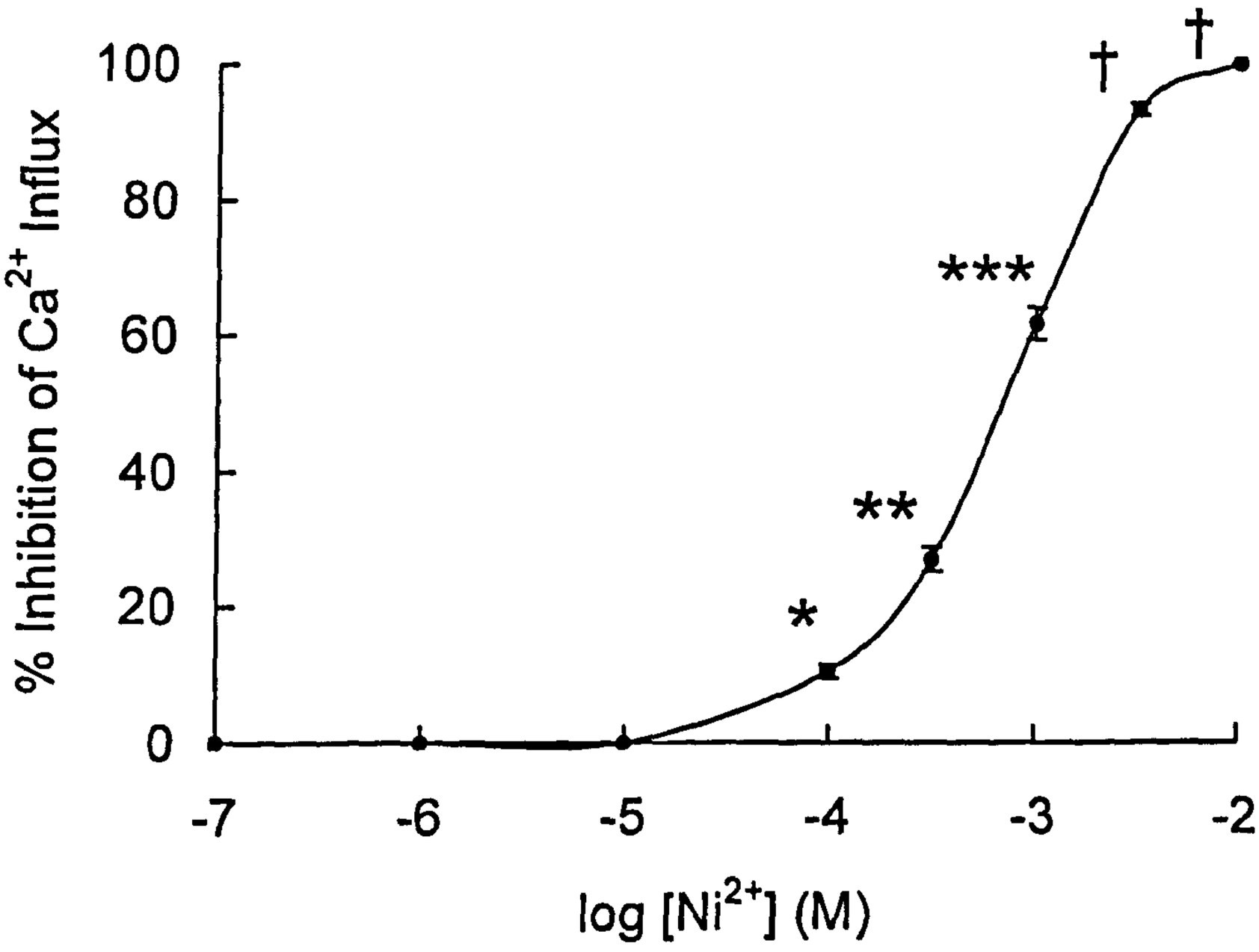
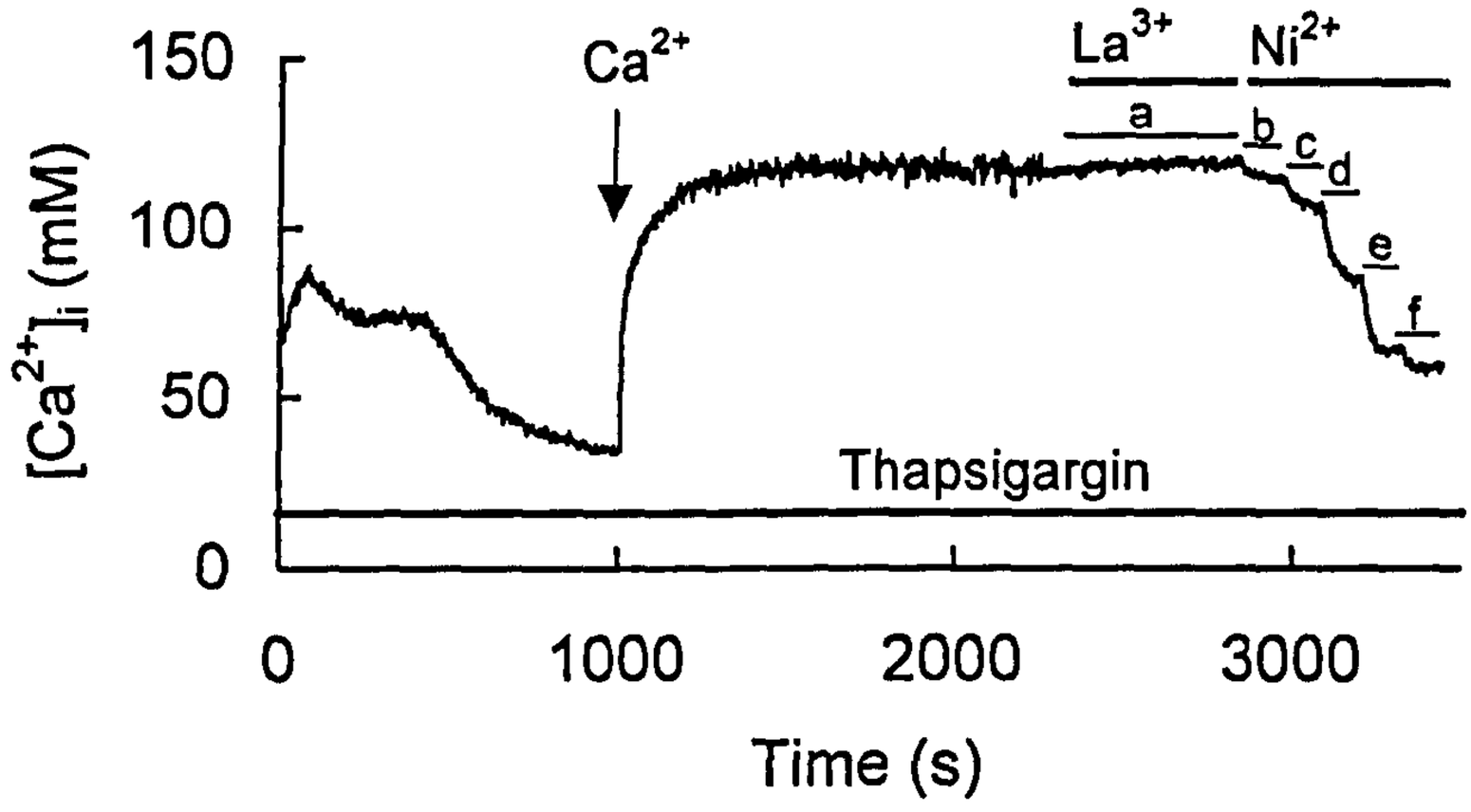


Figure 3.11 The effect of Ni^{2+} on thapsigargin-evoked CCE.
Fura-2 fluorometry was used to measure changes in $[Ca^{2+}]_i$ in DDT_1 MF-2 cells. Internal Ca^{2+} stores were initially depleted with 1 μM thapsigargin in Ca^{2+}-free medium. Re-addition of 1 mM Ca^{2+} to the external milieu produced a rise in $[Ca^{2+}]_i$ reflecting the activation of CCE. ***A***, a sample trace of the effect of Ni^{2+} on $[Ca^{2+}]_i$ at a concentration of: 0.1-10 μM (**a**), 100 μM (**b**), 300 μM (**c**), 1 mM (**d**), 3 mM (**e**), 10 mM (**f**). ***B***, the effect of Ni^{2+} on thapsigargin-evoked Ca^{2+} influx (n=3). Data represented as the mean % inhibition of Ca^{2+} influx (±S.E.M.).
Significant difference from Ni^{2+} (0.1 μM): **P*<0.05, ***P*<0.01, ****P*<0.001, †P<0.0001 (one way ANOVA).

A



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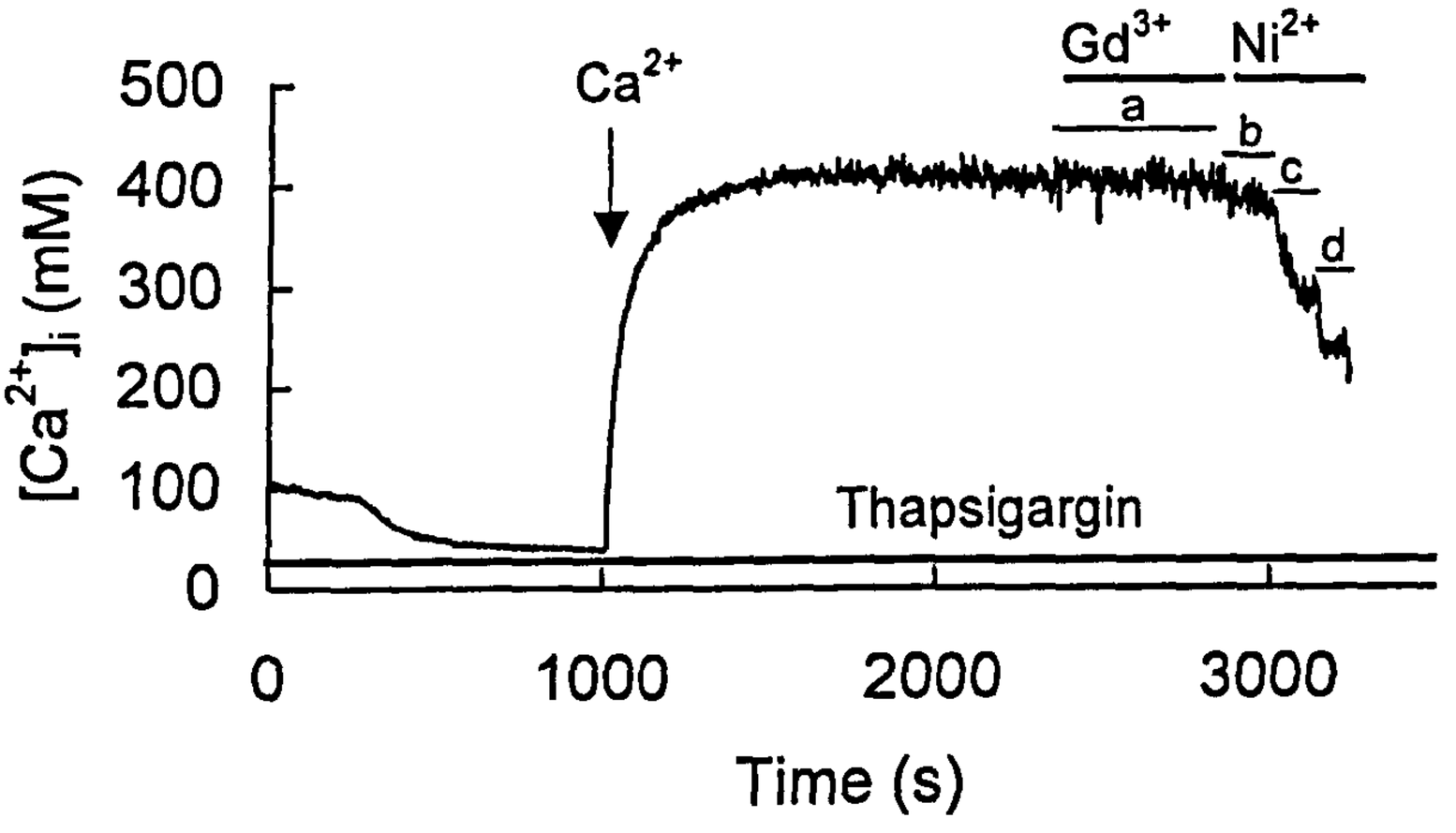

Figure 3.12 The effect of La^{3+} and Gd^{3+} on thapsigargin-evoked CCE.
Fura-2 fluorometry was used to measure changes in $[Ca^{2+}]_i$ in DDT_1 MF-2 cells. Internal Ca^{2+} stores were initially depleted with 1 μM thapsigargin in Ca^{2+}-free medium. Re-addition of 1 mM Ca^{2+} to the external milieu produced a rise in $[Ca^{2+}]_i$ reflecting the activation of CCE. Sample traces of $[Ca^{2+}]_i$ in the presence of *A*, La^{3+} (n=3) at a concentration ranging from 0.01 to 100 μM (**a**) and subsequent addition of Ni^{2+} 100 μM (**b**), 300 μM (**c**), 1 mM (**d**), 3 mM (**e**), 10 mM (**f**); *B*, Gd^{3+} (n=3) at a concentration ranging from 0.01 to 100 μM (**a**) and subsequent addition of Ni^{2+} 100 μM (**b**), 300 μM (**c**), 1 mM (**d**).

produced by 10 μM CP 55,940 to 7.5 ±3.6 pA/ pF (Fig. 3.13, n=9, P<0.0001). When applied alone 1 μM Gd^{3+} produced no observable change in membrane currents (n=5).

These results suggest that CP 55,940 may increase $[Ca^{2+}]_i$ through a CCE pathway, so the effect of Gd^{3+} on thapsigargin-evoked CCE in DDT_1 MF-2 cells was also ascertained. Even at a maximum concentration of 100 μM, Gd^{3+} had no effect on thapsigargin-induced $[Ca^{2+}]_i$, although subsequent Ni^{2+} application clearly inhibited the Ca^{2+} influx (Fig. 3.12B, n=3). This again is consistent with the idea that CP 55,940 activates a Ca^{2+} influx pathway distinct from CCE.

3.1.5 The role of phospholipase A_2 (PLA_2) in AA production

PLA_2 can generate free AA and cannabinoid-induced mobilisation of AA in WI-38 lung fibroblasts has been shown to involve the activities of the $cPLA_2$ subtype (Wartmann *et al.*, 1995).

Arachidonyl trifluoromethyl ketone (ATK), a specific inhibitor of $cPLA_2$ (Street *et al.*, 1993), has been shown to produce significant inhibition of AA release in agonist-stimulated neutrophils (Susztak *et al.*, 1997). At a concentration of 15 μM, ATK had no significant effect on basal AA release in these cells (Susztak *et al.*, 1997). In DDT_1 MF-2 cells, 15 μM ATK alone evoked a transient outward current, which peaked at 25.9 ±4.2 pA/ pF (Fig. 3.14A and C, n=6). Application of ATK significantly reduced the outward current evoked by subsequent administration of 10 μM CP 55,940 to 6.6 ±2.4 pA/ pF (Fig. 3.14A and C, n=7, P<0.0001). However, ATK has been shown to bind to the CB_1 receptor (Koutek *et al.*, 1994) suggesting the decrease in the outward current may be due to an inability of CP 55,940 to successfully bind to the CB_1 receptor. Moreover, the stimulant effect of ATK alone in

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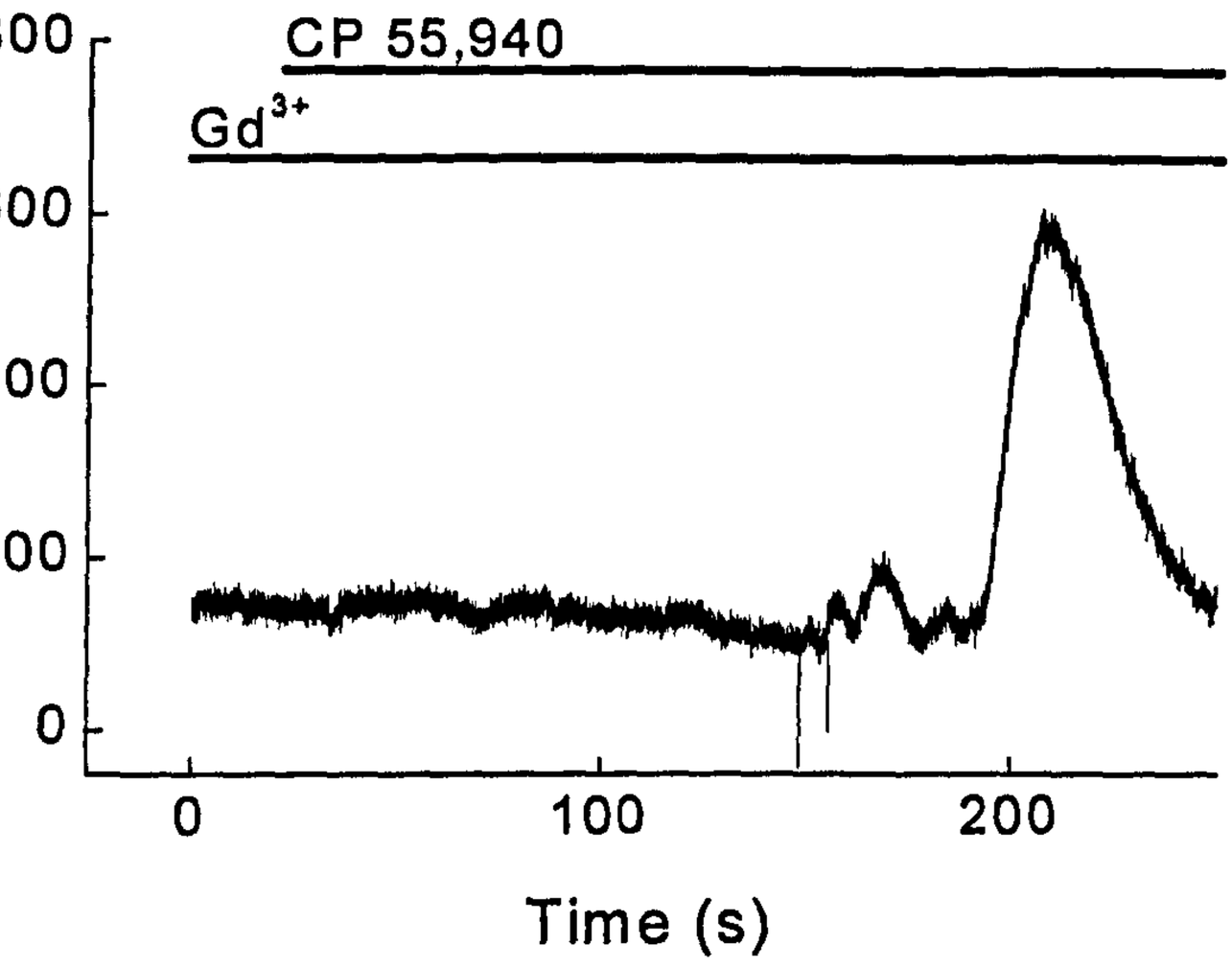

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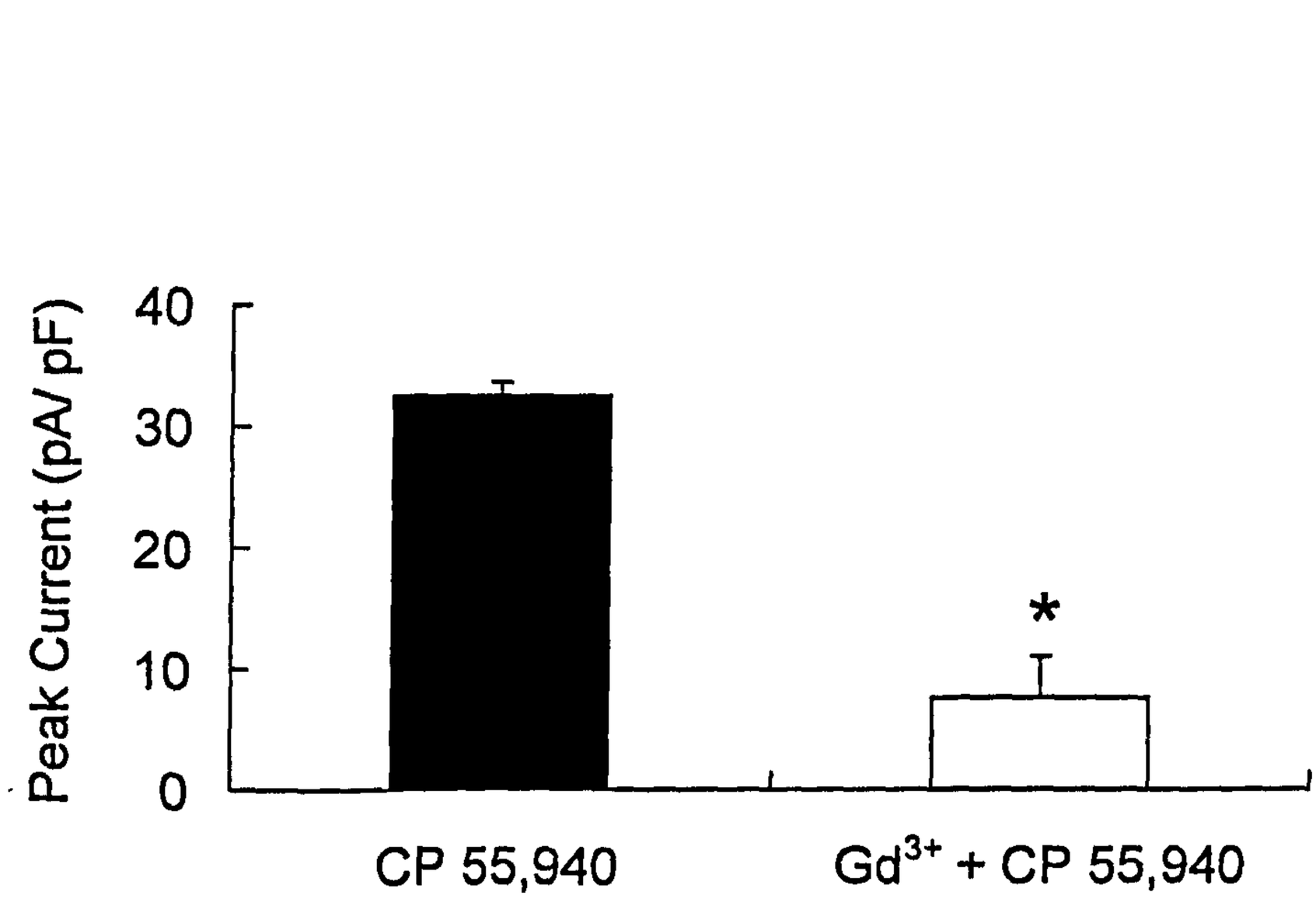

Figure 3.13 The effect of Gd^{3+} on currents evoked by CP 55,940.
Gd^{3+} (1 μM) was applied for 5 minutes prior to CP 55,940 (10 μM) application. *A*, a sample trace of the effect of Gd^{3+} on the CP 55,940-evoked outward current. Horizontal bars indicate the presence of ligands. ***B***, the effect of Gd^{3+} on the mean peak current (±S.E.M.) evoked by CP 55,940 (n=9).
Significant difference from CP 55,940 control: * $P<0.0001$.

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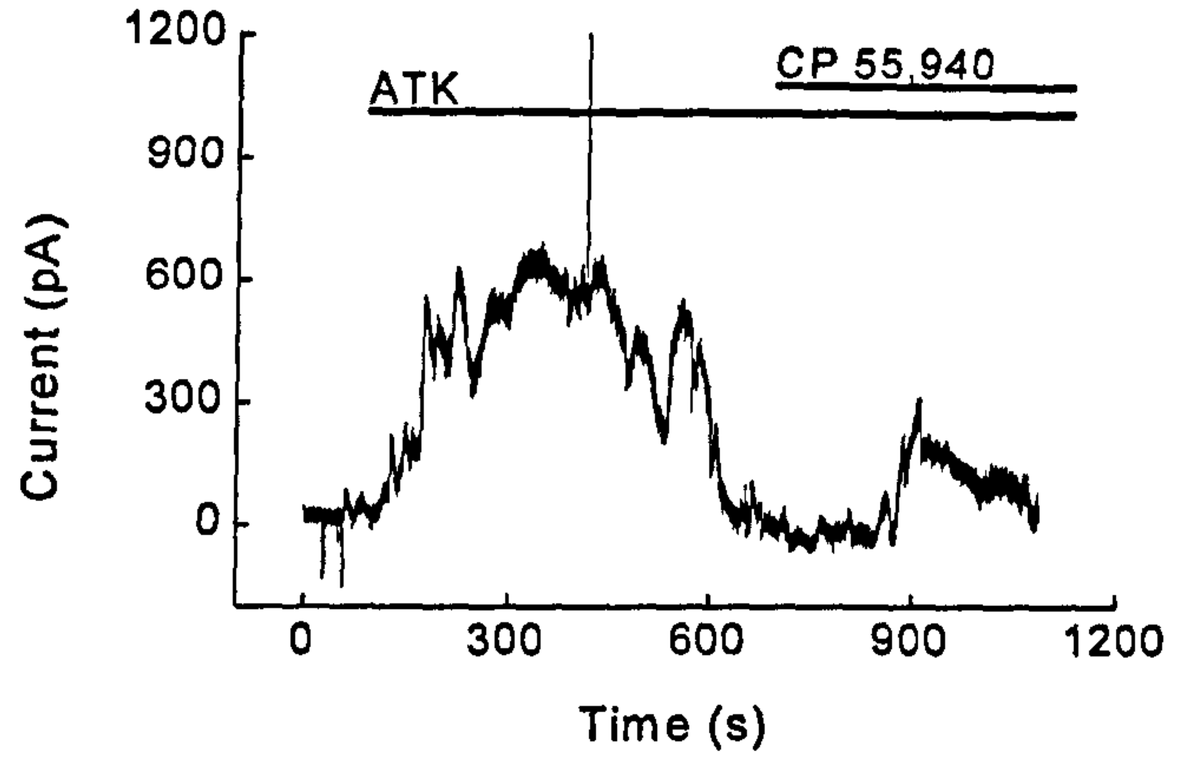

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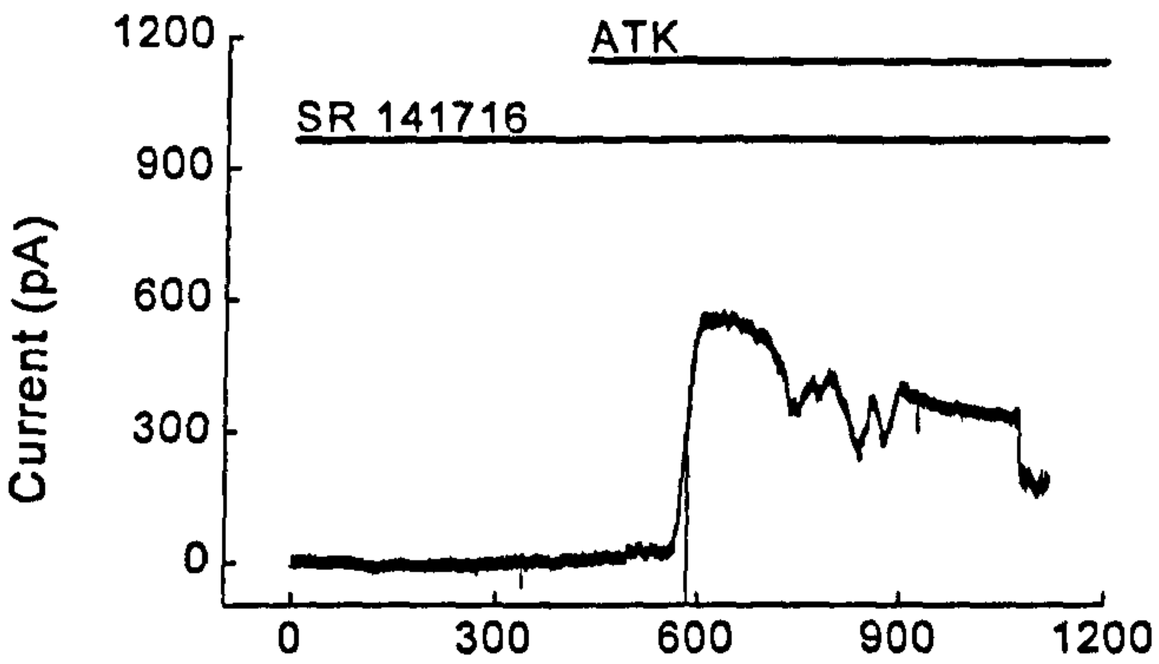

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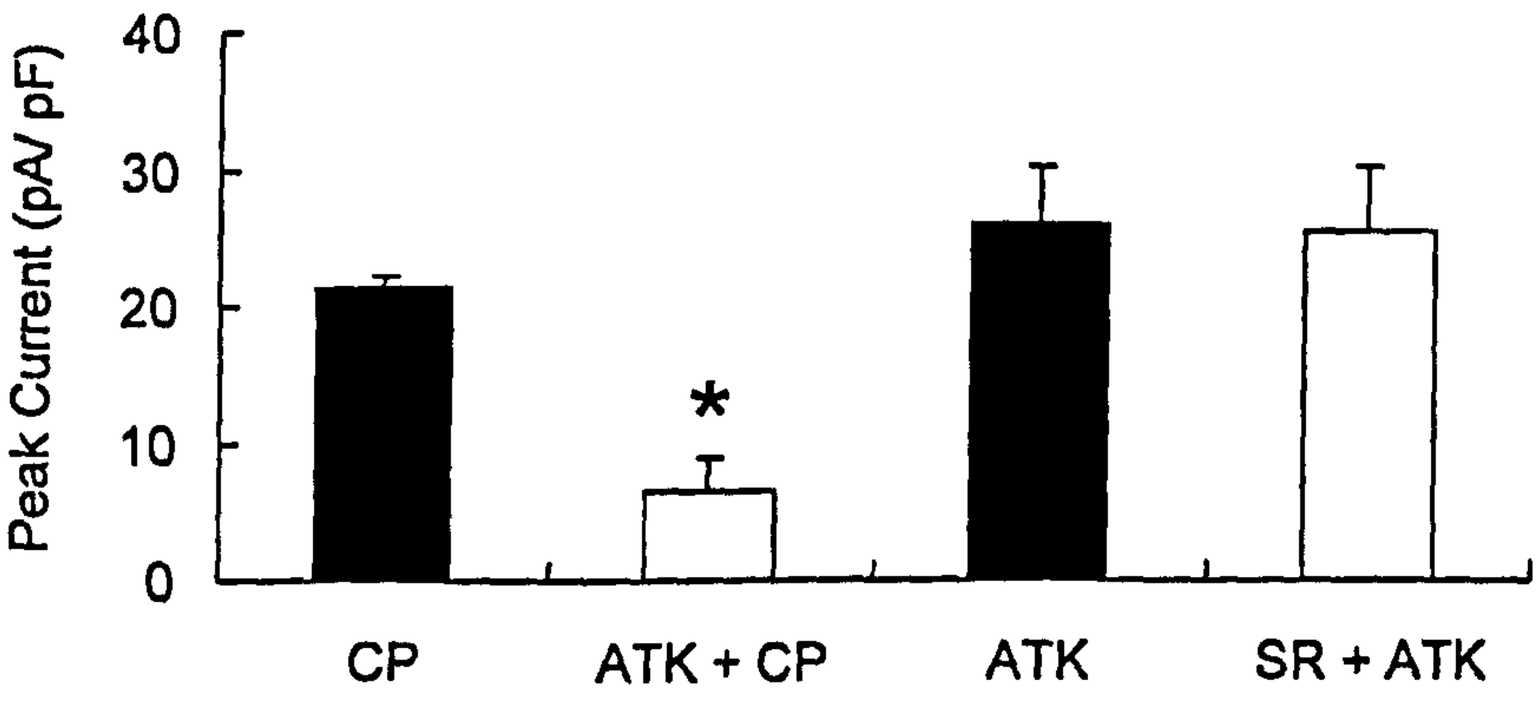


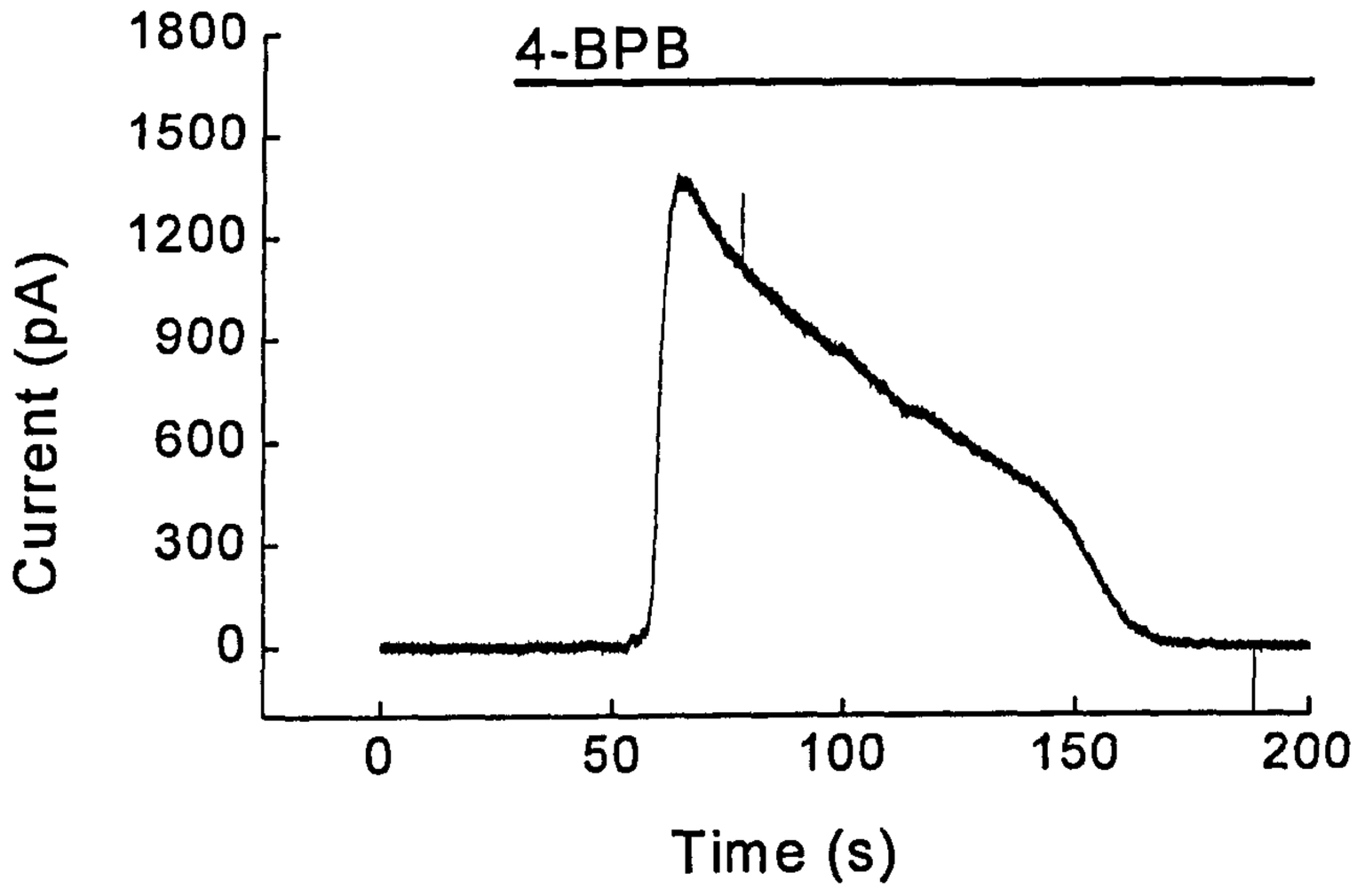
Figure 3.14 The effect of ATK on CP 55,940-evoked currents.
ATK (15 μM) was applied to cells for 10 minutes prior to CP 55,940 (CP, 10 μM) application. SR 141716 (SR, 1 μM) was applied to cells for 5 minutes prior to ATK application. ***A***, a sample trace of the current evoked by ATK (n=6) and its effect on the CP 55,940-evoked current. ***B***, a sample trace of the effect of SR 141716 on the ATK-evoked response. Horizontal bars indicate the presence of ligands. ***C***, the effect of ATK and SR 141716 on the mean peak current (±S.E.M.) evoked by CP 55,940 (n=7) and ATK (n=5) respectively.
Significant difference from CP 55,940 control: **P*<0.0001.

DDT_1 MF-2 cells may suggest the compound also has efficacy at the CB_1 receptor. To address this, the CB_1 receptor antagonist SR 141716 was used to try to inhibit the ATK-evoked response. 1 μM SR 141716 had no significant effect on the outward current evoked by ATK (25.4 ±9.4 pA/ pF, Fig. 3.14B and C, n=5). SR 141716 (1 μM) alone had no effect on membrane currents (n=5).

The non-specific PLA_2 inhibitor 4-bromophenacyl bromide (4-BPB) (Roberts *et al.*, 1977) was also used to investigate the activation of a PLA_2 signalling pathway. At a concentration of 10 μM, 4-BPB alone induced a transient outward current of peak amplitude 49.0 ±5.9 pA/ pF (Fig. 3.15A and D, n=7) and significantly inhibited the CP 55,940 (10 μM)-mediated outward current (6.3 ± 3.1 pA/ pF, Fig. 3.15B and D, n=9, $P<0.0001$). 4-BPB has been shown to activate Ca^{2+} influx in human gingival fibroblasts at concentrations lower than that used to inhibit PLA_2 (Ogata *et al.*, 2002). To further explore the actions of 4-BPB and help establish if the reduction of the CP 55,940-induced response may be due to the inhibition of PLA_2, the effect of CP 55,940 on the outward current induced by 4-BPB was investigated. CP 55,940 (10 μM) significantly reduced the outward current evoked by 10 μM 4-BPB to 8.4 ±4.4 pA/ pF (Fig. 3.15C and D, n=5, $P<0.001$) suggesting that both CP 55,940 and 4-BPB stimulate similar signalling events that would lead to a cross-desensitisation of their responses.

The effects of a second non-specific PLA_2 inhibitor, quinacrine (Lu *et al.*, 2001), were explored. At concentrations of 10 μM and 30 μM, quinacrine inhibited the outward current evoked by 10 μM CP 55,940 to 20.7 ±2.6 pA/ pF (Fig. 3.16C, n=5, $P<0.001$, one way ANOVA) and 20.6 ±2.1 pA/ pF (Fig. 3.16A and C, n=9, $P<0.01$, one way ANOVA) respectively. However, at 100 μM, quinacrine showed no significant effect on the outward current (31.0 ±4.7 pA/ pF, Fig. 3.16B and C, n=6,

A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1500
1200
900
600
300
0
Current (pA)
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50
100
150
200
Time (s)

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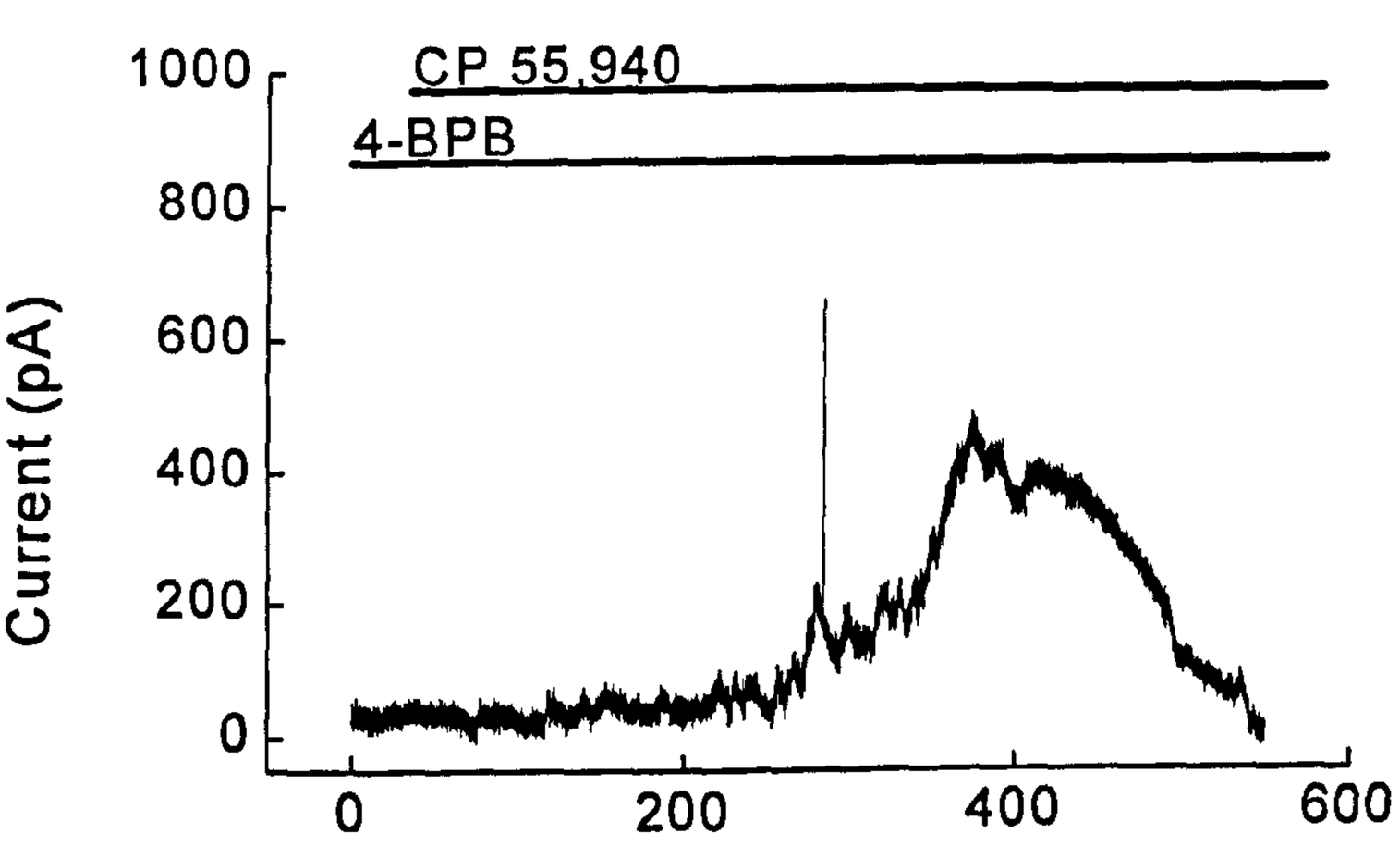
CP 55,940
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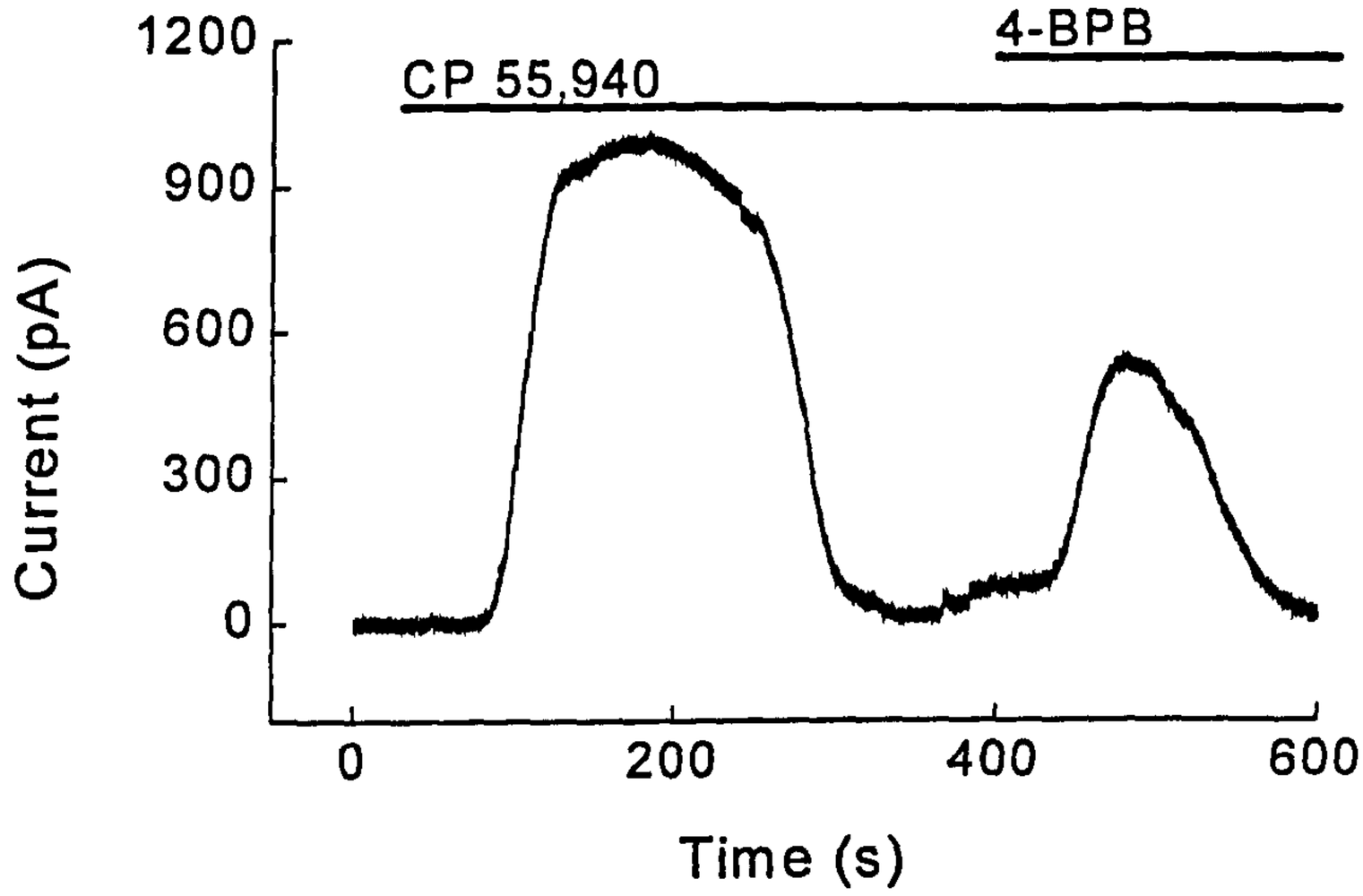
800
600
400
200
0
Current (pA)
0
200
400
600
Time (s)

C



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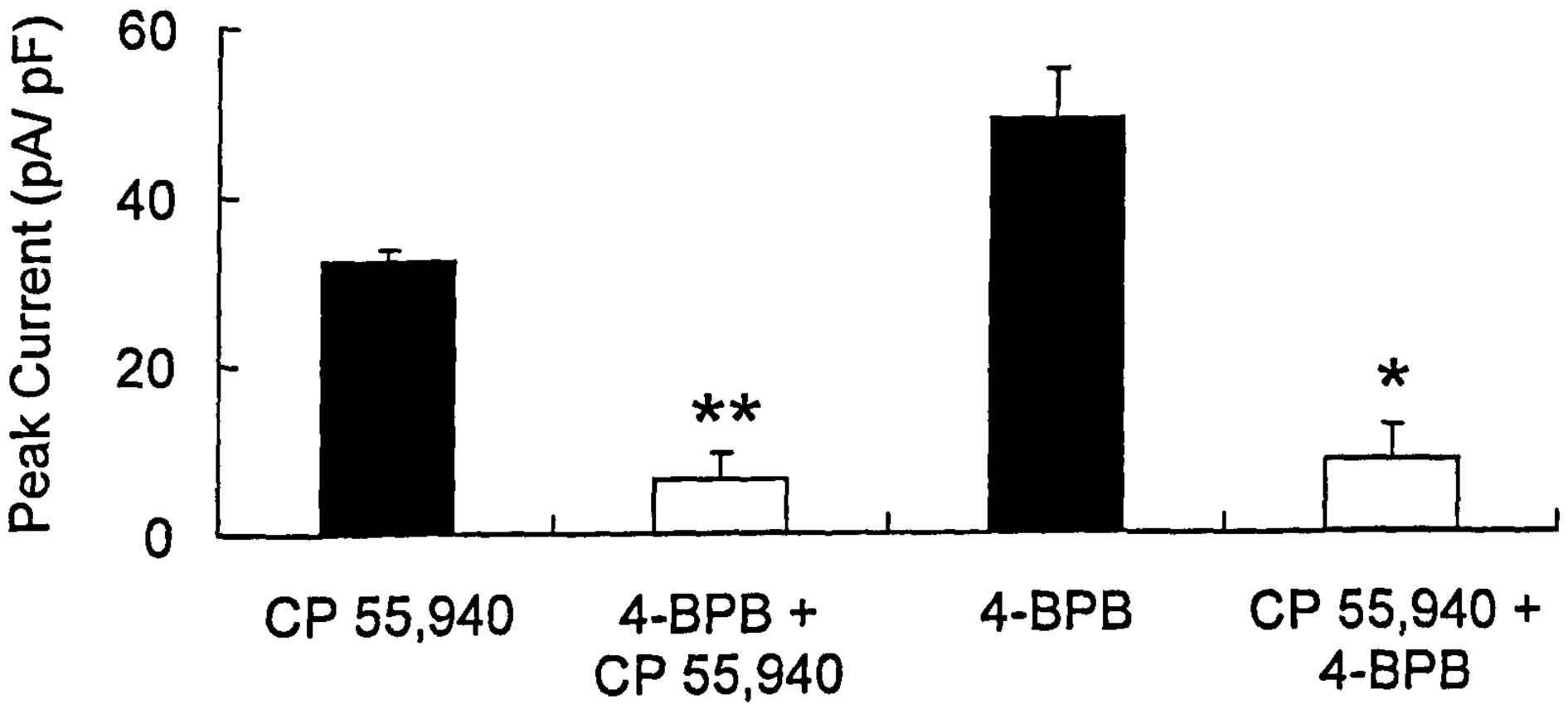
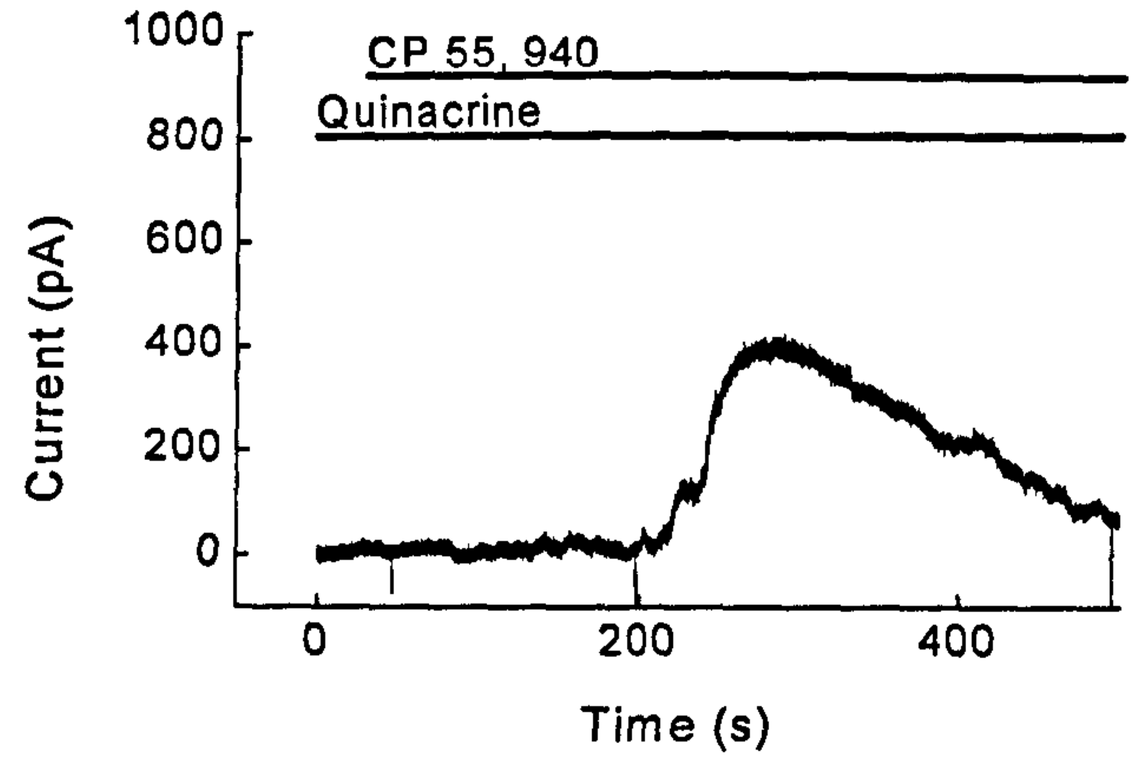


Figure 3.15 The effect of 4-BPB on currents evoked by CP 55,940.
Cells were pretreated with 4-BPB (10 μM) for 10 minutes prior to CP 55,940 (10 μM) application. To determine the effects of CP 55,940 (10 μM) on the 4-BPB (10 μM)-induced response, 4-BPB was applied immediately after CP 55,940-evoked currents had returned to baseline. ***A***, a sample trace of the effect of 4-BPB on resting membrane current. ***B***, a sample trace of the effect of 4-BPB on the outward current evoked by CP 55,940. ***C***, a sample trace of the effect of CP 55,940 on the outward current evoked by 4-BPB. Horizontal bars indicate the presence of ligands. ***D***, the effect of 4-BPB on membrane currents (n=7) and currents induced by CP 55,940 (n=9). The effect of CP 55,940 on currents evoked by 4-BPB (n=5). Data represented as the mean peak current (±S.E.M.).
Significant difference from 4-BPB control: $*P<0.001$.
Significant difference from CP 55,940 control: $**P<0.0001$.

A



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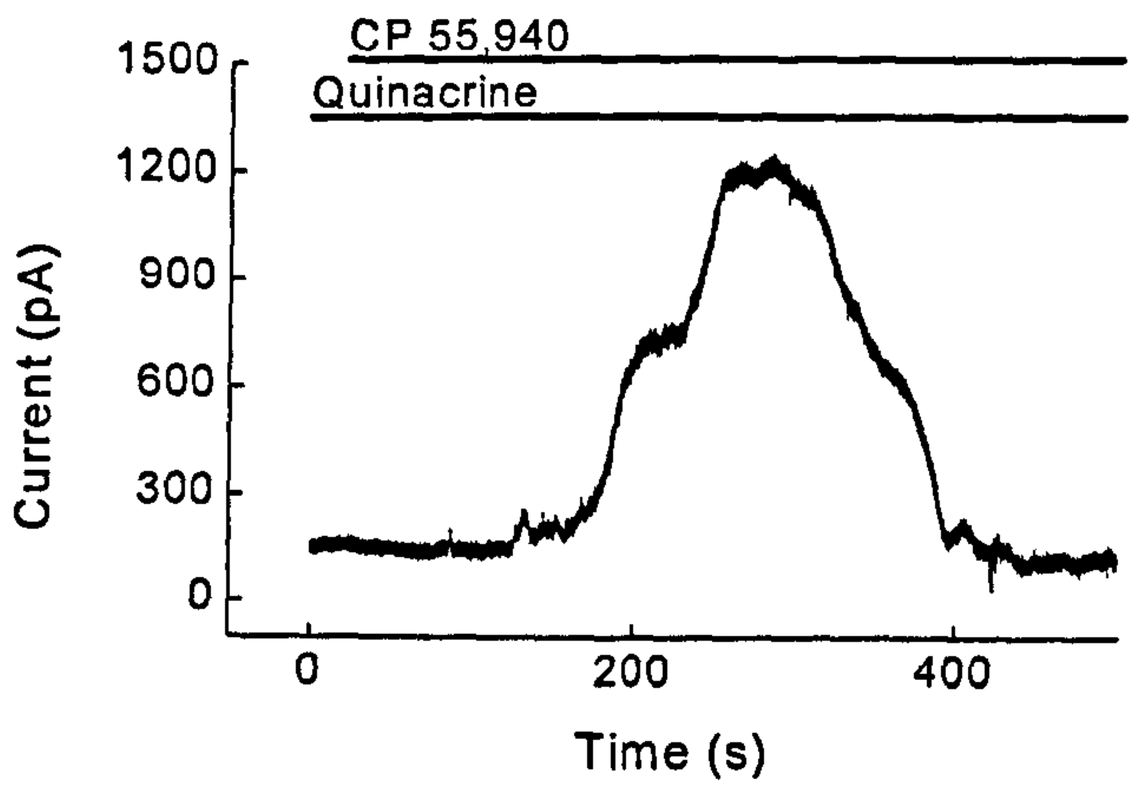

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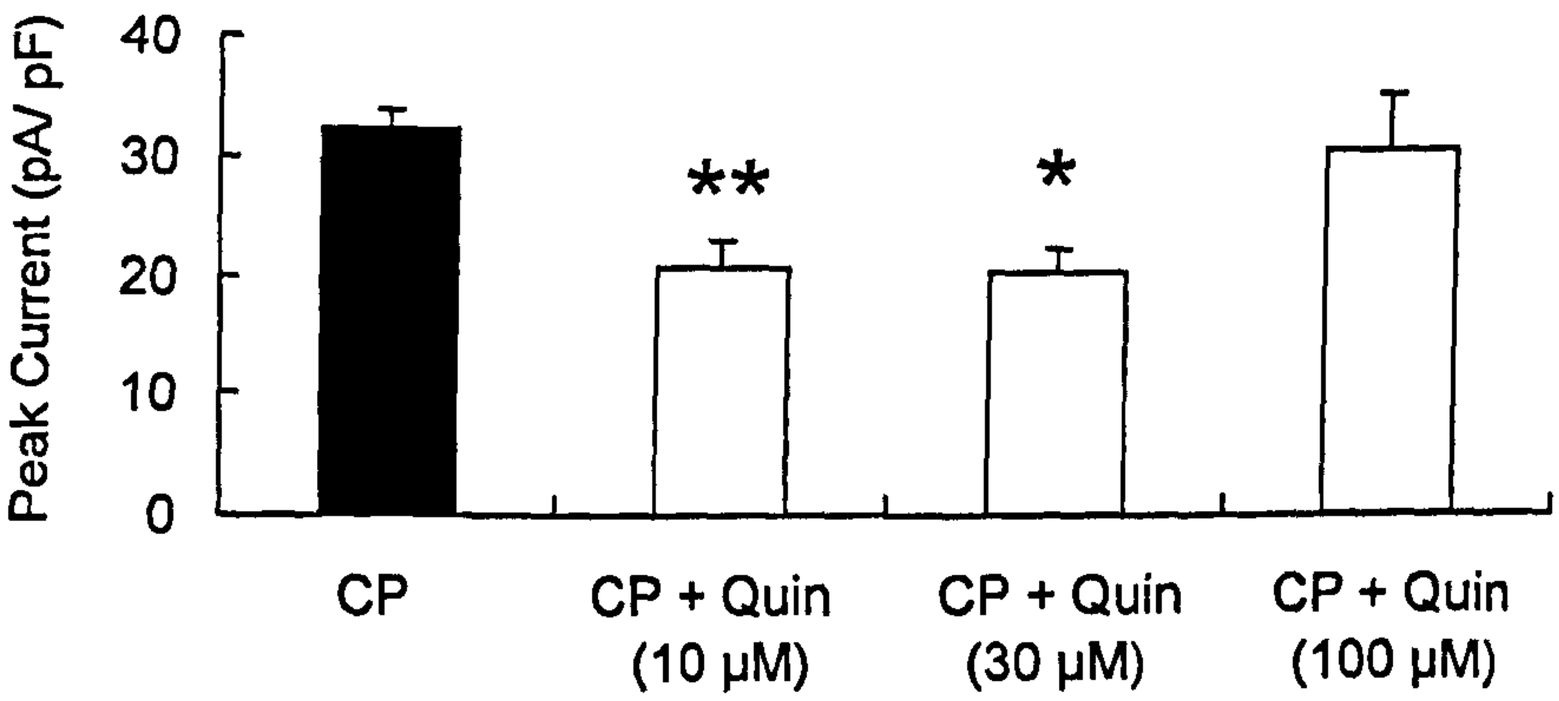


Figure 3.16 The effect of quinacrine on outward currents evoked by CP 55,940. Quinacrine (Quin, 10-100 μM) was applied to cells for 10 minutes prior to CP 55,940 (CP, 10 μM) application. ***A***, a sample trace of the effect of quinacrine (30 μM) on the outward current induced by CP 55,940. ***B***, a sample trace of the effect of quinacrine (100 μM) on the CP 55,940-induced response. Horizontal bars indicate the presence of ligands. ***C***, the effect of quinacrine, at a concentration of 10 μM (n=5), 30 μM (n=9) and 100 μM (n=6), on the mean peak current (±S.E.M.) evoked by CP 55,940. Significant difference from CP 55,940 control: **P*<0.01, ***P*<0.001.

one way ANOVA). Quinacrine (100 μM) alone had no effect on membrane currents in DDT_1 MF-2 cells (n=4). Previous reports have shown that quinacrine can directly modulate $I_{K,Ca}$ (Vanheel *et al.*, 1999). To add further support to the electrophysiological data, and hence a CB_1 receptor-mediated activation of PLA_2, the effect of quinacrine on CP 55,940-evoked [^{3}H]AA release was established (an assay independent of $I_{K,Ca}$ activation). Quinacrine (100 μM) abolished the CP 55,940 (1 μM)-evoked AA release (107.8 ±10.4% of basal, Fig. 3.17, n=11, $P<0.05$). Alone quinacrine (100 μM) had no significant effect on basal [^{3}H]AA efflux (116.9 ±19.8% of basal, Fig. 3.17, n=10).

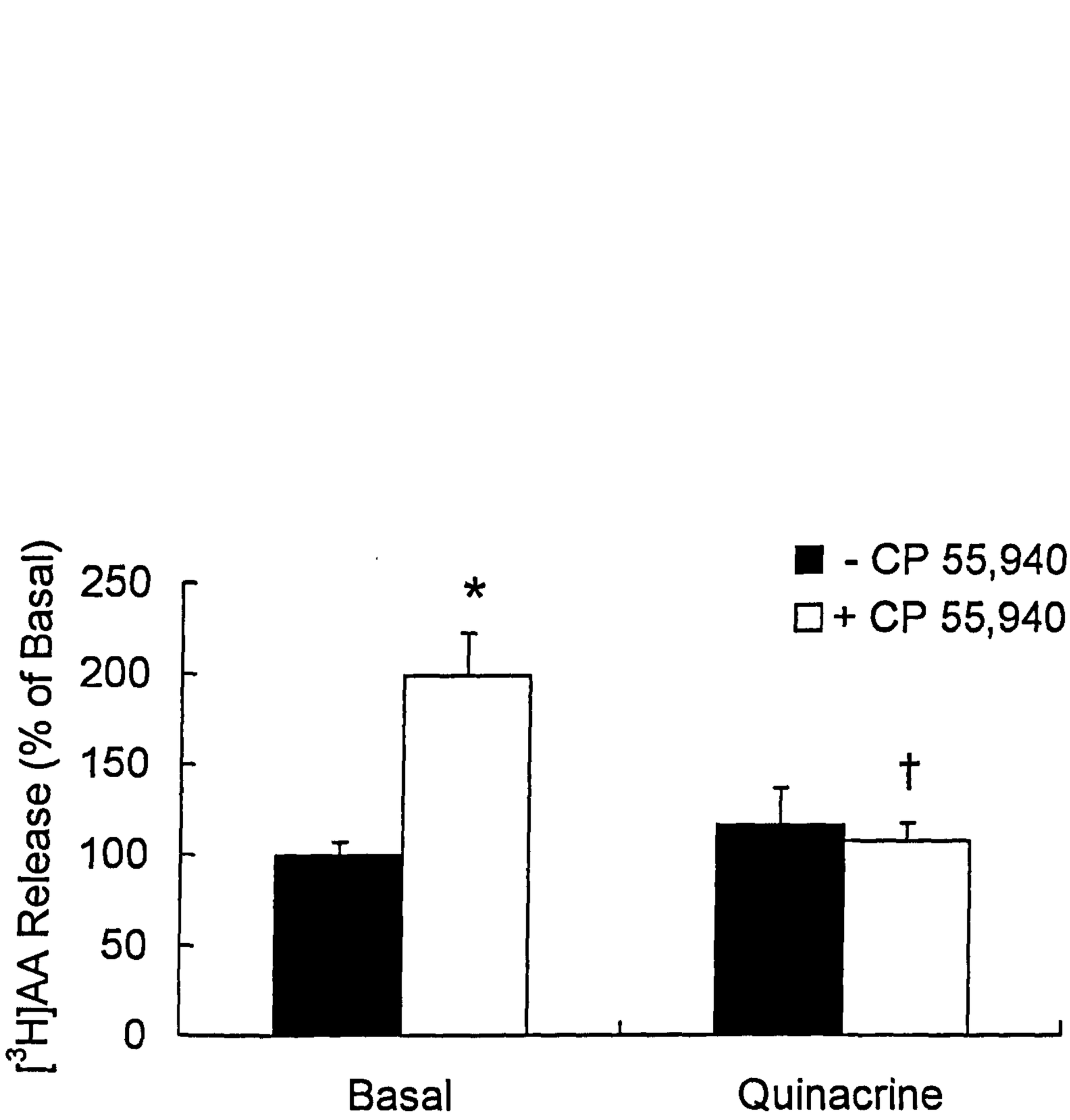


Figure 3.17 The effect of quinacrine on the CP 55,940-induced release of [^{3}H]AA. [^{3}H]AA-pre-labelled DDT_1 MF-2 cells were stimulated with CP 55,940 (1 μM) and the release of radiation measured (n=30). These results were compared to [^{3}H]AA measurements taken from cells pre-treated with quinacrine (100 μM; for 15 minutes), before stimulation with CP 55,940 (n=11). To establish the effects of quinacrine, [^{3}H]AA release was measured from cells treated with quinacrine alone (n=10). Basal AA release was taken as the amount of [^{3}H]AA released in response to vehicle (DMSO, 0.1%). Data represented as the mean release of [^{3}H]AA (±S.E.M.), taken as a % of basal.
Significant difference from basal (DMSO control): *P<0.01.
Significant difference from CP 55,940 control: †P<0.05.

3.2 Western blot analysis of p42/44 MAP kinase in DDT_1 MF-2 cells

Previous studies have demonstrated a CB_1 receptor-mediated activation of p42/44 MAP kinase (Bouaboula *et al.*, 1995b, 1999; Davis *et al.*, 2003). To confirm the activation of a p42/44 MAP kinase in DDT_1 MF-2 cells, in response to CB_1 receptor stimulation, western blot analysis was carried out on lysates prepared from cells treated with CP 55,940 (10 μM). Cells were stimulated for times ranging between 30 seconds and 30 minutes and a specific phospho-antibody was used to detect the phosphorylation of p42/44 MAP kinase.

An upward trend was observed with CP 55,940 (10 μM) in ethanol (Fig. 3.18A, n=3), reflecting a possible time-dependent activation of p42/44 MAP kinases. Densitometry was used to quantify the effects of CP 55,940 on MAP kinase phosphorylation, revealed by western blot analysis (Fig. 318B). However, these results showed that there was no significance change in p42/44 phosphorylation at any of the time points tested, when compared to control (phosphorylation at t=0).

The effects of vehicle (ethanol 0.1%) on p42/44 phosphorylation were established. Ethanol was also shown to phosphorylate p42/44 MAP kinase (Fig. 3.18A, n=3). In all cases the phosphorylation was as intense, if not greater, when compared to the results for corresponding time points for CP 55,940-activated cells. This suggests that the possible effects observed with CP 55,940 could be due to the activity of the ethanol.

To determine if the phosphorylation was due to stimulation of the CB_1 receptor, SR 141716 (1 μM) was used to try and inhibit the increase in phosphorylation observed in the presence of CP 55,940 (data not shown). However, these results were inconsistent as SR 141716 was shown to have varying effects on

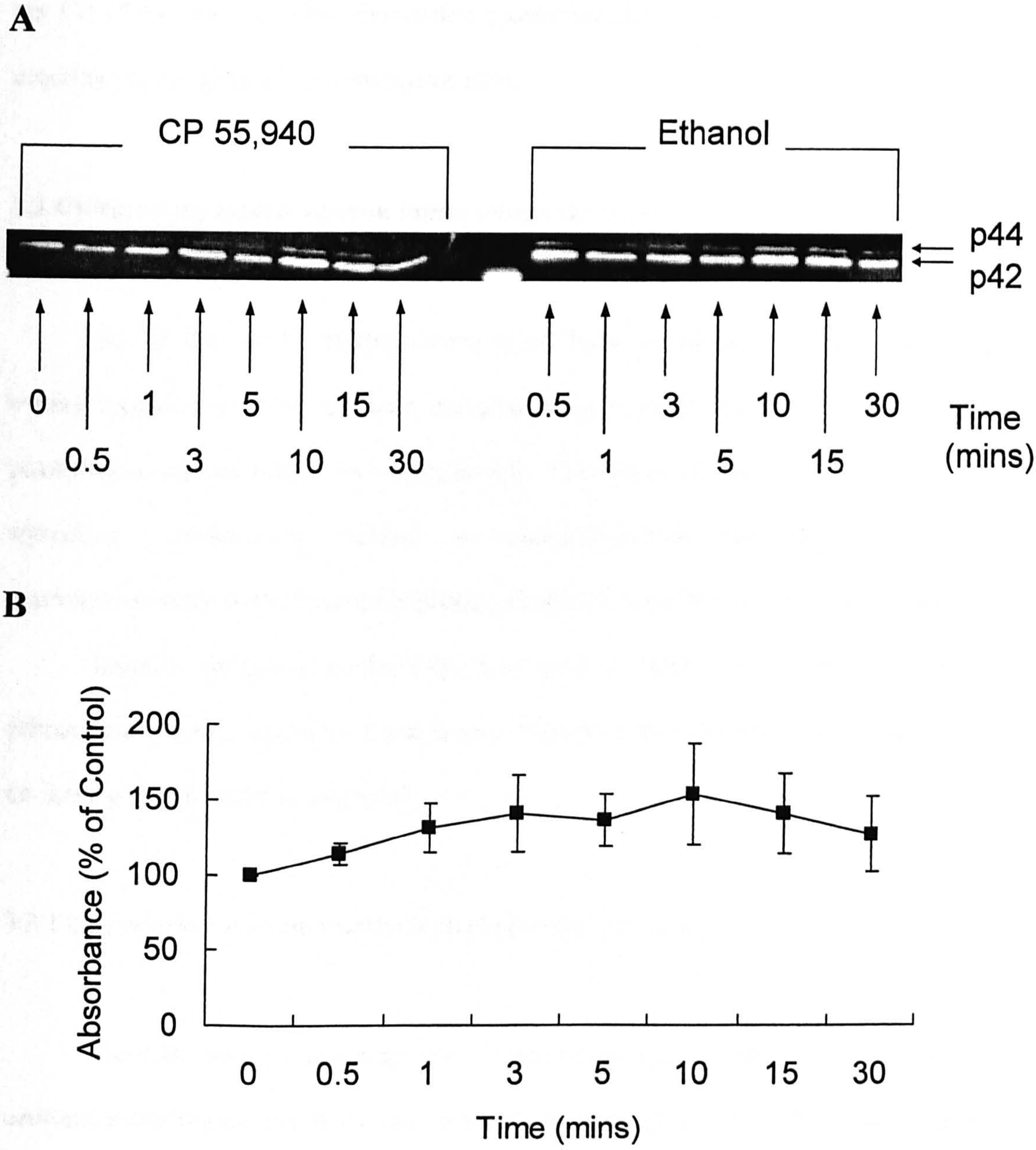


Figure 3.18 Western blot analysis showing the time-dependent effect of CP 55,940 on p42/44 MAP kinase phosphorylation, in DDT_1 MF-2 cells.
A, DDT_1 MF-2 cells were stimulated with either CP 55,940 (10 μM, n=3) or ethanol (0.1%, n=3) at time points ranging between 30 seconds and 30 minutes. Cell lysates were then separated by SDS-PAGE electrophoresis and probed with anti-phospho-p42/44 antibody. ***B***, densitometry was used to quantify CP 55,940-mediated changes in p42/44 MAP kinase phosphorylation, revealed by western blot analysis. Data represented as the mean absorbance (±S.E.M.) as a % of control (absorbance at t=0).

the CP 55,940-induced phosphorylation (potentiate, inhibit or have no effect), which could never be repeated on subsequent blots.

3.3 Cultured myenteric neuron immunohistochemistry

So far the results of the current study have concentrated on the signalling events mediated by CB_1 receptor stimulation on smooth muscle cells, a possible postsynaptic site of action for cannabinoids. The following work, investigating the signalling mechanisms behind a cannabinoid-mediated modulation of neurotransmission in the myenteric plexus, identifies possible neuronal sites of action.

Initially, immunohistochemistry was used to validate myenteric neurons in primary culture as a model for those *in situ*. Following this, the effect of cannabinoids on these cultures could be explored.

3.3.1 CB_1 receptor immunoreactivity on cholinergic neurons

The CB_1 receptor antibody showed dense labelling of all cultured myenteric neurons, including the cell body and neuronal processes (Fig. 3.19). Neuronal cultures also exhibited dual labelling with both the ChAT and CB_1 receptor antibody suggesting the expression of CB_1 receptors on cholinergic neurons (Fig. 3.20).

The number of ChAT-positive cells was counted, and the proportion of these that were also positive for CB_1 receptor protein was determined. 97.5 ±1.5% of ChAT-positive cells expressed CB_1 receptors (n=556 ChAT-positive cells from 5 different cultures with a minimum of 52 cells/ culture), while 100% of CB_1-positive cells were cholinergic.

Figure 3.19 Immunostaining of cultured myenteric neurons with CB_1 receptor antibody.

C-terminus CB_1 receptor antibody was used to label myenteric neurons in primary culture. Intense staining was observed over the entire neurons, including the cell bodies and the neuronal processes.

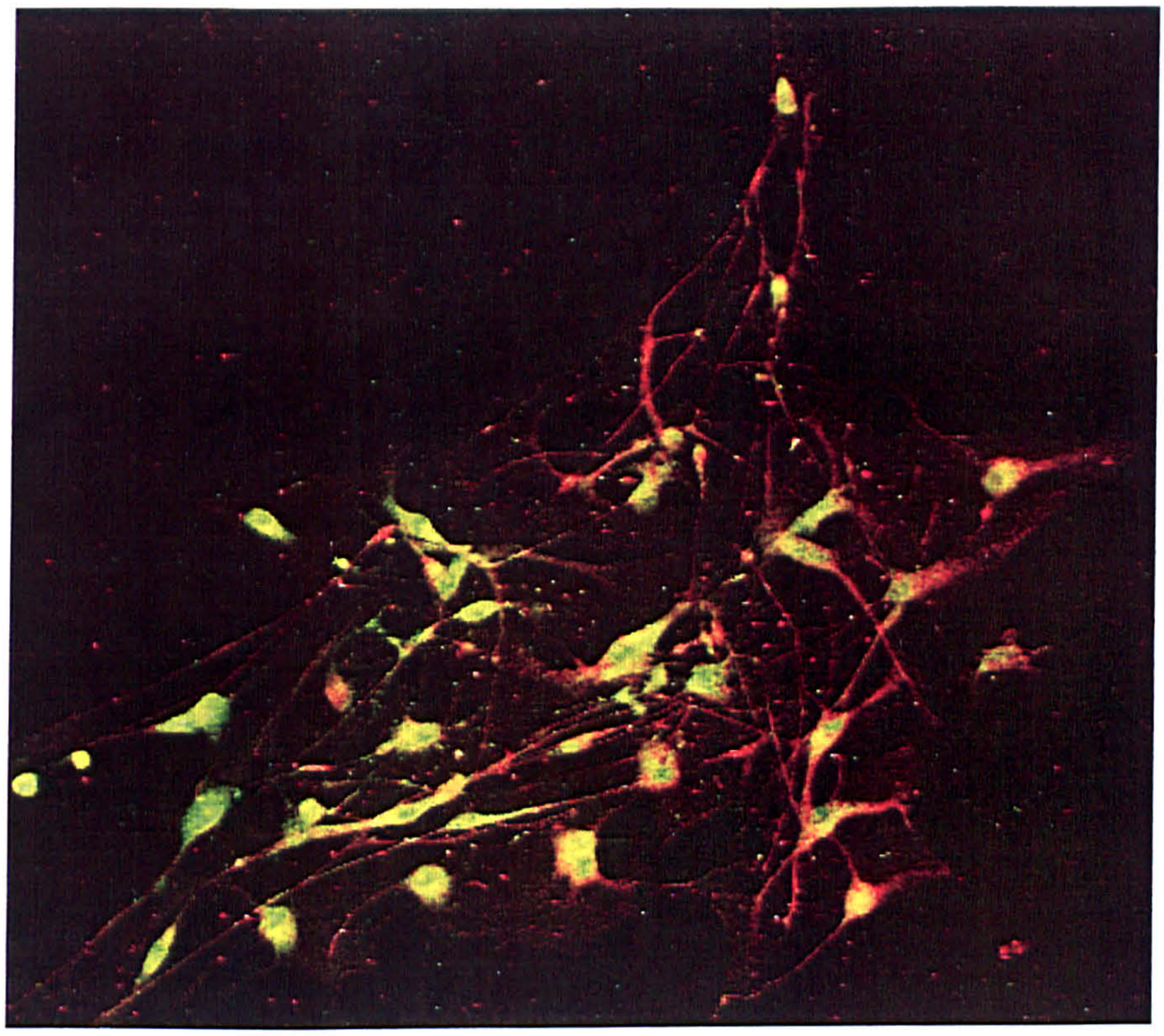


Figure 3.20 Dual labelling of cultured myenteric neurons with CB_1 receptor antibody and markers for cholinergic neurons.
Cultured myenteric neurons were labelled with ChAT antibody (in green) and then co-labelled with C-terminus CB_1 receptor antibody (in red). Yellow corresponds to overlap of the two antibodies and therefore co-localisation.
Cholinergic neurons are present in culture and are shown to express cannabinoid CB_1 receptors.

3.3.2 Neurofilament (NF) immunostaining

Immunostaining of NF protein was performed in cultured myenteric neurons as, *in situ*, cells labelled with the NFP-200 antibody show the cell morphology much more clearly than antibodies for CB_1 receptors or ChAT. The NF antibody labelled a variety of cultured myenteric neurons, different in size, morphology and intensity of labelling of the soma (Fig. 3.21A).

There were generally 3 types of neurons present: (1) neurons that showed bright, dense labelling of the soma, leaving a defined cell outline (Fig. 3.21A) (2) cells with less dense cytoplasmic labelling but with clear staining of individual intracellular neurofilaments and the outline of a large, ovid nucleus (Fig. 3.21B and C) (3) neurons that did not label with NFP-200 antibody at all (Fig. 3.21C).

3.3.3 Double labelling of CB_1 receptor immunoreactivity with antibody against NF protein

Virtually all NFP-labelled cells expressed CB_1 receptors (Fig. 3.21C) but only a subset of CB_1-positive cells expressed NF protein (Fig. 3.21D), while several CB_1-positive cells did not label for NF protein at all (Fig. 3.21C). Due to the variation in the type of NFP labelling, which suggests different types of cells, and the great variability in proportion of cells labelled in different fields, it was not possible to give an accurate quantification of the total prevalence of NFP-labelled cells.

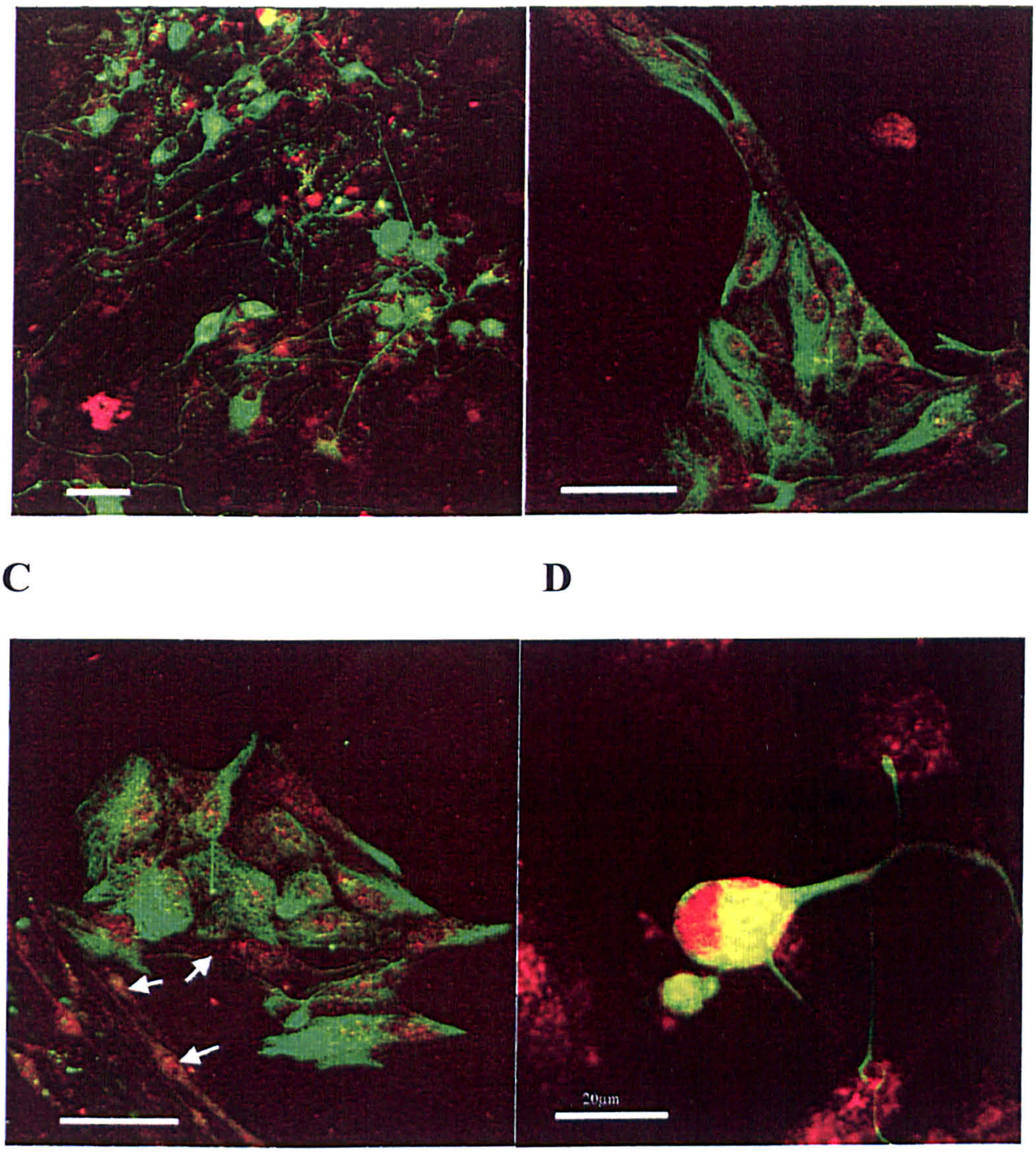


Figure 3.21 Characterisation of cultured myenteric neurons by double labelling with neurofilament (NF) antibody and CB_1 antibody

Cultured myenteric neurons were labelled with C-terminus CB_1 receptor antibody and NFP-200. Red corresponds to CB_1 receptor label and green to NF. Yellow corresponds to overlap of the two antibodies and therefore co-localisation. ***A***, NFP labels a variety of cells, showing clear differences in size and morphology. ***B***, NFP-positive cells express CB_1 receptors. ***C***, several CB_1-positive cells do not express NF protein (indicted by arrows). ***D***, enlarged image of a single cell, labelled with both antibodies. Scale bars = 50 μM unless otherwise stated.

3.4 Patch clamping of myenetric neurons in primary culture

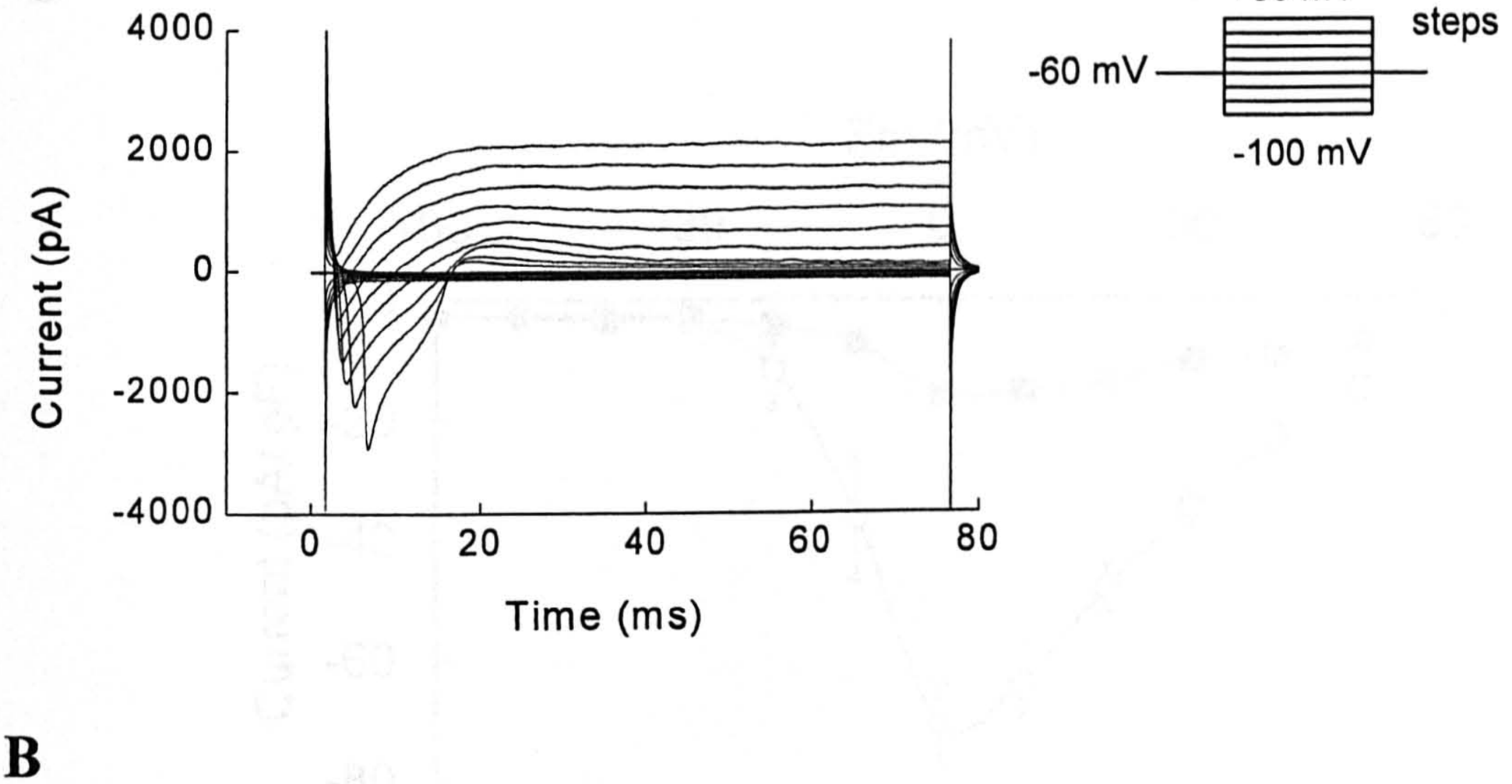
3.4.1 Identification of voltage-dependent ion channels: isolation of Ca^{2+} currents

A voltage step protocol was used to determine the voltage-dependent ion channels present in cultured myenteric neurons. Membrane potentials were stepped from –100 mV to +50 mV, in 10 mV increments, while the resulting effect on membrane current was recorded. The voltage steps evoked a fast inward current, followed by a slower, more prolonged inward current, and finally an outward current (Fig. 3.22A). Fast inward currents were blocked by the voltage-dependent Na^+ channel inhibitor TTX (300 nM), while outward currents were blocked by a combination of Cs^+ in the pipette solution and TEA (10 mM), suggesting the activation of voltage-dependent K^+ channels (Fig. 3.22B). Slower-activating, more prolonged, inward Ca^{2+} currents were then unmasked (Fig. 3.22B) and inhibited by Cd^{2+} (0.1 mM, Fig. 3.22C and D, n=3), a non-specific Ca^{2+} channel antagonist (Bian *et al.*, 2004). Threshold for the activation of Ca^{2+} currents was at –30 mV, and the peak inward current was recorded at a test potential of 0 mV (71.3 ±6.8 pA/ pF, Fig. 3.22D, n=11).

3.4.2 Action potential generation

Under current clamp, action potentials were evoked in myenteric neurons by increasing current steps that depolarised the membrane (~20 mV membrane deflection by each current step) (Fig. 3.23, n=9). Even at strong depolarisations only a single action potential was generated by each current step. This may be due to the long AHP

A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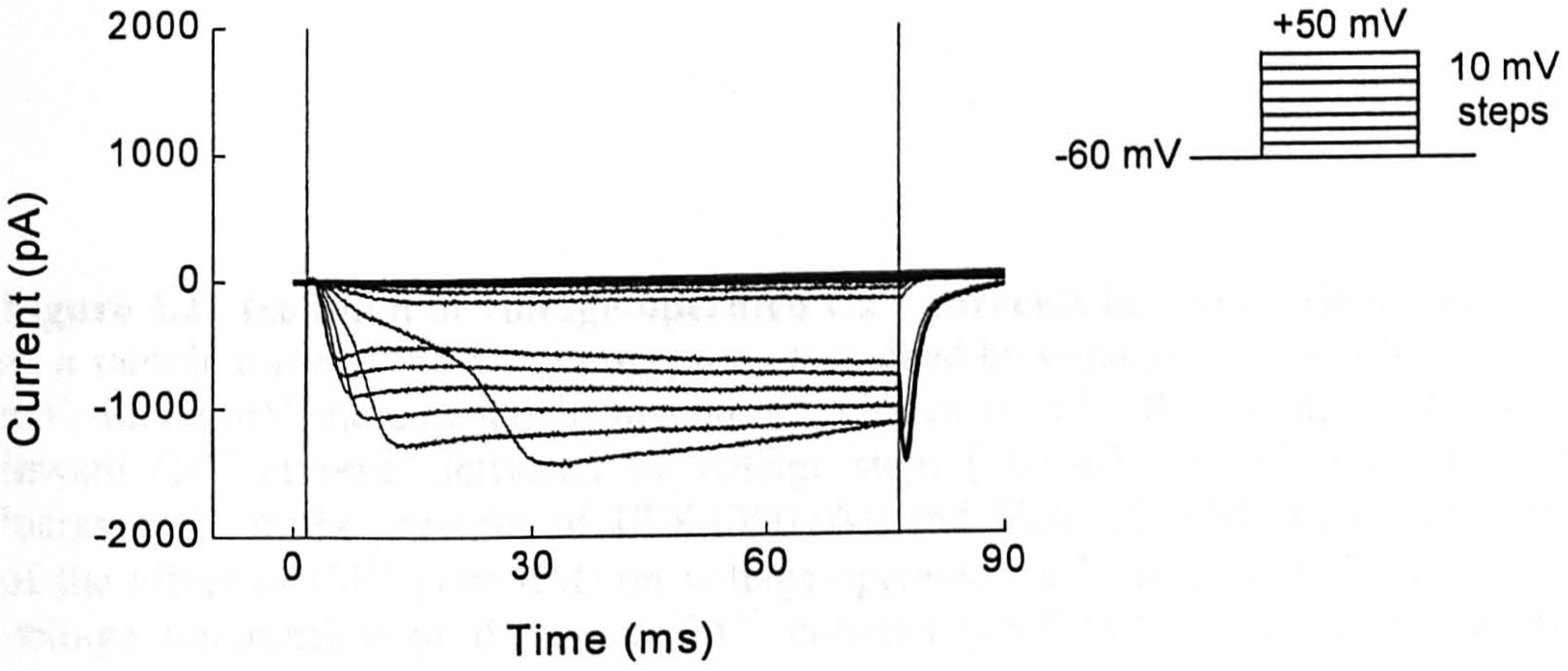
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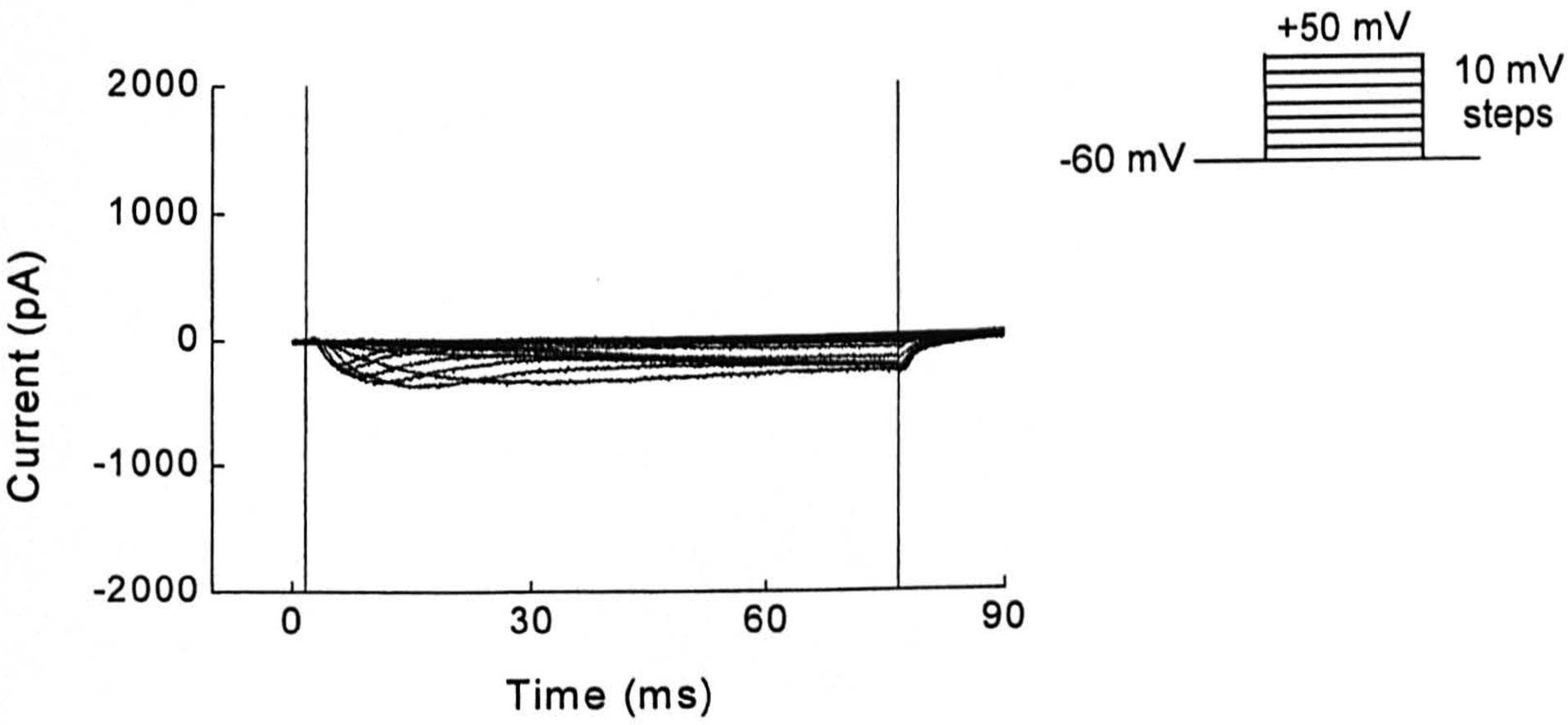
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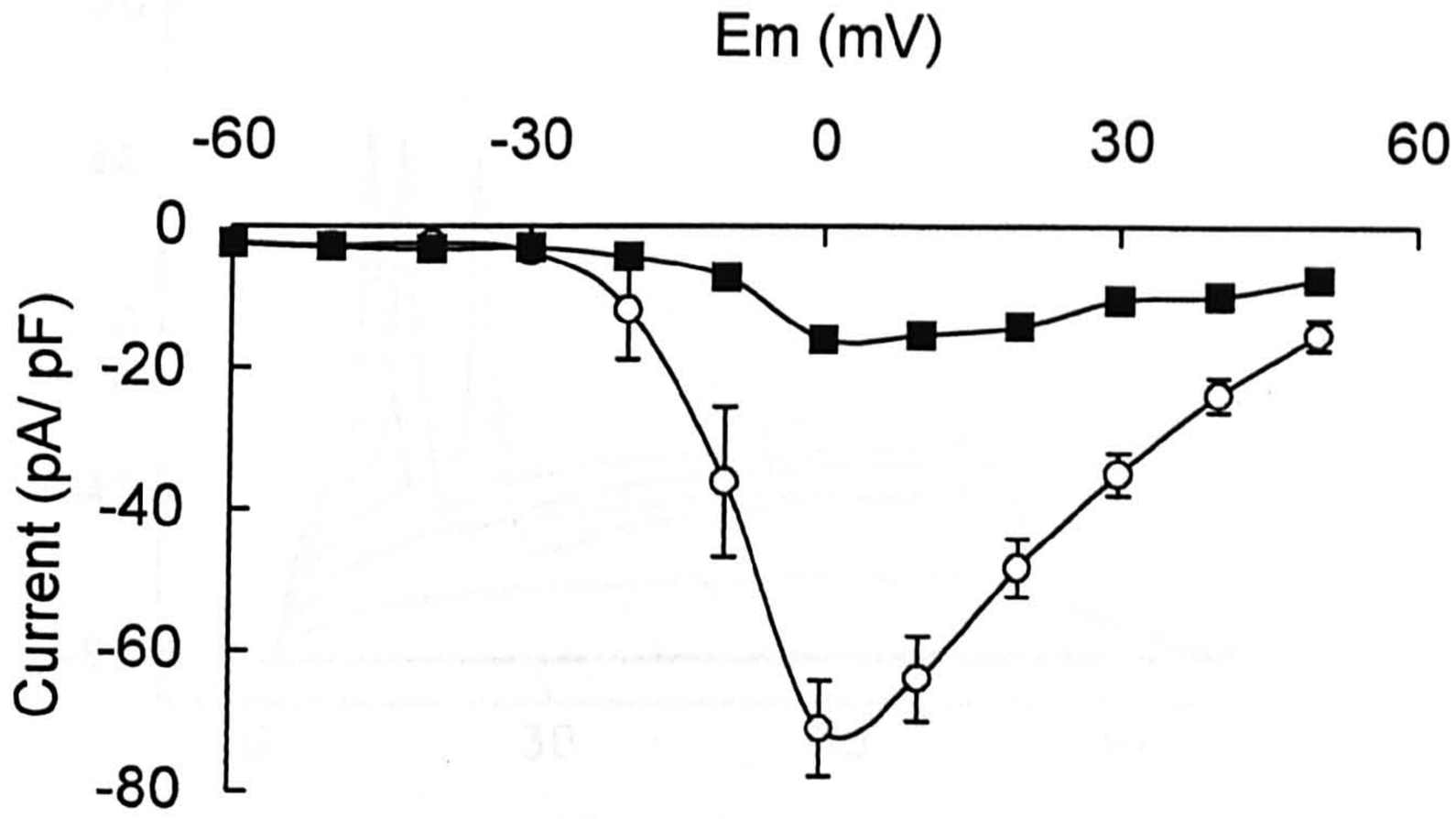


Figure 3.22 Isolation of voltage-operated Ca^{2+} currents in myenteric neurons. *A*, a sample trace of the membrane currents evoked by voltage steps (–100 mV to +50 mV, in 10 mV increments) in myenteric cultures (n=12). ***B***, a sample trace of the inward Ca^{2+} currents activated by voltage steps (–60 mV to +50 mV, in 10 mV increments), in the presence of TTX (300 nM) and TEA (10 mM). ***C***, a sample trace of the effect of Cd^{2+} (100 μM) on voltage-operated Ca^{2+} currents (n=3). ***D***, current-voltage relationship of the mean Ca^{2+} currents (±S.E.M.) evoked in the absence (○, n=11) and presence of Cd^{2+} (■, n=3).

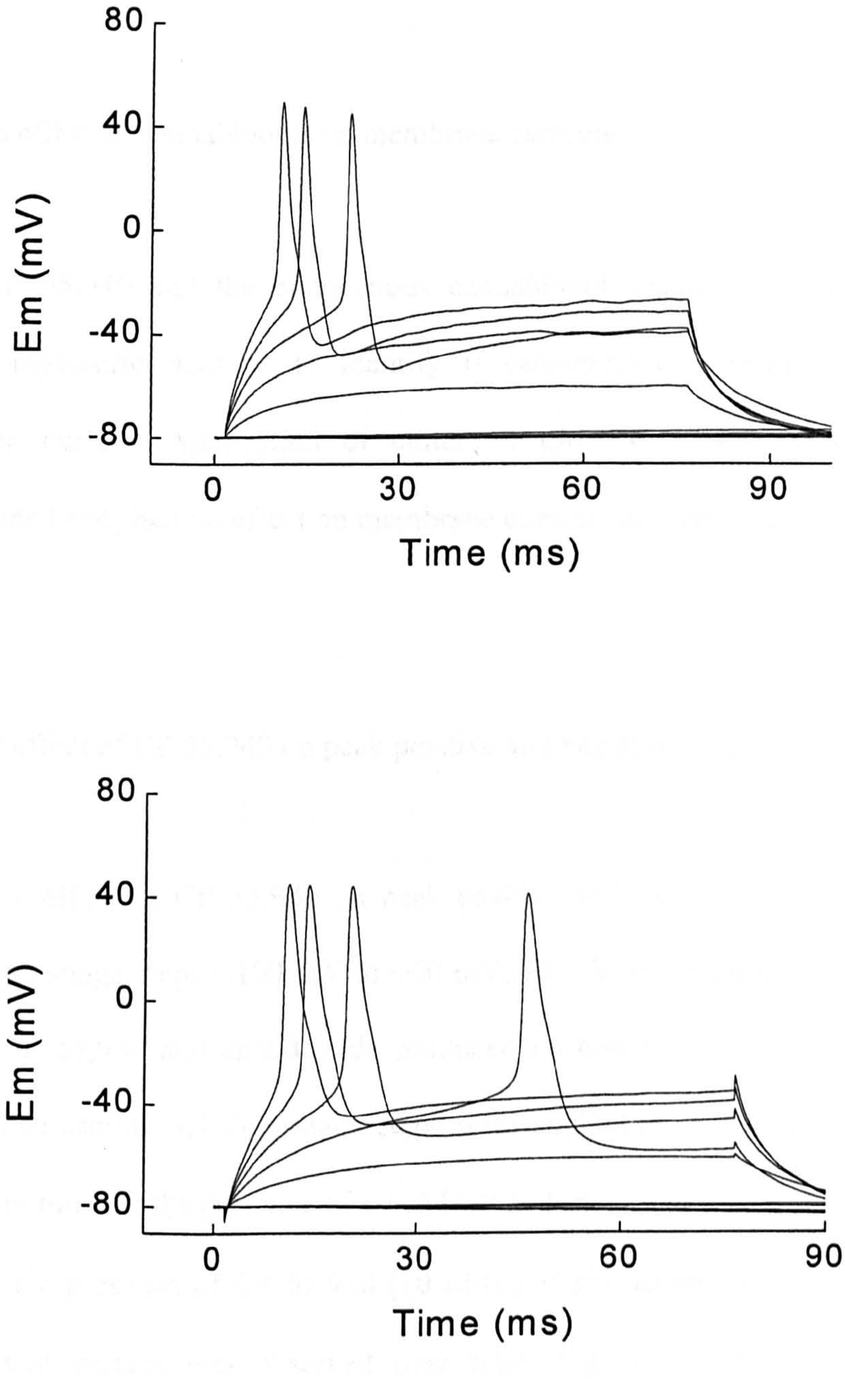


Figure 3.23 Action potential generation in myenteric neurons.
Under current clamp, action potentials were evoked in myenteric neurons by increasing current steps (~20 mV membrane deflection by each current step) that depolarised the membrane (n=9). Each trace was recorded from a separate cell, held initially at around –80 mV (membrane potential), by sustained current injection.

observed after the propagation of each action potential, which persisted till the end of current injection.

3.4.3 The effect of cannabinoids on membrane currents

CP 55,940 and the endogenous cannabinoid anandamide were applied to cultured myenteric neurons to identify if cannabinoids evoked any change in membrane current. Application of either 10 μM CP 55,940 (n=4) or 10 μM anandamide (n=4) had no effect on membrane currents in myenteric neurons (data not shown).

3.4.4 The effect of CP 55,940 on peak positive and negative membrane currents

The effect of CP 55,940 on peak positive and negative membrane currents evoked by voltage steps (-100 mV to +50 mV, 10 mV increments) was investigated. As both CP 55,940 and anandamide produced no observable change in membrane currents (see section 3.4.3), voltage steps were applied at 30 second intervals for a total of 5 minutes in the presence of 10 μM CP 55,940.

In the presence of CP 55,940 (10 μM) a slight decline in both peak positive and negative current was observed over time (Fig. 3.24, n=9). However, these reductions in peak positive and negative membrane current were not significant, when compared to currents evoked in the absence of CP 55,940 (Fig. 3.24).

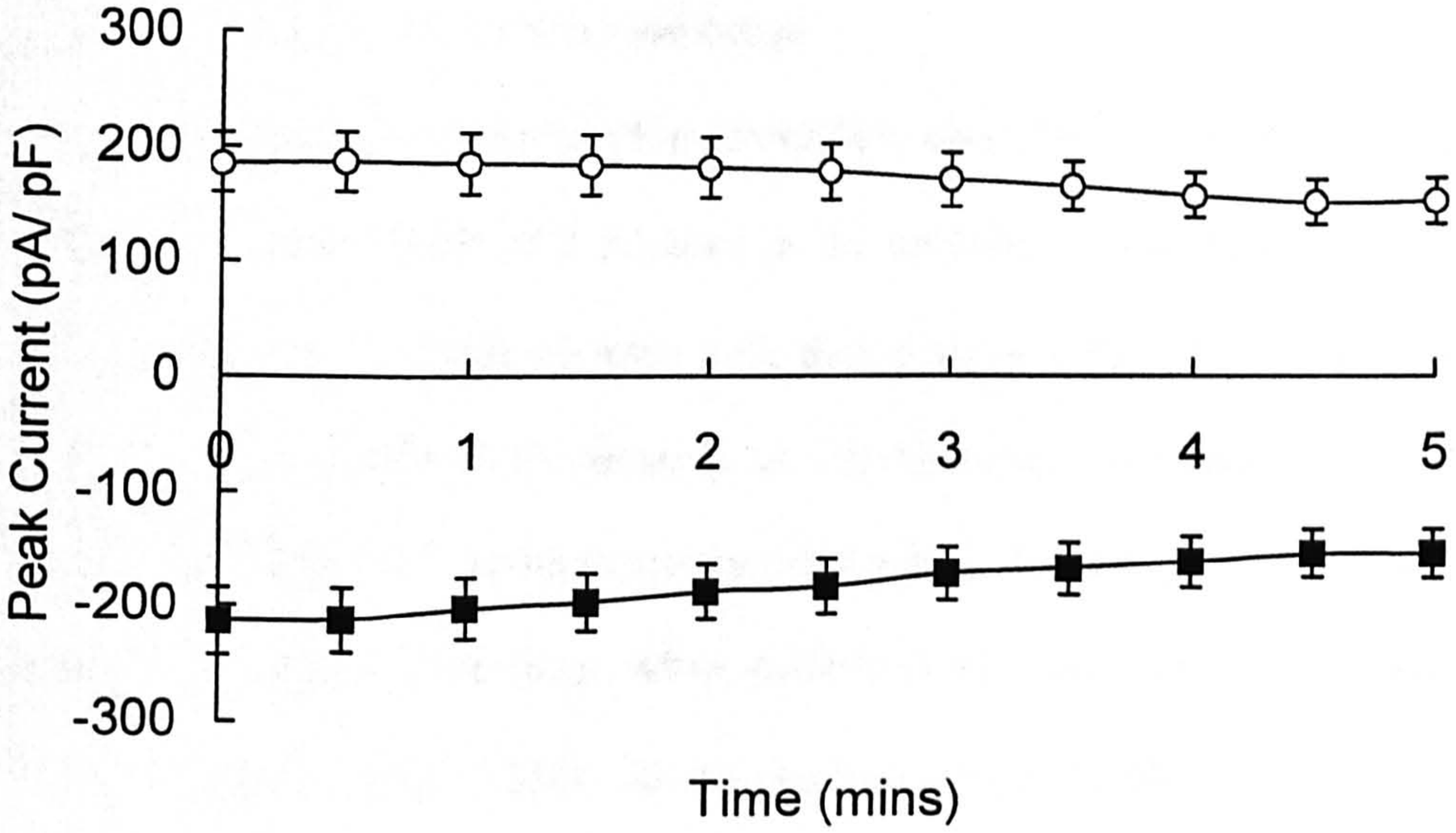


Figure 3.24 The effect of CP 55,940 on peak positive and negative membrane currents evoked in cultured myenteric neurons.

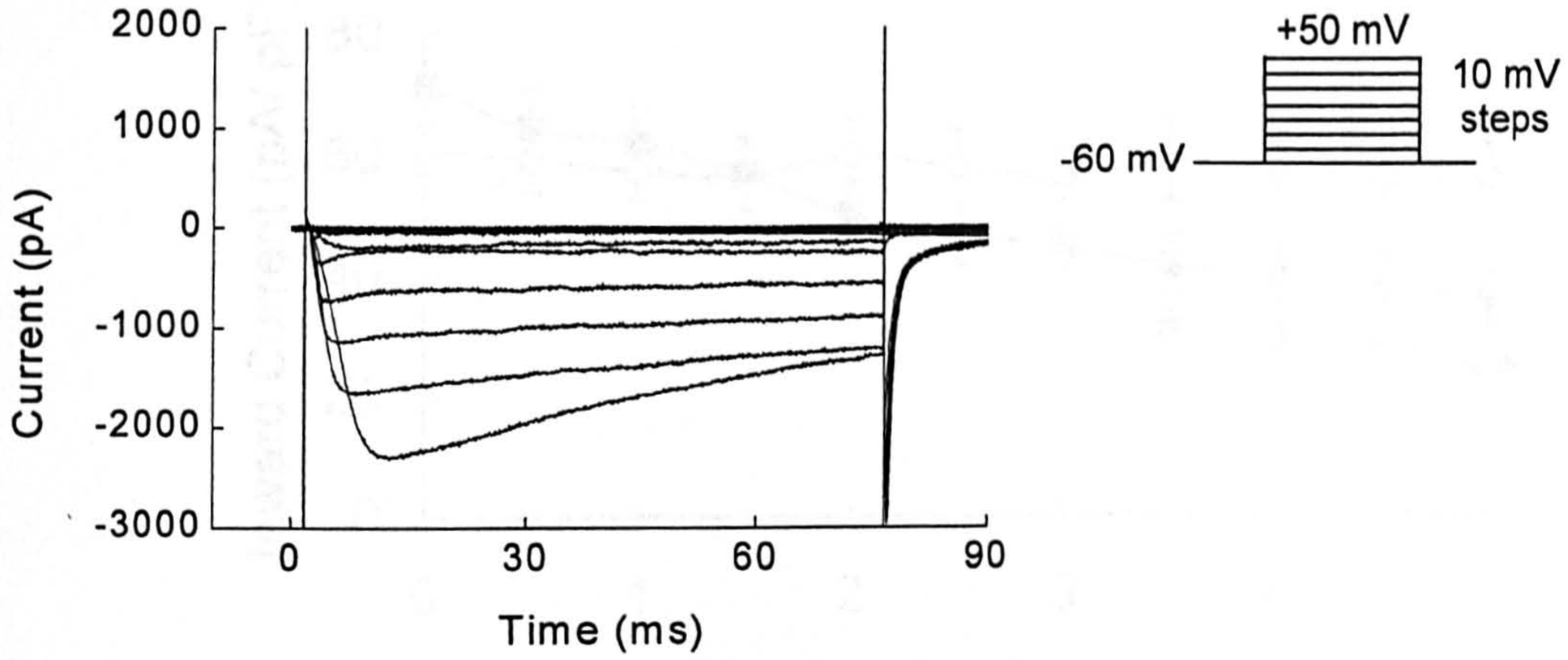
Peak positive (○) and negative (■) membrane currents were recorded in neurons stepped from –100 mV to +50 mV, in 10 mV increments. The voltage step protocol was subsequently applied every 30 seconds for a total of 5 minutes, in the presence of 10 μM CP 55, 940 (n=9). Data represented as the mean peak current (±S.E.M.).

3.4.5 The effect of CP 55,940 on voltage-operated Ca^{2+} channels (VOCCs)

Previous studies have shown that activation of the CB_1 receptor can inhibit VOCCs, including N-, L- and P/Q-type Ca^{2+} channels (Mackie *et al.*, 1995; Twitchell *et al.*, 1997; Hampson *et al.*, 1998; Gebremedhin *et al.*, 1999) and this could be a mechanism by which cannabinoids inhibit neurotransmitter release in the myenteric plexus. Therefore the effect of CP 55,940 on voltage-dependent Ca^{2+} currents in cultured myenteric neurons was established.

Ca^{2+} currents were evoked as previously described (see section 3.4.1), every 30 seconds over a period of 5 minutes in the presence of CP 55,940 (10 μM, Fig. 3.25A and B, n=11). These currents were then compared to Ca^{2+} currents evoked in the presence of vehicle (0.1% ethanol), at corresponding time points (Fig. 3.25C, D and E, n=10). Peak Ca^{2+} currents (evoked at 0 mV) in the presence of ethanol did not significantly change over time, when compared to peak currents induced in the absence of ethanol (Fig. 3.25D). In the presence of CP 55,940 (10 μM), peak Ca^{2+} currents were significantly inhibited after 3.5 minutes compared to ethanol controls, and at 5 minutes the greatest inhibition was observed (36.7% inhibition compared to corresponding ethanol time-point, Fig. 3.25D). The absolute decrease in peak current (taken as the difference in current at t=0 and t=5 minutes) was significantly greater in the presence of 10 μM CP 55,940 (35.8 ±6.5 pA /pF, n=11) compared to ethanol (5.1 ±2.1 pA/ pF, Fig. 3.25E, n=10, $P<0.001$).

A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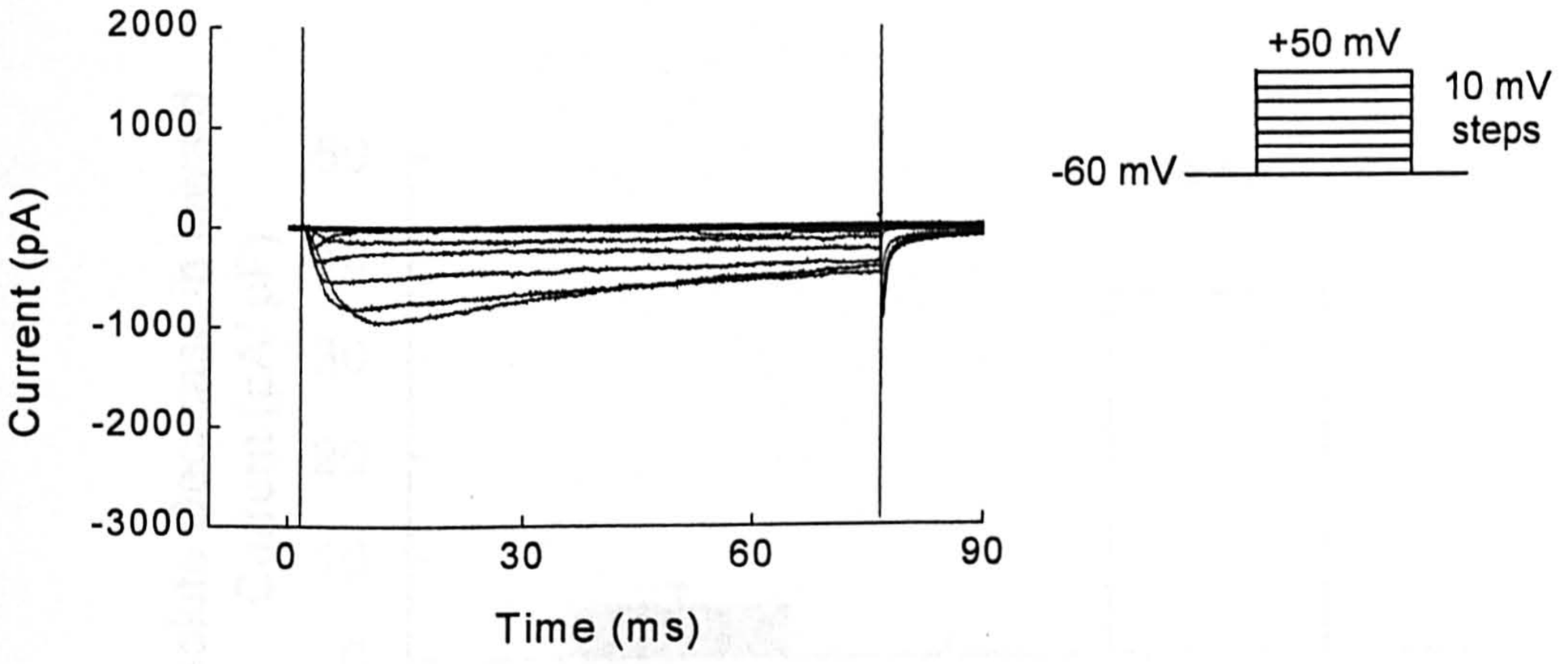
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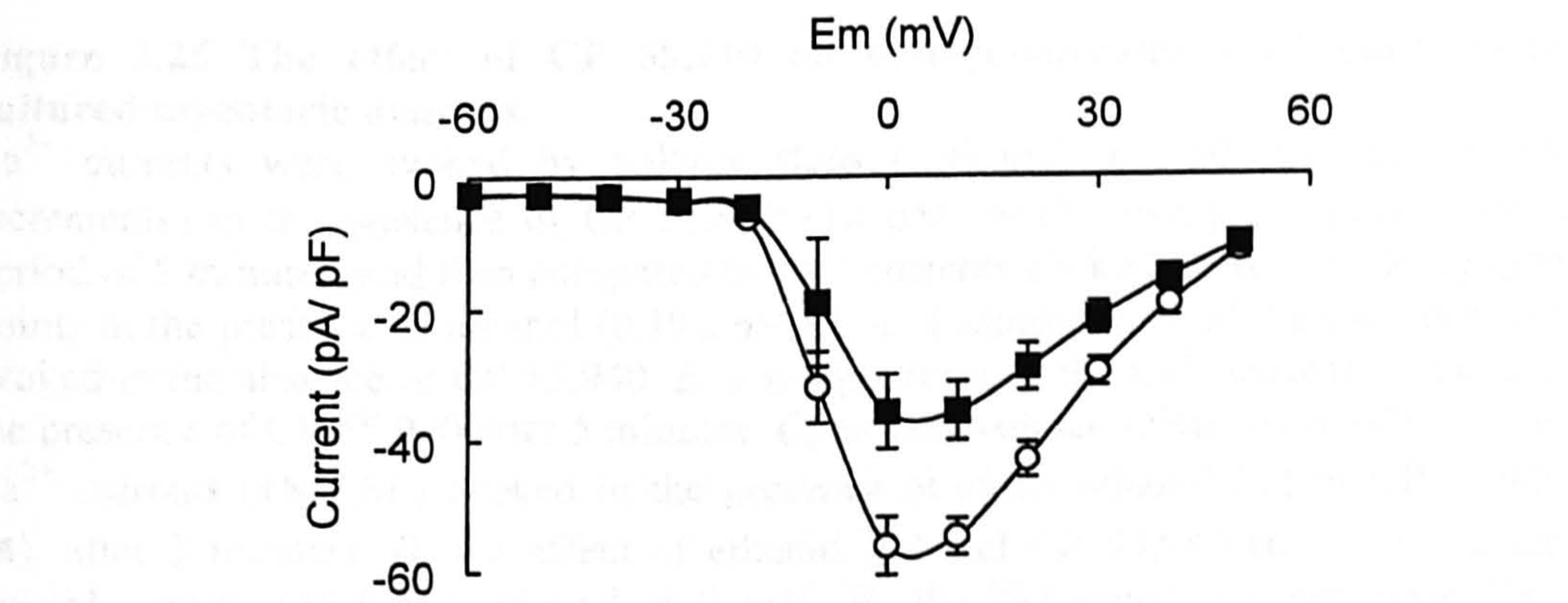
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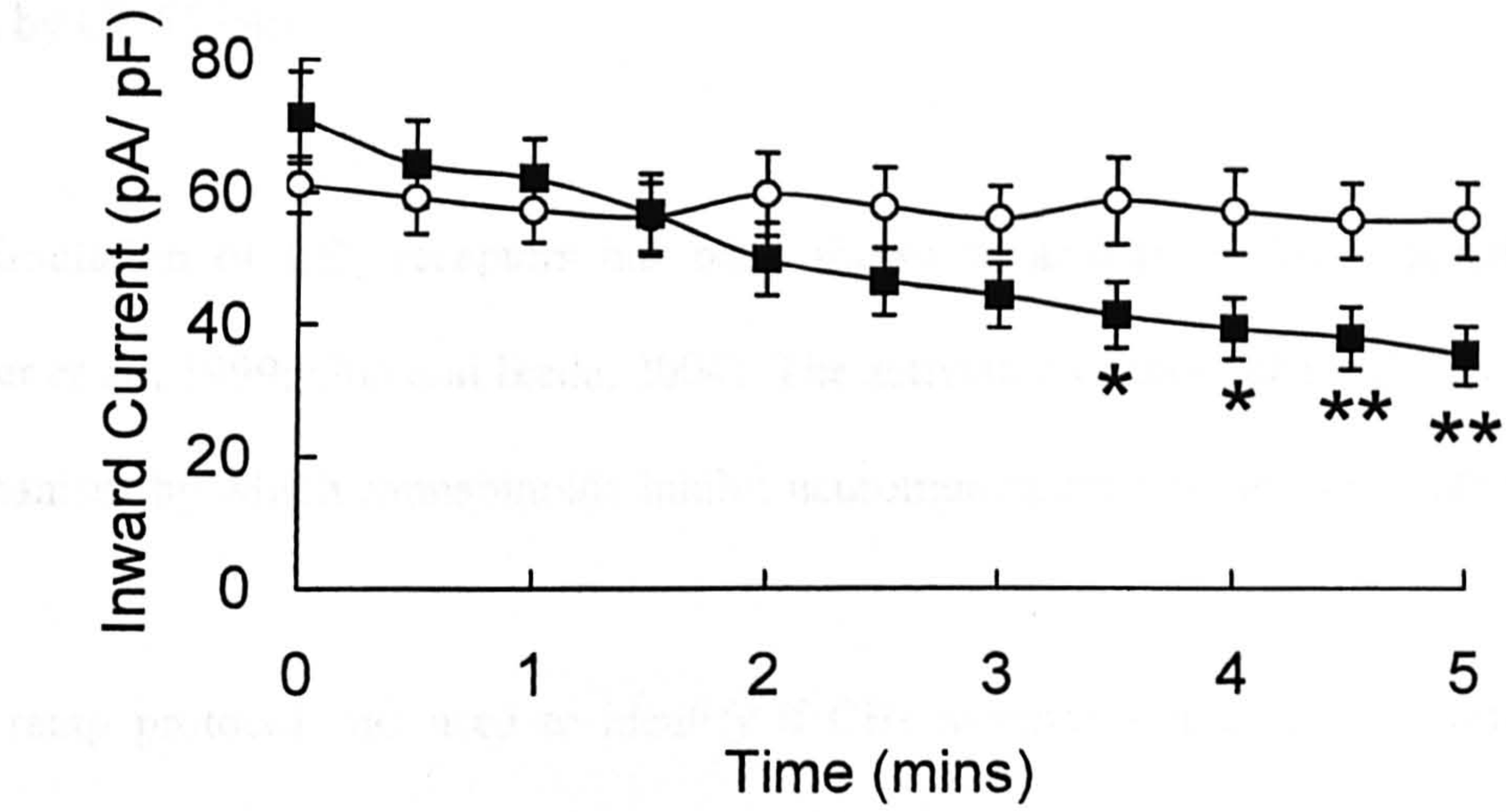

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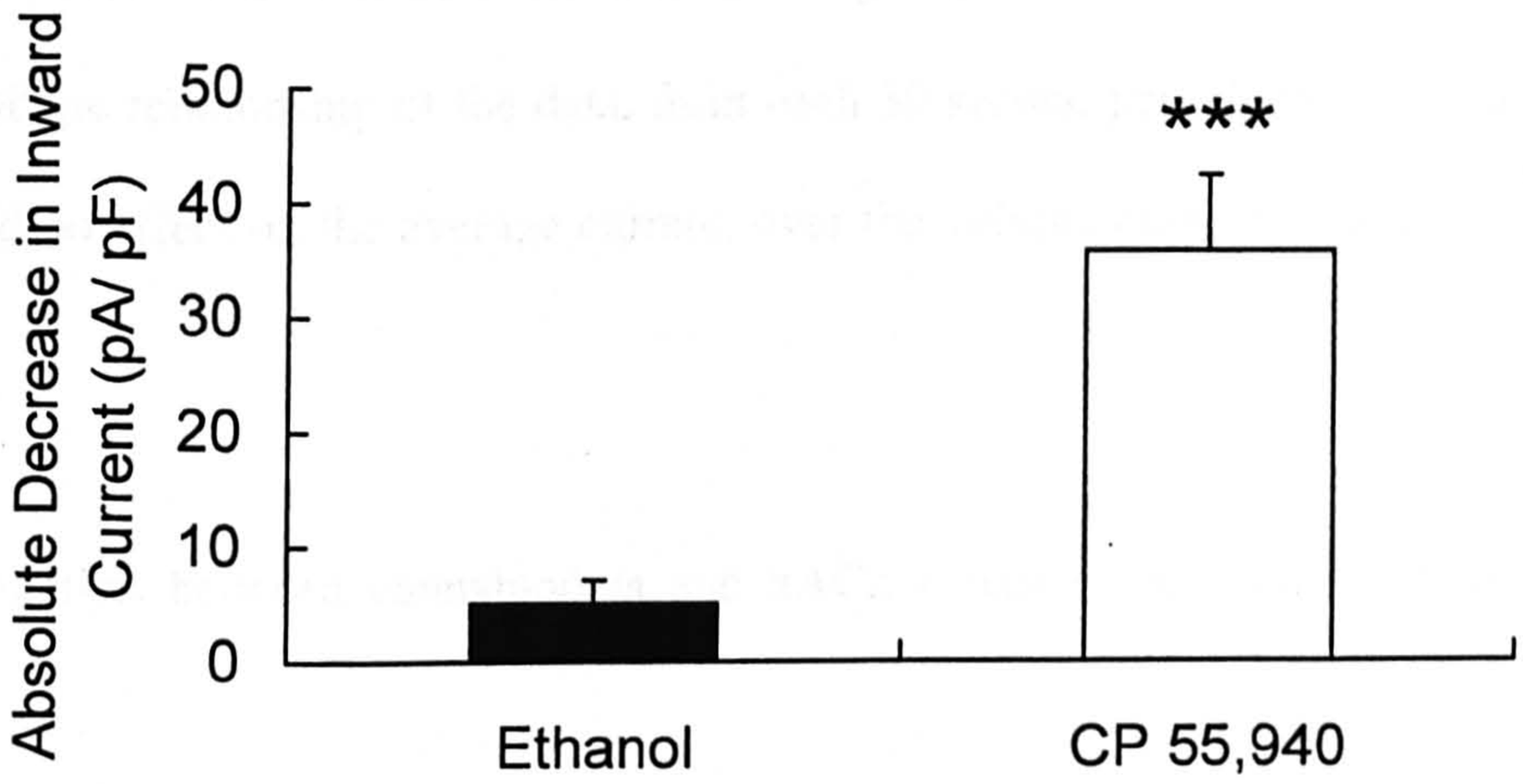


Figure 3.25 The effect of CP 55,940 on voltage-operated Ca^{2+} currents in cultured myenteric neurons.

Ca^{2+} currents were evoked by voltage steps (–60 mV to +50 mV, in 10 mV increments) in the presence of CP 55,940 (10 μM, n=11), every 30 seconds for a period of 5 minutes, and then compared to Ca^{2+} currents evoked at corresponding time points in the presence of ethanol (0.1%, n=10). ***A***, a sample trace of the Ca^{2+} currents evoked in the absence of CP 55,940. ***B***, a sample trace of the Ca^{2+} currents evoked in the presence of CP 55,940 after 5 minutes. ***C***, current-voltage relationship of the mean Ca^{2+} currents (±S.E.M.) evoked in the presence of either ethanol (○) or CP 55,940 (■), after 5 minutes. ***D***, the effect of ethanol (○) and CP 55,940 (■) on the mean inward current (±S.E.M.) evoked at 0 mV. ***E***, the difference between peak Ca^{2+} currents at t=0 and t=5 minutes was taken as the absolute decrease in Ca^{2+} current. Data represented as the mean absolute decrease in inward current (±S.E.M.).
Significant difference from ethanol control: **P*<0.05, ***P*<0.01, ****P*<0.001.

3.4.6 The identification of G-protein inwardly rectifying K^+ (GIRK) channel activation by CP 55,940

Stimulation of CB_1 receptors has been shown to activate GIRK channels. (McAllister *et al.*, 1999; Guo and Ikeda, 2004). The activation of these channels could be a mechanism by which cannabinoids inhibit neurotransmitter release in myenteric neurons.

A ramp protocol was used to identify if CB_1 receptor stimulation activates GIRK channels in cultured myenteric neurons. Ramp responses were taken at 30 second intervals for a total of 5 minutes in the presence of 10 μM CP 55,940. A current-voltage relationship of the data, from each 30 second period, showed that CP 55,940 had no effect on the average current, over the voltage range tested (Fig. 3.26, n=9).

3.4.7 Interaction between cannabinoids and nACh receptors in cultured myenteric neurons

3.4.7.1 The effect of nicotine on membrane currents

Endogenous application of nicotine to myenteric neurons in primary culture has been shown to produce a concentration-dependent, desensitising, inward current (Zhou *et al.*, 2002). Consistent with this data, nicotine (1 mM) induced a transient, inward current, with peak amplitude of 29.5 ±5.2 pA/ pF after 33.6 ±1.8 seconds (Fig. 3.27, n=17).

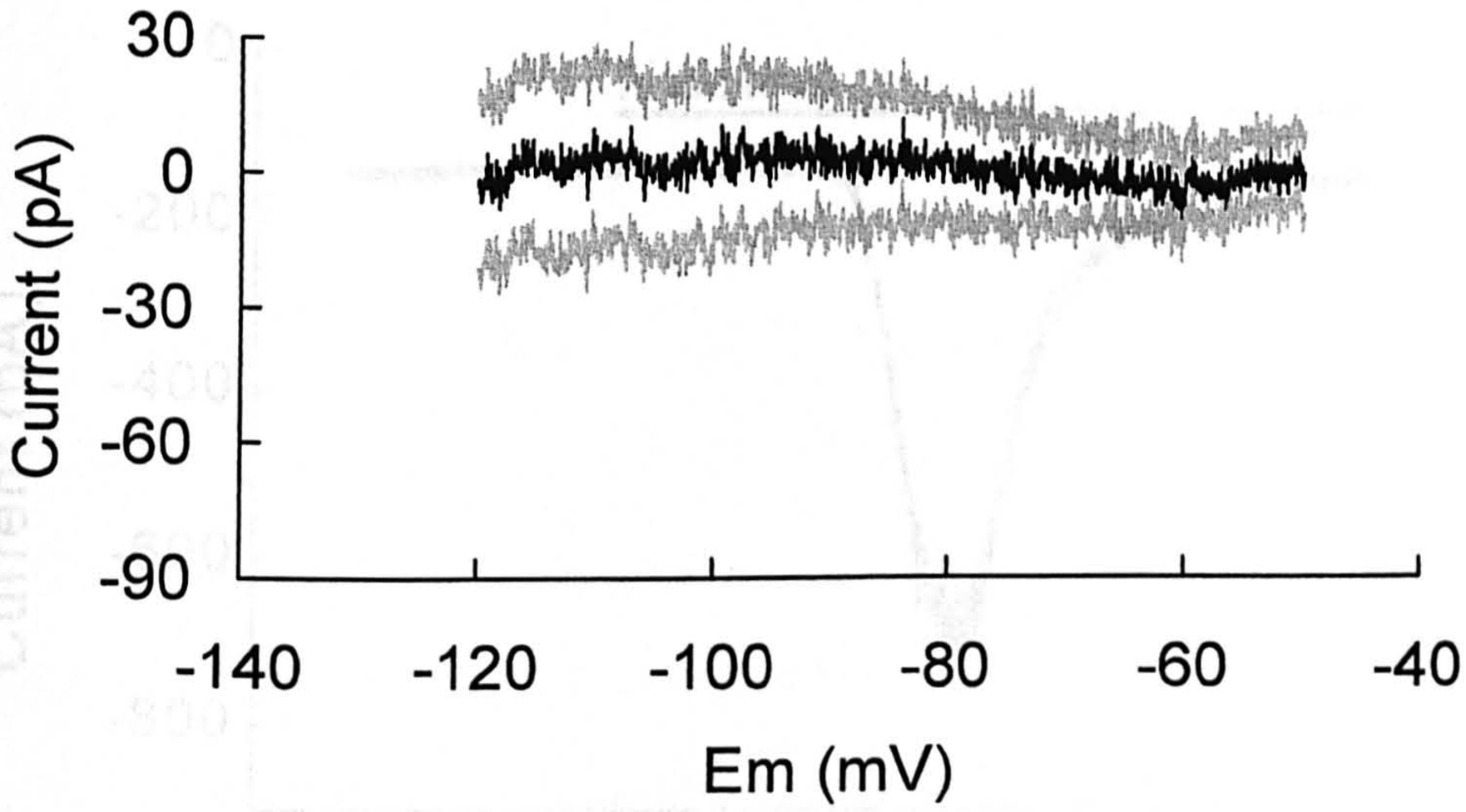


Figure 3.26 Determination of GIRK channel activation by CP 55,940 in cultured myenteric neurons.

A ramp protocol was used to identify the activation of GIRK channels by CP 55,940 (10 μM), present in myenteric cultures. The results were calculated by subtracting the ramp response (-120 mV to -50 mV over 800 ms) under control conditions from the ramp in the presence of CP 55,940, after 5 minutes (n=9). The results are typical of those obtained at earlier time points. The black trace represents the mean current with ±S.E.M. represented by the grey traces.

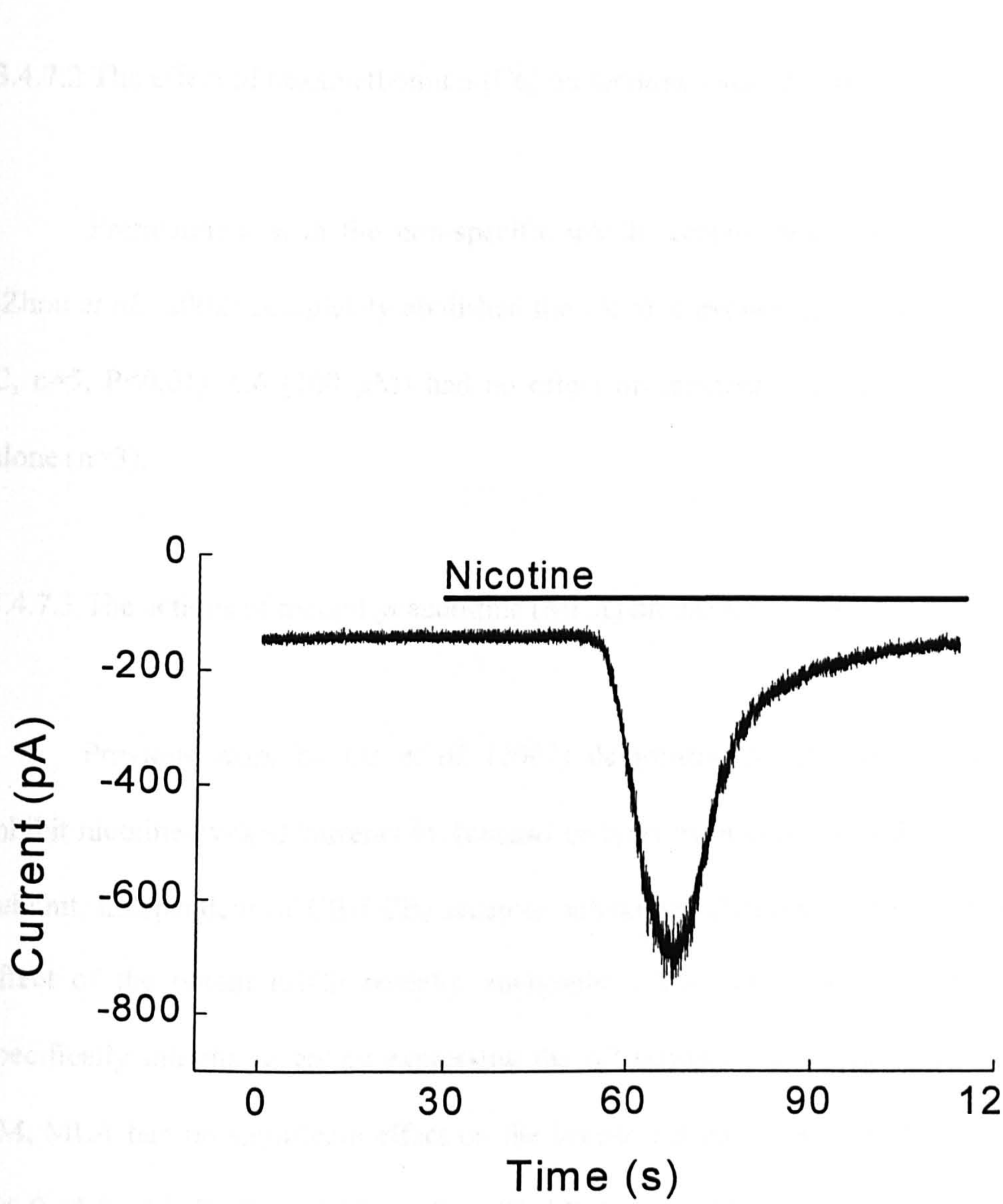


Figure 3.27 The effect of nicotine on membrane currents in myenteric neurons.
A sample trace of the effect of nicotine (1 mM) on resting membrane current in cultured myenteric neurons (n=17). Horizontal bar indicates the presence of ligand.

3.4.7.2 The effect of hexamethonium (C6) on nicotine-evoked currents

Pretreatment with the non-specific nACh receptor antagonist C6 (100 μM) (Zhou *et al.*, 2002) completely abolished the nicotine-evoked current (Fig. 3.28A and C, n=5, P<0.01). C6 (100 μM) had no effect on membrane currents when applied alone (n=3).

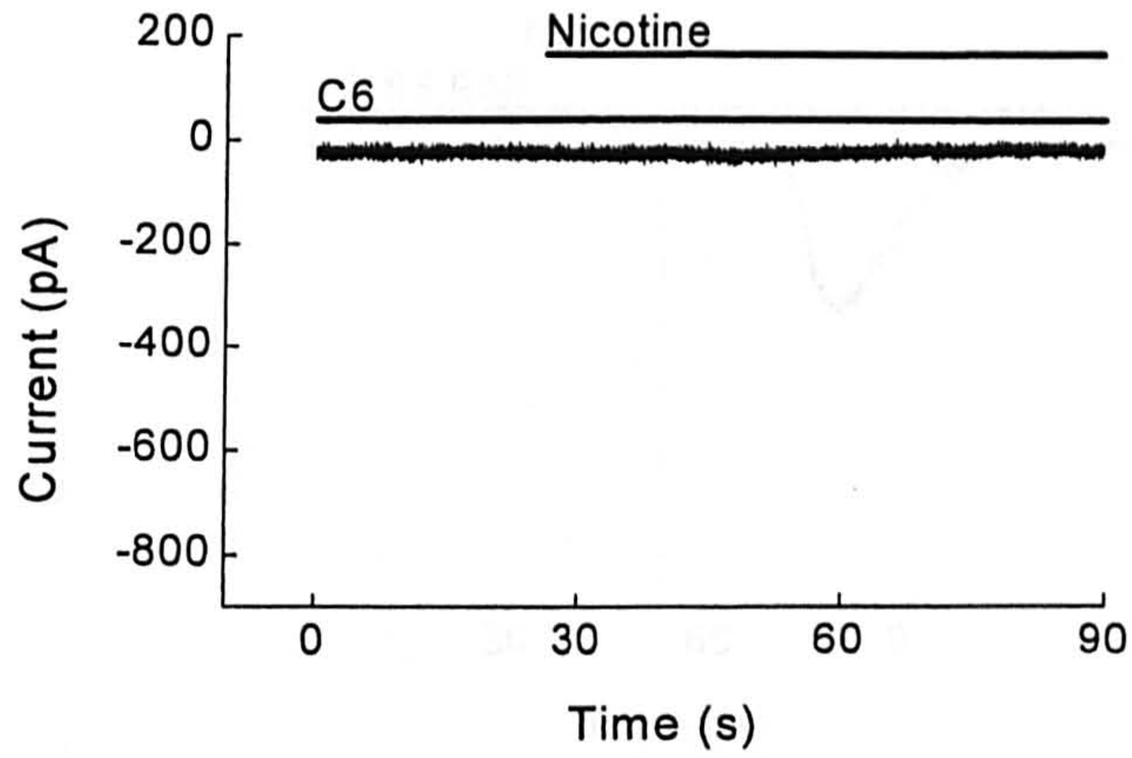
3.4.7.3 The actions of methyllycaconitine (MLA) on nicotine-evoked currents

Previous work by Oz *et al.* (2003) demonstrated that cannabinoids could inhibit nicotine-evoked currents in *Xenopus* oocytes transfected with the nicotinic α7 subunit, independent of CB_1/ CB_2 receptor activation. Therefore we investigated the effect of the potent nACh receptor antagonist MLA (Turek *et al.*, 1995), which specifically inhibits receptors expressing the α7 subunit. At a concentration of 100 nM, MLA had no significant effect on the inward current evoked by 1 mM nicotine (26.9 ±5.9 pA/ pF, Fig. 3.28B and C, n=5). MLA (100 nM) alone produced no change in membrane currents (n=4).

3.4.7.4 The modulation of nicotine-evoked currents by cannabinoids

At concentrations of 1 μM and 10 μM the synthetic cannabinoid agonist CP 55,940 significantly reduced the response to nicotine (1 mM) to 12.9 ±3.9 pA/ pF (Fig. 3.29A and C, n=14, P<0.05, one way ANOVA) and 3.9 ±2.0 pA/ pF (Fig. 3.29B and C, n=9, P<0.01, one way ANOVA) respectively.

A



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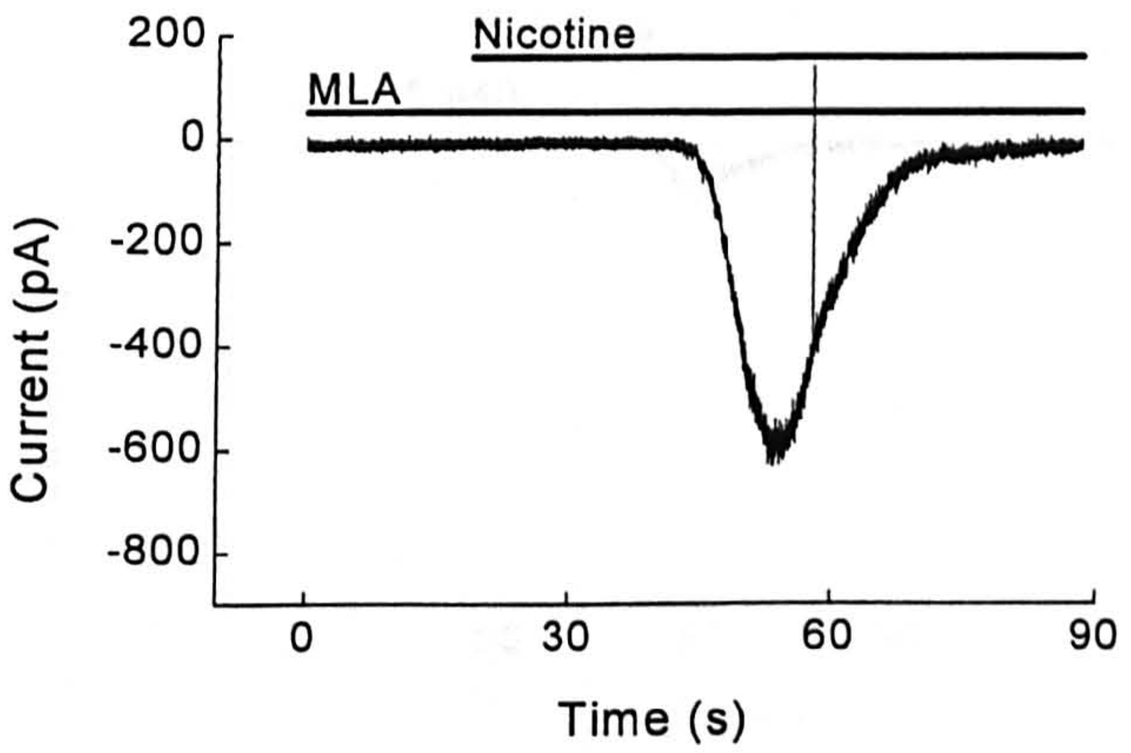

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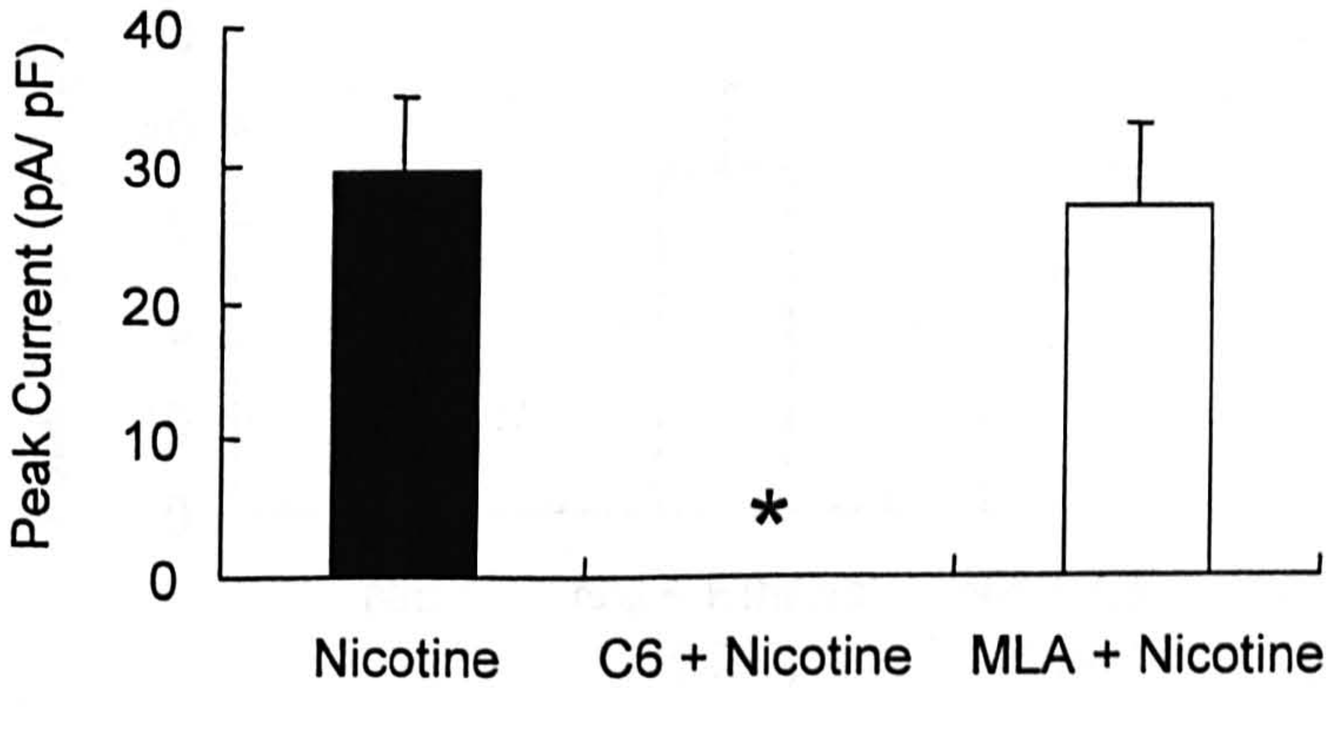


Figure 3.28 The effect of hexamethonium and MLA on nicotine-evoked currents in myenteric neurons.

Hexamethonium (C6, 100 μM) and MLA (100 nM) were applied to neurons for 5 minutes prior to nicotine (1 mM) application. ***A***, a sample trace of the effect of C6 on the nicotine-evoked inward current. ***B***, a sample trace of the effect of MLA on nicotinic currents. Horizontal bars indicate the presence of ligands. ***C***, the effect of C6 (n=5) and MLA (n=5) on the mean peak current (±S.E.M.) evoked by nicotine. Significant difference from nicotine control: **P*<0.01

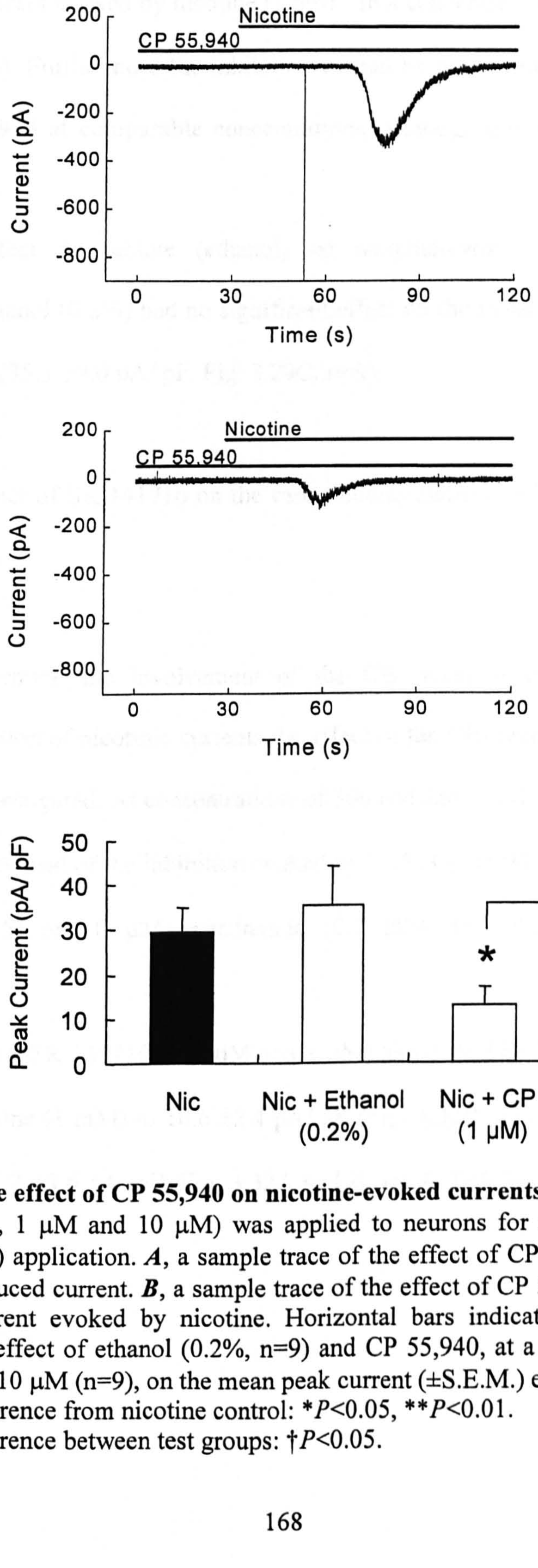


Figure 3.29 The effect of CP 55,940 on nicotine-evoked currents.
CP 55,940 (CP, 1 μM and 10 μM) was applied to neurons for 5 minutes prior to nicotine (1 mM) application. ***A***, a sample trace of the effect of CP 55,940 (1 μM) on the nicotine-induced current. ***B***, a sample trace of the effect of CP 55,940 (10 μM) on the inward current evoked by nicotine. Horizontal bars indicate the presence of ligands. ***C***, the effect of ethanol (0.2%, n=9) and CP 55,940, at a concentration of 1 μM (n=14) and 10 μM (n=9), on the mean peak current (±S.E.M.) evoked by nicotine. Significant difference from nicotine control: **P*<0.05, ***P*<0.01.
Significant difference between test groups: †*P*<0.05.

The endogenous cannabinoid anandamide (AEA) also significantly inhibited the outward current evoked by nicotine (1 mM), in a concentration dependent-manner (Fig. 3.30, n≥8). Furthermore the inhibition caused by anandamide was greater than that of CP 55,940 at comparable concentrations, although this was not statistically significant.

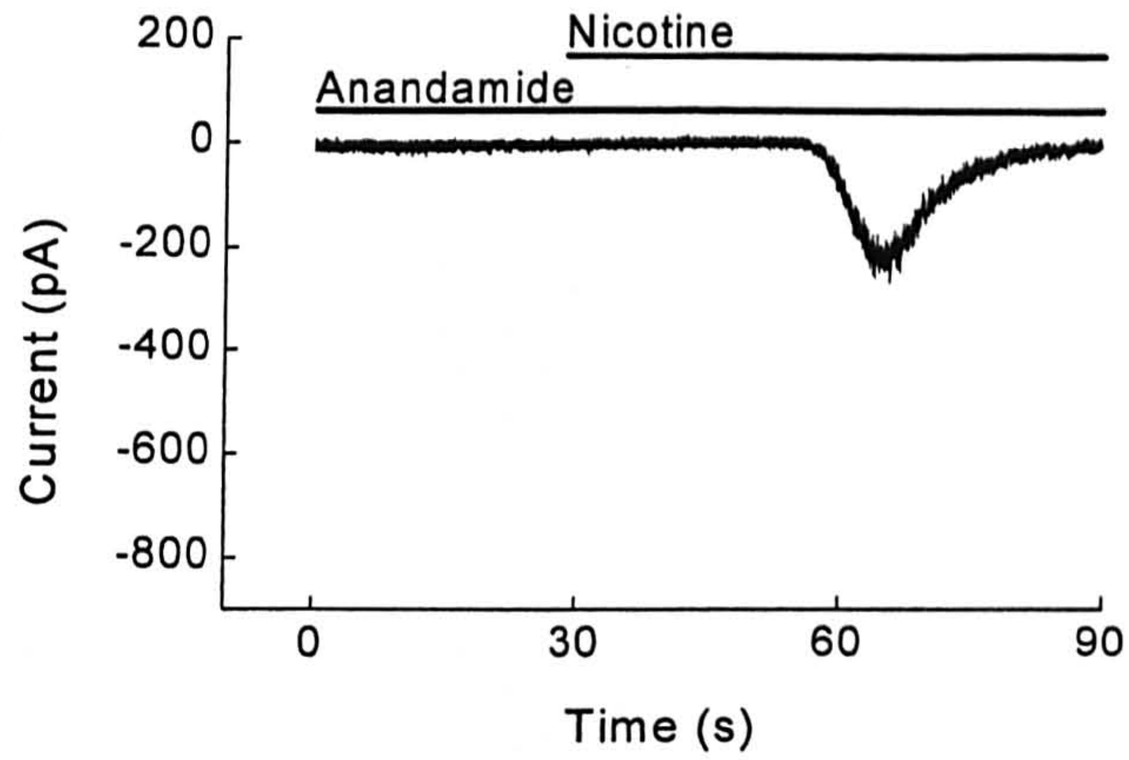
The effect of vehicle (ethanol) on nicotine-evoked currents was also established. Ethanol (0.2%) had no significant effect on the inward current evoked by 1 mM nicotine (35.3 ±9.0 pA/ pF, Fig. 3.29C, n=9).

3.4.7.5 The effect of SR 141716 on the cannabinoid-mediated inhibition of nicotine-evoked currents

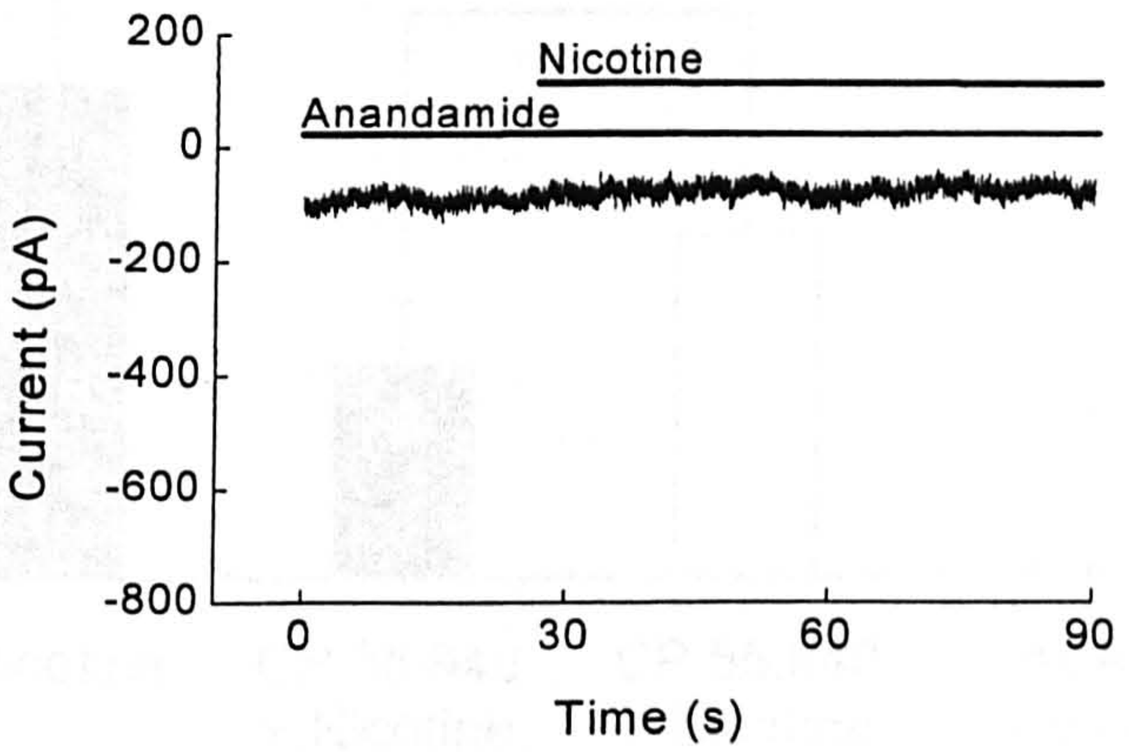
To determine the involvement of the CB_1 receptor in the cannabinoid-mediated inhibition of nicotinic currents the effect of the CB_1 receptor antagonist, SR 141716 was investigated. At concentrations of 300 nM and 1 μM, SR 141716 showed no significant reversal of the inhibition evoked by 1 μM CP 55,940 (20.6 ±5.6 pA/ pF, Fig. 3.31, n=15) or 10 μM anandamide (0.3 ±0.4 pA/ pF, Fig. 3.31, n=10) respectively.

Moreover, SR 141716 (300 nM and 1 μM) alone inhibited the inward current evoked by nicotine (1 mM) to 10.6 ±2.4 pA/ pF (Fig. 3.32B, n=12, $P<0.01$, one way ANOVA) and 7.7 ±3.6 pA/ pF (Fig. 3.32A and B, n=10, $P<0.01$, one way ANOVA) respectively.

A



B



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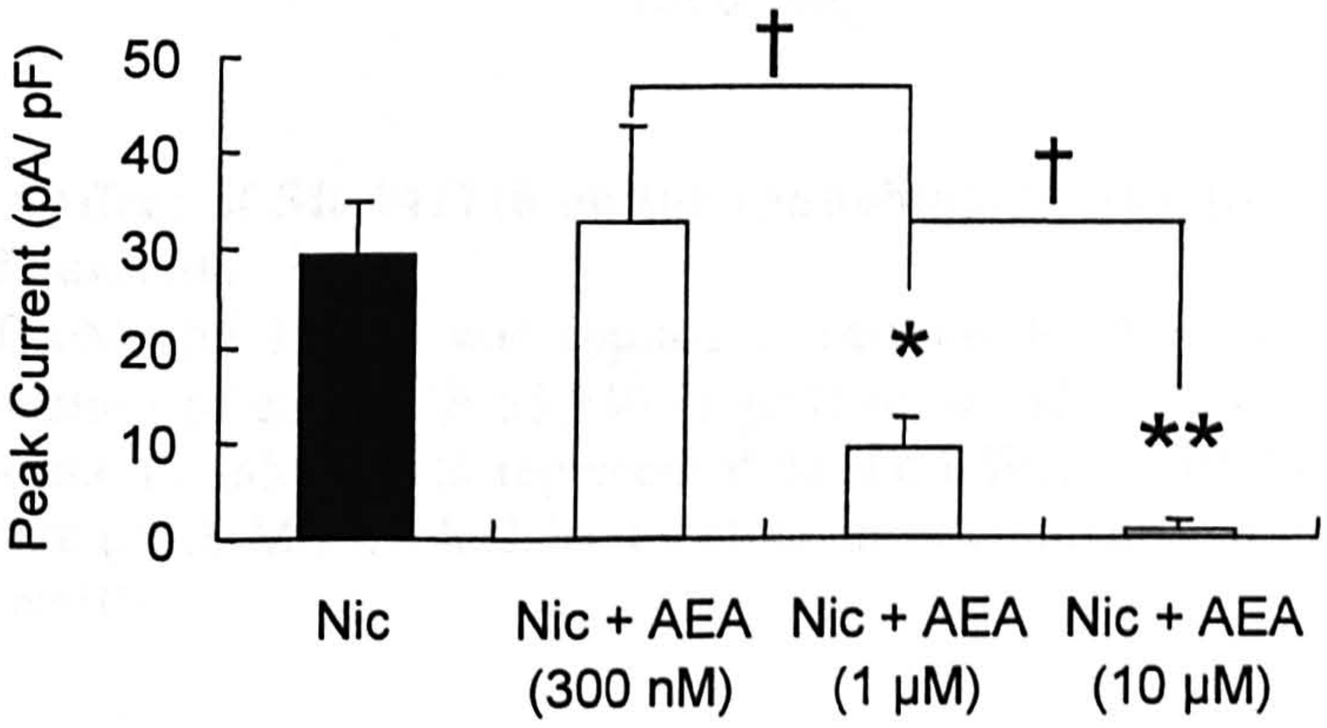


Figure 3.30 The effect of anandamide on nicotine-evoked currents.
Anandamide (AEA, 300 nM-10 μM) was applied to neurons for 10 minutes prior to nicotine (Nic, 1 mM) application. ***A***, a sample trace of the effect of AEA (1 μM) on nicotine-induced currents. ***B***, a sample trace of the effect of AEA (10 μM) on nicotine-induced currents. Horizontal bars indicate the presence of ligands. ***C***, the effect of AEA on the mean peak current (±S.E.M.) evoked by nicotine (n≥8).
Significant difference from nicotine control: **P*<0.05, **P*<0.01 (one way ANOVA).
Significant difference between test groups: †*P*<0.05.

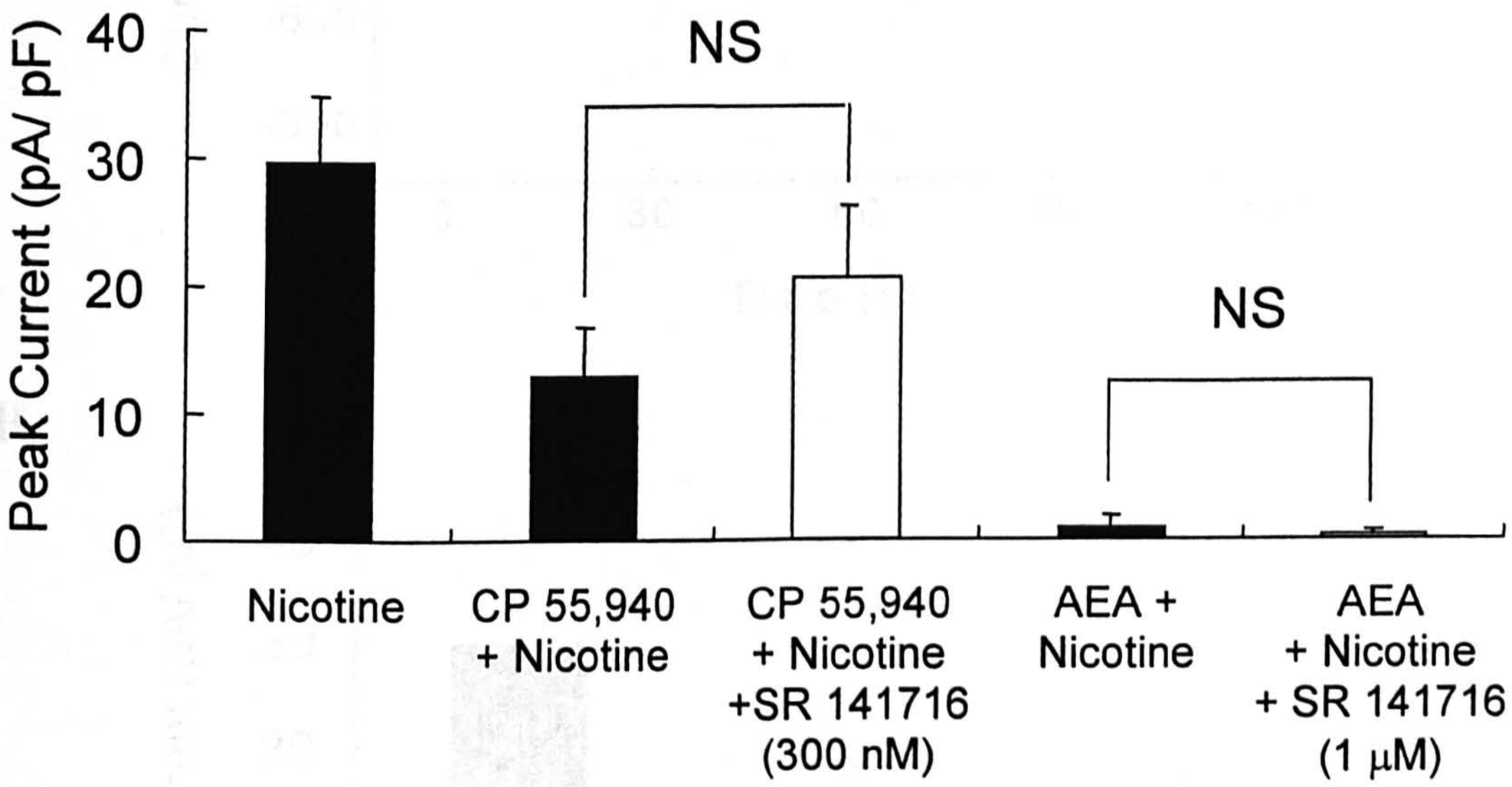
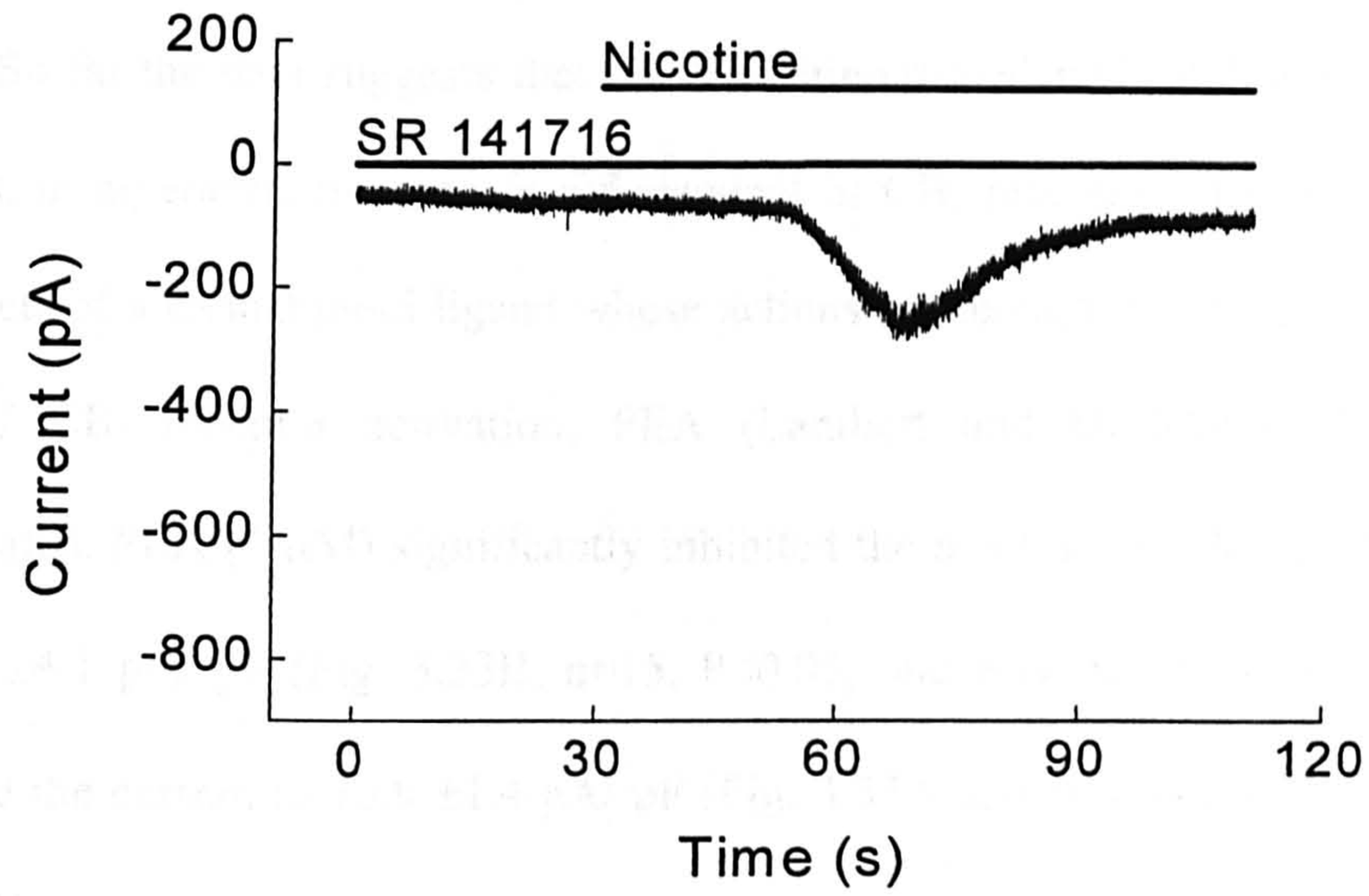


Figure 3.31 The effect of SR 141716 on the cannabinoid-mediated inhibition of nicotine-evoked currents.

SR 141716 (300 nM and 1 μM) was applied to neurons for 5 minutes prior to a subsequent application of either CP 55,940 (1 μM) or anandamide (AEA, 10 μM), followed by nicotine (1 mM). Data represented as the effect of SR 141716 on the mean peak current (±S.E.M.) evoked by nicotine in combination with CP 55,940 (n=15) or AEA (n=10).

A



B

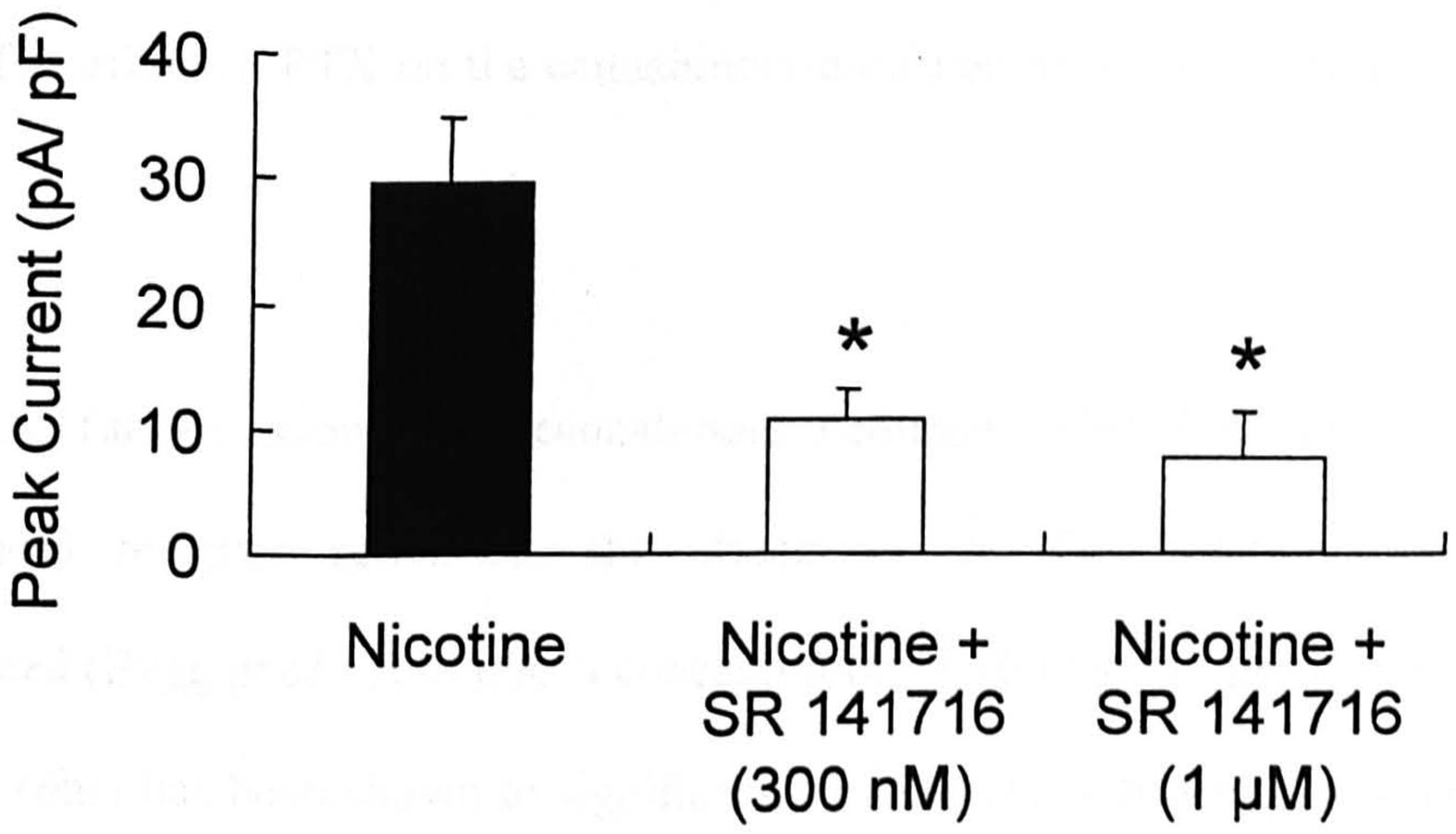


Figure 3.32 The effect of SR 141716 on nicotine-evoked currents.
Neurons were treated with SR 141716 (300 nM and 1 μM) for 5 minutes prior to nicotine (1 mM) application. ***A***, a sample trace of the effect of 1 μM SR 141716 on the nicotine-evoked inward current. Horizontal bars indicate the presence of ligands. ***B***, the effect of SR 141716, at a concentration of 300 nM (n=12) and 1 μM (n=10), on the mean peak current (±S.E.M.) induced by nicotine.
Significant difference from nicotine control: **P*<0.01.

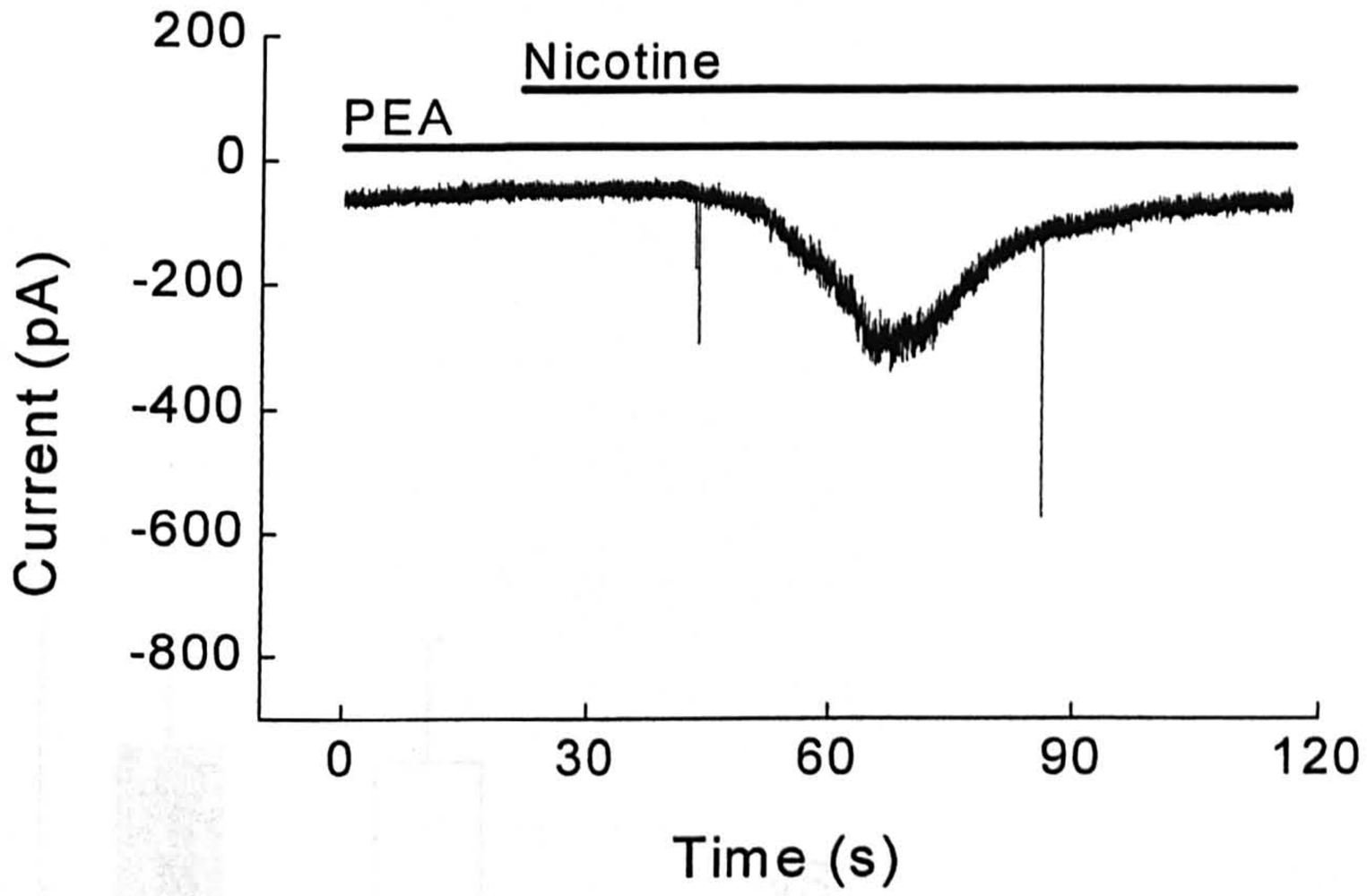
3.4.7.6 The actions of palmitoylethanolamide (PEA) on inward currents induced by nicotine

So far the data suggests that the cannabinoid-mediated inhibition of nicotinic currents, in myenteric neurons, is independent of CB_1 receptor activation. Therefore the effects of a cannabinoid ligand whose actions are thought to occur independently of CB_1/ CB_2 receptor activation, PEA (Lambert and Di Marzo, 1999), were investigated. PEA (1 μM) significantly inhibited the nicotine (1 mM)-evoked current to 15.6 ±4.1 pA/ pF (Fig. 3.33B, n=15, $P<0.05$, one way ANOVA) and at 10 μM inhibited the current to 10.9 ±1.4 pA/ pF (Fig. 3.33A and B, n=12, $P<0.01$, one way ANOVA).

3.4.7.7 The effect of PTX on the cannabinoid-mediated inhibition of nicotine-evoked currents

To further support a cannabinoid-mediated inhibition, independent of cannabinoid receptor activation, the effects of the $G_{i/o}$ inhibitor, PTX were investigated (Begg *et al.*, 2001). At a concentration of 100 ng/ ml, pre-incubation with PTX for 16hrs has been shown to significantly inhibit the outward current evoked by CB_1 receptor activation in DDT_1 MF-2 cells (Begg *et al.*, 2001). Myenteric neurons in primary culture were pre-incubated with PTX (100 ng/ ml) for at least 18 hrs prior to being patch clamped. PTX (100 ng/ ml) alone had no significant effect on the inward current evoked by 1 mM nicotine (28.3 ±8.0 pA/ pF, Fig. 3.34, n=14). The inhibition of nicotine (1 mM)-evoked currents, by 10 μM CP 55,940 (2.1 ±1.4 pA/ pF, n=6) or

A



B

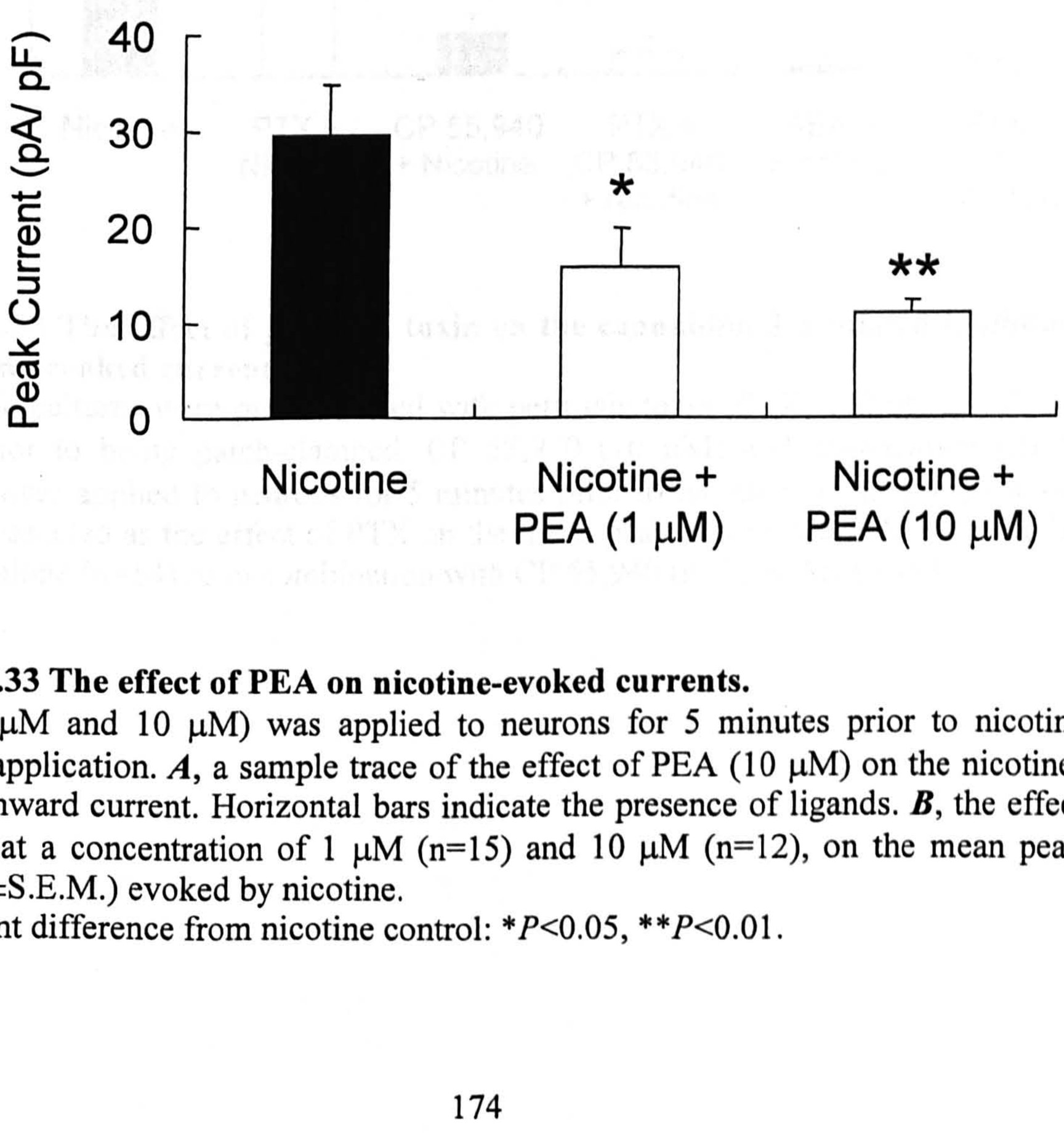


Figure 3.33 The effect of PEA on nicotine-evoked currents.
PEA (1 μM and 10 μM) was applied to neurons for 5 minutes prior to nicotine (1 mM) application. ***A***, a sample trace of the effect of PEA (10 μM) on the nicotine-evoked inward current. Horizontal bars indicate the presence of ligands. ***B***, the effect of PEA, at a concentration of 1 μM (n=15) and 10 μM (n=12), on the mean peak current (±S.E.M.) evoked by nicotine.
Significant difference from nicotine control: **P*<0.05, ***P*<0.01.

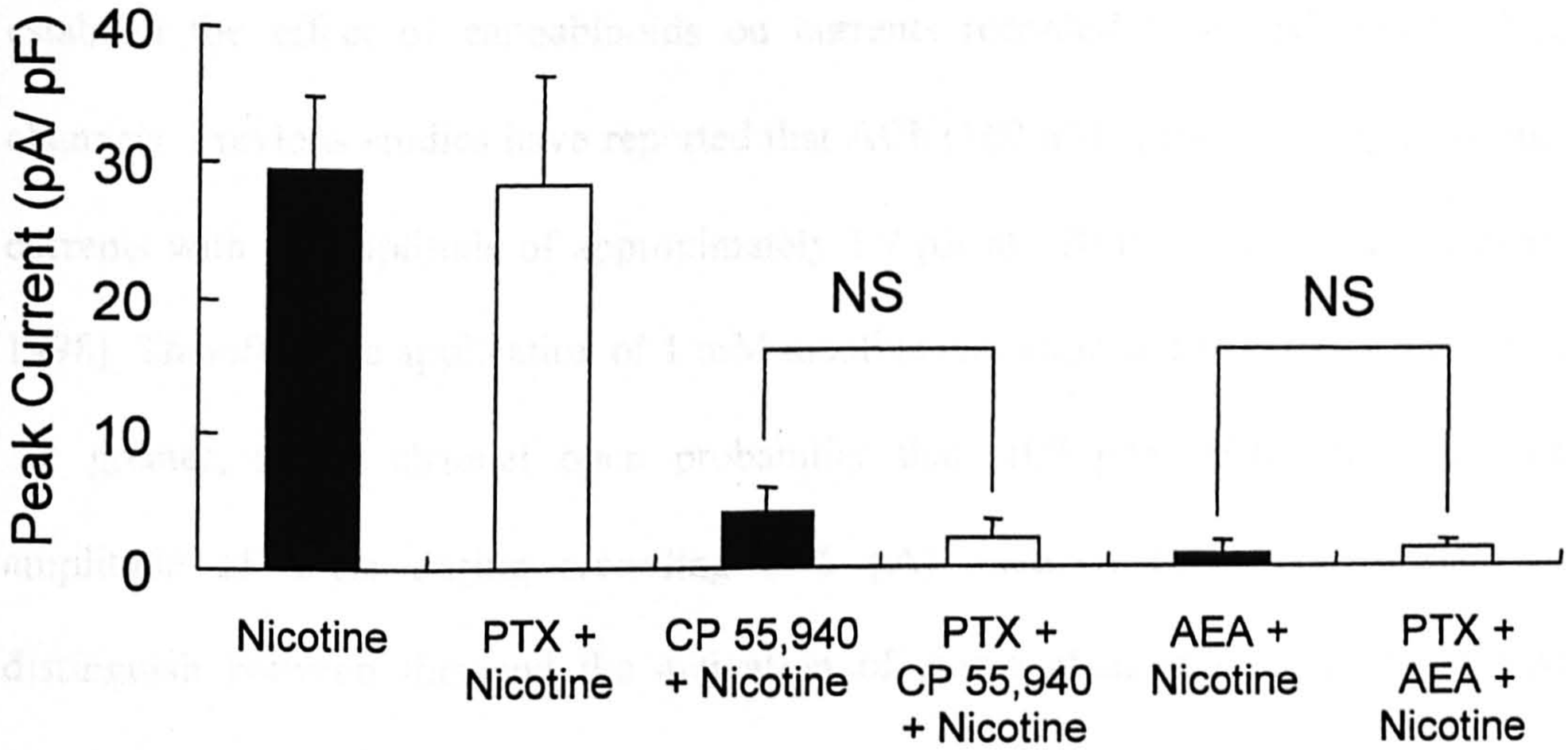


Figure 3.34 The effect of pertussis toxin on the cannabinoid-mediated inhibition of nicotine-evoked currents.
Myenteric cultures were pre-incubated with pertussis toxin (PTX, 100 ng/ ml) for 18 hours prior to being patch-clamped. CP 55,940 (10 μM) and anandamide (AEA, 10 μM) were applied to neurons for 5 minutes prior to nicotine (1 mM) application. Data represented as the effect of PTX on the mean peak current (±S.E.M.) evoked by nicotine alone (n=14) or in combination with CP 55,940 (n=6) or AEA (n=8).

10 μM anandamide (1.3 ±0.6 pA/ pF, n=8), were not significantly reversed in the presence of PTX (100 ng/ ml) (Fig. 3.34).

3.4.7.8 Single channel recording of nACh receptors

The results imply that cannabinoids inhibit nACh receptor activation independent of CB_1 receptor stimulation. To support the possibility that cannabinoids may be acting directly on the nACh receptor, single channel recording was used to establish the effect of cannabinoids on currents recorded from individual nACh channels. Previous studies have reported that ACh (100 μM) produces single channel currents with an amplitude of approximately 1.7 pA at –70 mV (Zhou and Galligan, 1998). Therefore the application of 1 mM nicotine was expected to evoke a similar, if not greater, single channel open probability than 100 μM ACh. However, the amplitude of noise during recording (4-5 pA) made it extremely difficult to distinguish between this and the activation of single channel currents by 1 mM nicotine (n=4, Fig. 3.35).

3.4.8 The effect of CP 55,940 on 5-HT-evoked inward currents in cultured myenteric neurons

Application of 5-HT has been shown to evoke a concentration-dependent inward current in cultured myenteric neurons (Zhou and Galligan, 1999). The inward current was biphasic, with an initial rapidly developing peak that desensitised in the presence of agonist, followed by a slower developing, sustained inward current (Zhou and Galligan, 1999). The 5-HT_3 antagonist ondansetron completely blocked the

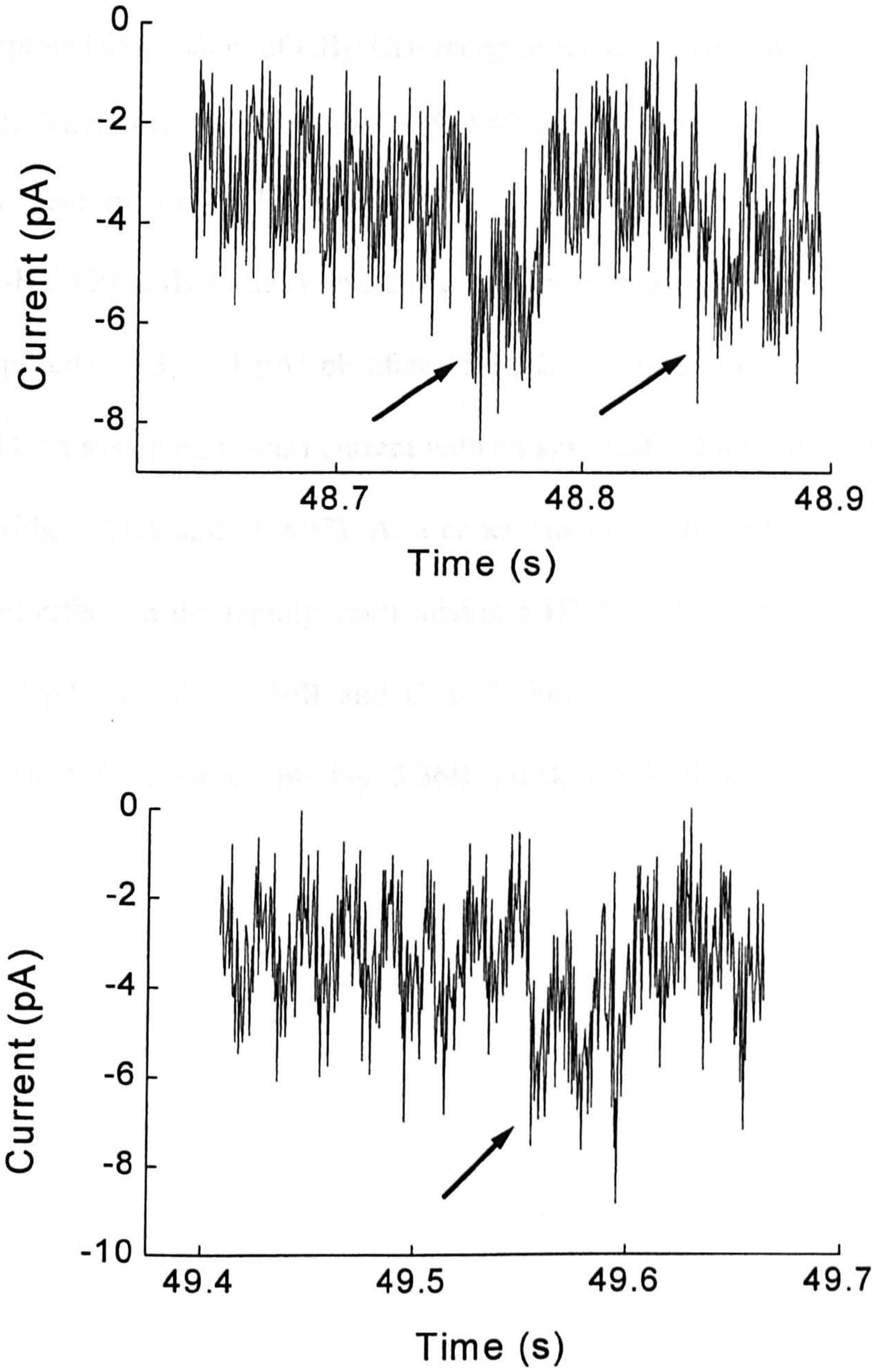
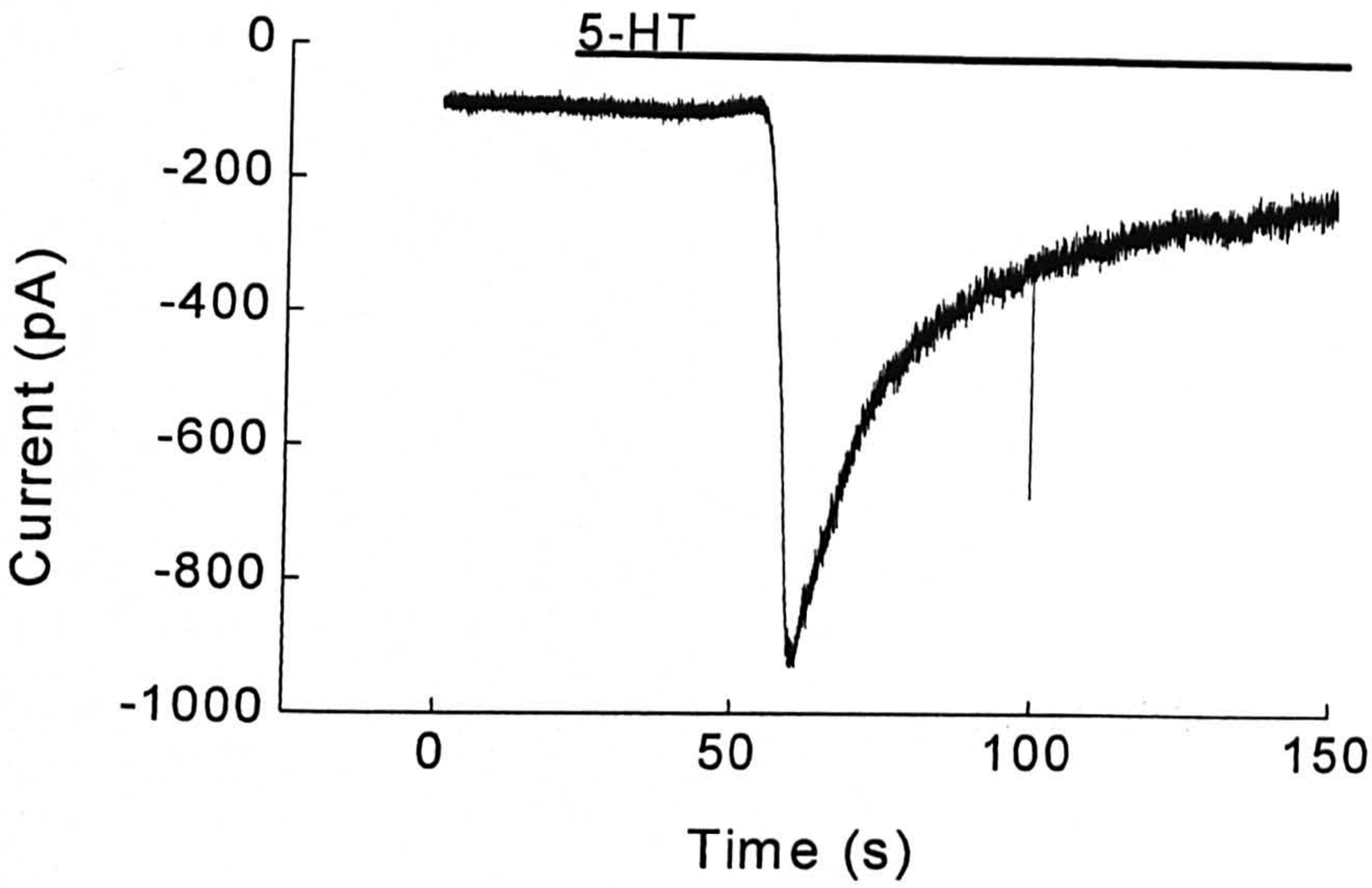


Figure 3.35 Single channel recording in cultured myenteric neurons.
The outside-out patch configuration was used to obtain single channel recordings in myenteric cultures, treated with 1 mM nicotine (n=4). Each trace represents a separate cell. Due to the amount of noise the baseline spans approximately 4-5 pA, which could easily mask the activation of smaller single channel currents. The arrows point to slight decreases in current, which may represent the opening of single nACh channels rather than fluctuations in the baseline as a result of noise.

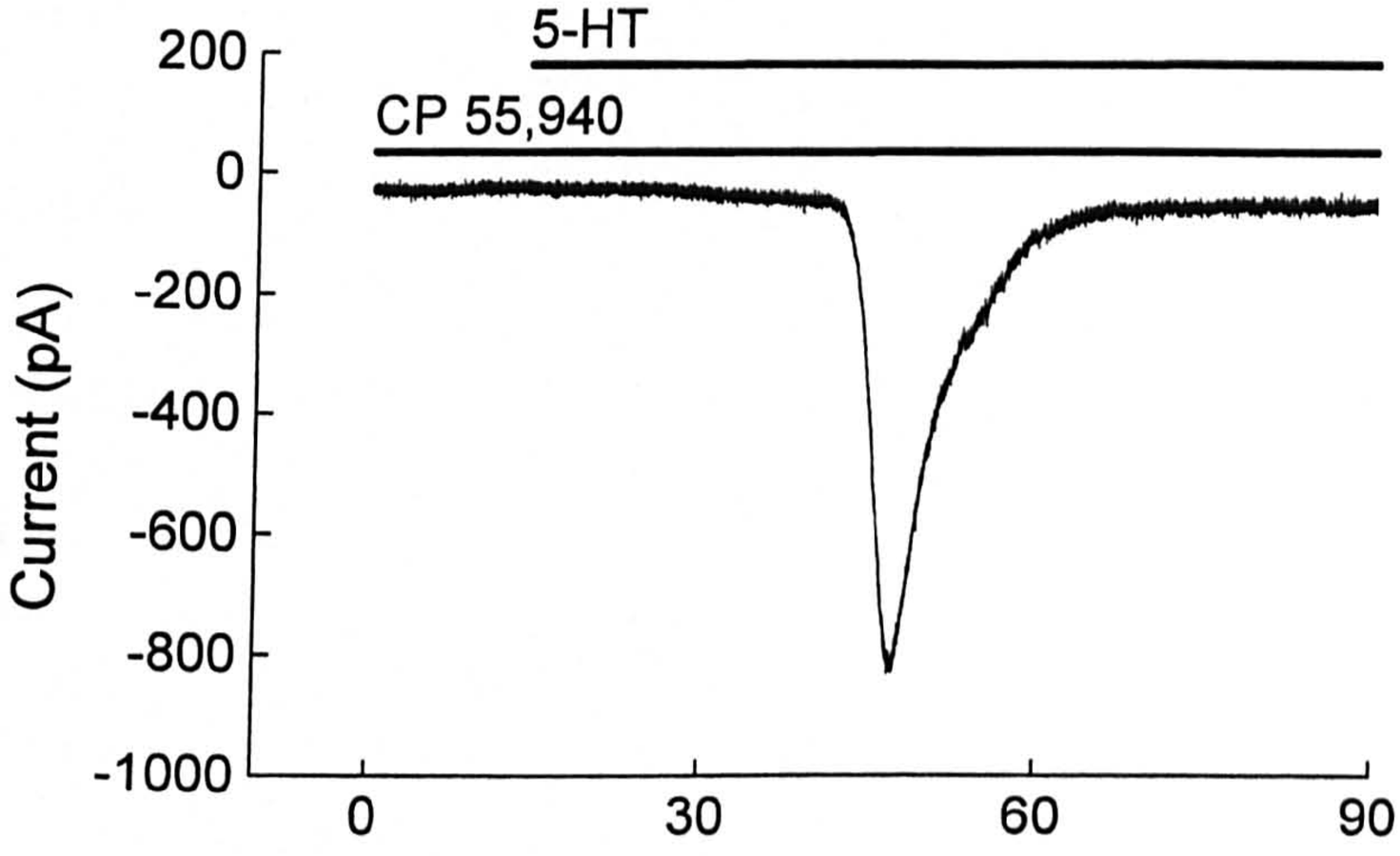
rapidly developing inward current but had no effect on the sustained current (Zhou and Galligan, 1999). In addition, it has been reported that cannabinoids can inhibit 5-HT_3 receptors independent of CB_1/ CB_2 receptor activation (Barann *et al.*, 2002; Oz *et al.*, 2002). Therefore the effect of CP 55,940 on 5-HT-induced currents in cultured myenteric neurons was established.

5-HT (50 μM) initially evoked a rapidly desensitising inward current, with peak amplitude 27.3 ±2.4 pA/ pF after 35.0 ±2.5 seconds (Fig. 3.36A and C, n=7) followed by a sustained inward current with an amplitude of 8.8 ±0.8 pA/ pF after 75 seconds (Fig. 3.36A and D, n=7). At a concentration of 10 μM, CP 55,940 had no significant effect on the rapidly desensitising 5-HT (50 μM)-induced inward current (29.3 ±7.7 pA/ pF, Fig. 3.36B and C, n=5) but virtually abolished the sustained inward current (0.5 ±0.4 pA/ pF, Fig. 3.36B and D, n=5, P<0.0001).

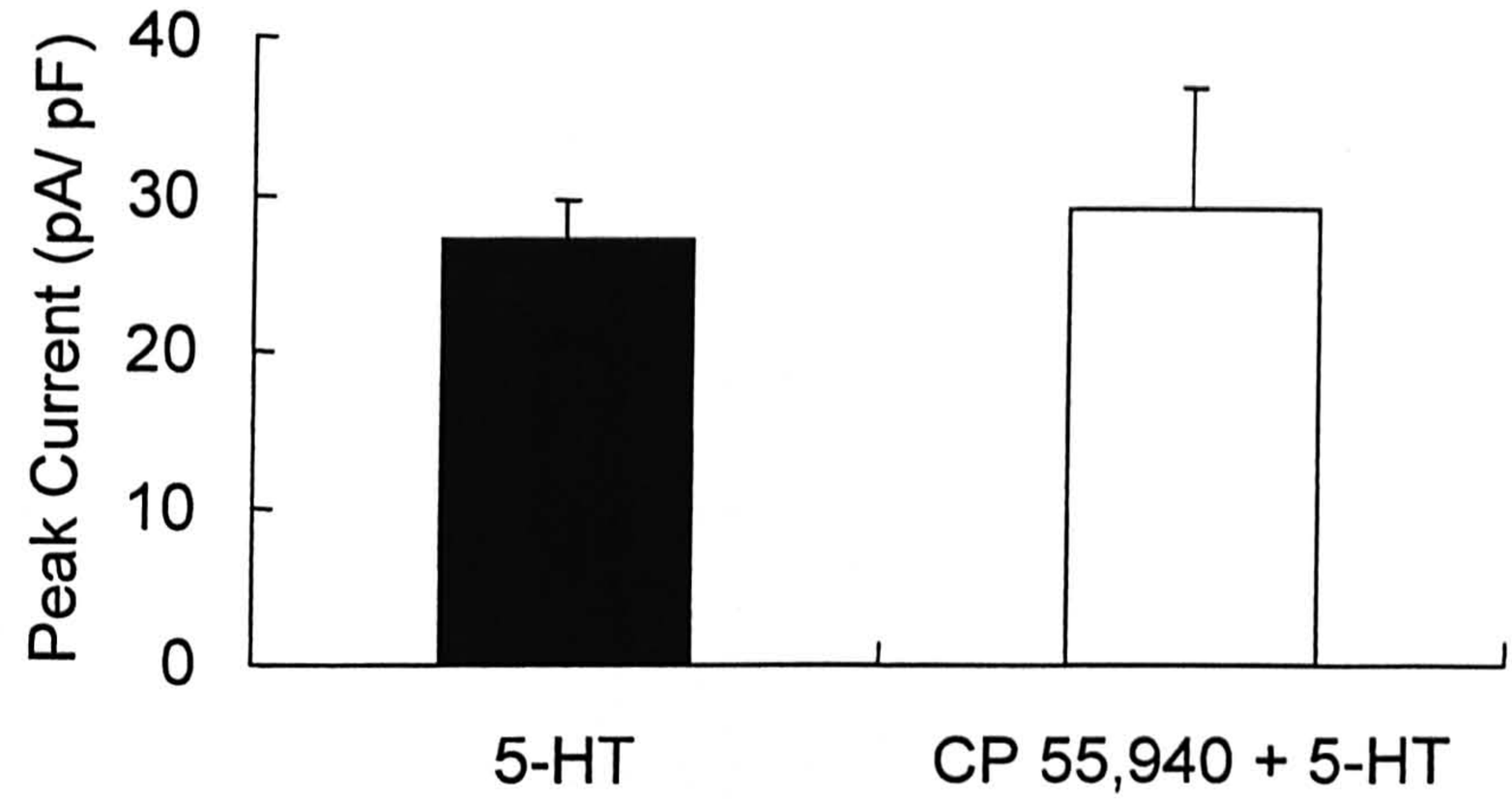
A


5-HT
0
-200
-400
-600
-800
-1000
Current (pA)
0
50
100
150
Time (s)

B


5-HT
CP 55,940
200
0
-200
-400
-600
-800
-1000
Current (pA)
0
30
60
90
Time (s)

C



D

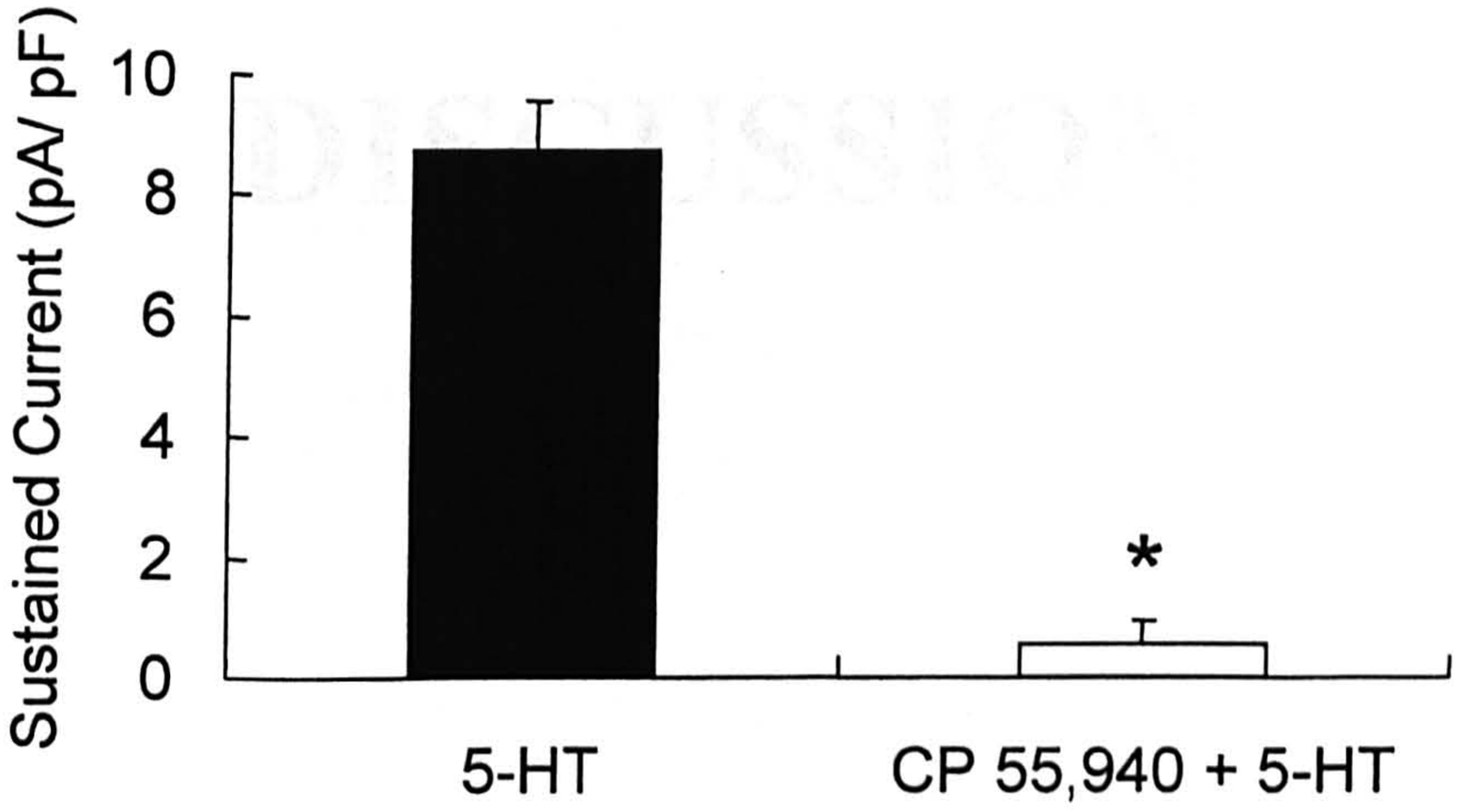


Figure 3.36 The effect of CP 55,940 on currents evoked by 5-HT in myenteric neurons.

Neurons were treated with CP 55,940 (10 μM) for 5 minutes prior to 5-HT (50 μM) application. ***A***, a sample trace of the effect of 5-HT on resting membrane current in cultured myenteric neurons (n=7). ***B***, a sample trace of the effect of CP 55,940 on the 5-HT-induced current. ***C***, the effect of CP 55,940 on the mean peak current (±S.E.M.) evoked by 5-HT (n=5). ***D***, the effect of CP 55,940 on the mean sustained current (±S.E.M.) induced by 5-HT (n=5).
Significant difference from 5-HT control: **P*<0.0001.

DISCUSSION

4.1 DDT_1 MF-2 smooth muscle cells

4.1.1 CB_1 receptor-mediated increase in $[Ca^{2+}]_i$

The present study sought to further define the signal transduction mechanisms mediating CB_1 receptor-induced increases in $[Ca^{2+}]_i$ in DDT_1 MF-2 smooth muscle cells. Previous work has shown that the cannabinoid agonist CP 55,940 evokes a transient outward current in this cell line, mediated by $I_{K,Ca}$ (Begg *et al.*, 2001). The CP 55,940-evoked current is sensitive to inhibition by the CB_1 receptor antagonist SR 141716A and is completely abolished by the removal of Ca^{2+} from the bathing solution (Begg *et al.*, 2001). Similarly, we showed an increase in $[Ca^{2+}]_i$ by CP 55,940, but only at high concentrations ($\geq$ 10 μM). The increase was abolished by the removal of extracellular Ca^{2+} suggesting that the CP 55,940-induced increase in $[Ca^{2+}]_i$ is entirely dependent on Ca^{2+} influx from the extracellular space, although Ca^{2+} release from thapsigargin-sensitive stores is also shown to play a role (Begg *et al.*, 2001). The lack of a significant increase in $[Ca^{2+}]_i$ at lower CP 55,940 concentrations is surprising, especially in light of studies by Begg *et al.* (2001) who demonstrated that 1 μM CP 55,940 evoked a significant outward current in DDT_1 MF-2 cells, in an SR 141716A-sensitive manner. One explanation could be that smaller CP 55,940 concentrations evoke a localised increase in $[Ca^{2+}]$, which activates Ca^{2+}-dependent K^+ channels close to the site of Ca^{2+} influx. A larger CP 55,940 concentration may produce a more 'global' increase in cytosolic Ca^{2+}, due to a greater stimulation of Ca^{2+} influx, which is measured as a significant increase in $[Ca^{2+}]_i$. Furthermore, the distinct increase in $[Ca^{2+}]_i$, seen at higher CP 55,940 concentrations ($\geq$ 30 μM), may be attributable to a combination of CB_1- and non-CB_1

receptor-dependent mechanisms. Further work is needed to establish the effect of SR 141716A on the CP 55,940-induced increase in $[Ca^{2+}]_i$.

Interestingly, 100 μM CP 55,940 significantly decreased $[Ca^{2+}]_i$, when extracellular Ca^{2+} was absent. This suggests that at least part of the Ca^{2+} transport mechanism is still operational (i.e. open Ca^{2+} channels) under these conditions but reversed due to the inverted driving force for Ca^{2+}.

4.1.2 The role of capacitative Ca^{2+} entry (CCE)

The requirement of both a release of Ca^{2+} from thapsigargin-sensitive stores and an influx of Ca^{2+} from the extracellular medium would imply a model for CCE, where the depletion of intracellular Ca^{2+} stores is coupled to the activation of membrane-bound store-operated Ca^{2+} channels (SOCCs) resulting in Ca^{2+} influx (Putney and McKay, 1999).

DDT_1 MF-2 cells have been used previously to study increases in $[Ca^{2+}]_i$ caused by the activation of histamine H_1 receptors, purine P_{2Y} receptors and α-adrenoceptors (Molleman *et al.*, 1990, 1991a). H_1 receptor stimulation increased the production of $InsP_3$ and $InsP_4$, which was accompanied by an elevation in cytoplasmic Ca^{2+} (Molleman *et al.*, 1991a). The generation of $InsP_3$ requires the activation of PLC and hence the cleavage of PIP_2 to DAG and $InsP_3$ (Begg *et al.*, 2001). The inhibitory effect of thapsigargin on the cannabinoid response may suggest a similar signalling pathway is utilised during CB_1 receptor stimulation. However, inhibition of PLC has no effect on the outward current evoked by CP 55,940 (Begg *et al.*, 2001) suggesting the CB_1 receptor-mediated increase in $[Ca^{2+}]_i$ is independent of $InsP_3$ generation. To

verify this conclusion the effect of 2-APB, a membrane-permeable $InsP_3$ receptor antagonist, on the outward current evoked by CP 55,940 was established.

2-APB had no significant effect on the CP 55,940-induced outward current at a concentration that was shown to significantly inhibit histamine-evoked currents. An $InsP_3$-independent increase in $[Ca^{2+}]_i$ has also been observed in MDCK tubular cells stimulated with CP 55,940 (Chou *et al.*, 2001). In comparison with DDT_1 MF-2 cells the increase in $[Ca^{2+}]_i$ involved a release of Ca^{2+} from thapsigargin-sensitive stores and was significantly reduced (but not abolished) with the removal of extracellular Ca^{2+}. The actions of CP 55,940 were shown to occur independently of the CB_1 receptor as the CB_1 receptor antagonist AM251 did not inhibit the increase in $[Ca^{2+}]_i$. The study did not investigate the effects of other cannabinoid agonists or antagonists, including those more specific to the CB_2 receptor, indicating that more work is needed to establish how CP 55,940 evokes a rise in $[Ca^{2+}]_i$ in MDCK cells.

In addition to its effects on the $InsP_3$ receptor, 2-APB has been shown to directly block SOCCs in human platelets at concentrations used in the present study (Dobrydneva and Blackmore, 2001). This suggests that CCE does not mediate the cannabinoid-induced rise in $[Ca^{2+}]_i$ in DDT_1 MF-2 cells. We therefore used the SOCC inhibitor SKF 96365 to support the data obtained with 2-APB. SKF 96365 had no effect on the CP 55,940-evoked current at a concentration shown to significantly inhibit CCE in other systems (Merritt *et al.*, 1990). Thus, together the SKF 96365 and 2-APB data argue against CCE as a mechanism for CB_1 receptor-evoked increases in $[Ca^{2+}]_i$. To ascertain if SKF 96365-sensitive SOCCs are present at all in DDT_1 MF-2 cells the effects of the inhibitor on the outward currents evoked subsequently by histamine were determined. Interestingly, SKF 96365 had no effect on the initial response to histamine but significantly reduced the response to a second histamine

application suggesting that SKF 96365-sensitive SOCCs are present in DDT_1 MF-2 cells. However, instead of mediating Ca^{2+} influx during the histamine response they may act to refill depleted internal Ca^{2+} stores between responses. Consistent with these results, Molleman *et al.* (1991a) showed that the removal of extracellular Ca^{2+} inhibited subsequent responses to histamine in DDT_1 MF-2 cells. In addition, the refilling of Ca^{2+} stores occurred independently of H_1 receptor-mediated Ca^{2+} influx as store-refilling could still be demonstrated in the presence of the H_1 receptor antagonist mepyramine (Dickenson and Hill, 1992). This is consistent with the idea that SOCC activation is dependent on the filling state of the stores and therefore independent of receptor occupation. Moreover, SKF 96365 has been shown to inhibit the refilling of Ca^{2+} stores following repeated M_3-muscarinic receptor stimulation in vascular smooth muscle (Weirich *et al.*, 2004).

4.1.3 Non-capacitative Ca^{2+} entry (NCCE): the role of arachidonic acid (AA)

The results obtained with 2-APB and SKF 96365 suggest that CCE does not mediate the increase in $[Ca^{2+}]_i$ observed during CB_1 receptor stimulation, although an influx of Ca^{2+} is clearly required. Recently an NCCE pathway has been described which operates independently of intracellular store depletion. In this pathway the rise in $[Ca^{2+}]_i$ occurs via Ca^{2+} influx activated by intracellular messengers including AA. AA-evoked Ca^{2+} influx has been described in a variety of cell types including Balb-C 3T3 mouse fibroblasts (Munaron *et al.*, 1997), rat aortic smooth muscle cells (Broad *et al.*, 1999), bovine aortic endothelial cells (Fiorio Pla and Munaron, 2001) and rat astrocytes (Sergeeva *et al.*, 2003). In HEK293 cells the channels responsible for the AA-mediated Ca^{2+} influx were investigated and the resulting membrane current

designated as I_{ARC} (arachidonate-regulated Ca^{2+} current) (Mignen and Shuttleworth, 2000). In addition, Van der Zee *et al.* (1995) demonstrated that AA initiated Ca^{2+} influx during H_1 receptor stimulation in DDT_1 MF-2 cells suggesting an NCCE pathway is operational in this cell line. This lends support to the possibility that CB_1 receptor stimulation induces a rise in $[Ca^{2+}]_i$ through a similar non-capacitative pathway.

Application of AA to DDT_1 MF-2 cells evoked a transient outward current, similar to that evoked by application of CP 55,940. Exogenous application of AA has been shown to induce Ca^{2+} influx in cells utilising NCCE (Munaron *et al.*, 1997; Broad *et al.*, 1999; Mignen and Shuttleworth, 2000; Fiorio Pla and Munaron, 2001) including DDT_1 MF-2 cells (Van der Zee *et al.*, 1995) so it was expected to produce an outward K^+ current. In order to determine if CB_1 receptor-induced increases in $[Ca^{2+}]_i$ occur through the activation of an ARC-like channel, the effects of AA on the CP 55,940-evoked current were established. AA concentration-dependently reduced the CP 55,940-evoked response. A similar result was obtained previously when AA was applied prior to histamine application in this cell line (Van der Zee *et al.*, 1995). This resulted in an abolition of the histamine-evoked Ca^{2+} influx (NCCE), reflected by a reduction in the outward current. The remaining current was due to a release of Ca^{2+} from intracellular stores as it was abolished by the $InsP_3$ antagonist heparin. These observations together also suggest that the Ca^{2+}-dependent K^+ channels were not desensitised as a result of AA-mediated Ca^{2+} influx, otherwise a complete abolition of the subsequent histamine-evoked current would have been observed. Hence, the data collectively implies that AA activates the same Ca^{2+} channels that mediate Ca^{2+} influx during both CB_1 and H_1 receptor stimulation.

To determine the presence of an AA-mediated NCCE pathway evoked by CB_1 receptor stimulation, [^{3}H]AA release was measured in response to CP 55,940 application. CP 55,940 evoked a concentration-dependent increase in [^{3}H]AA release suggesting that CB_1 receptor stimulation results in AA production. This was confirmed by using the CB_1 receptor antagonist SR 141716A, which blocked the CP 55,940-evoked AA release completely. This compound appears to act as a partial agonist in view of its enhancement of basal AA efflux. It is known that SR 141716A can exhibit partial agonist effects in other experimental preparations (Schivachar *et al.*, 1996; Smith *et al.*, 2000). Previous work using DDT_1 MF-2 cells has shown that SR 141716A reduces both Δ^9-THC-induced increases in $[Ca^{2+}]_i$ and CP 55,940-evoked outward currents (Filipeanu *et al.*, 1997; Begg *et al.*, 2001). Together with our data this suggests that CP 55,940 generates AA in a CB_1 receptor-dependent manner.

In N18 mouse neuroblastoma cells (Hunter and Burnstein, 1997) and rat brain astrocytes (Schivachar *et al.*, 1996) Δ^9-THC has been shown to mobilise AA. These effects were SR 141716A-sensitive, implying the involvement of the CB_1 receptor. The signalling implications for AA in the latter experimental preparation were not explored further but it is interesting that AA induces Ca^{2+} influx in primary rat astrocyte cell cultures (Sergeeva *et al.*, 2003). AA was thought to activate Ca^{2+} channels directly in DDT_1 MF-2 cells as inhibitors of the cyclo-oxygenase and lipoxygenase pathway did not affect the characteristics of the histamine-induced [^{3}H]AA release (Van der Zee *et al.*, 1995). In accordance with this we found that inhibitors of either of these two pathways had no effect on the CP 55,940-evoked outward current in DDT_1 MF-2 cells. Comparable results have also been described in other preparations, where NCCE is mediated by AA rather than its metabolites (Munaron *et al.*, 1997; Broad *et al.*, 1999; Fiorio Pla and Munaron, 2001). This

further supports the possibility that CB_1 receptor stimulation may initiate Ca^{2+} influx through AA-mediated NCCE.

AA directly activates ARC channels in HEK293 cells (Mignen and Shuttleworth, 2000; Luo *et al.*, 2001a) and aortic endothelial cells (Fiorio Pla and Munaron, 2001). However, other preparations have shown that an AA-mediated production of NO is a key regulator of the Ca^{2+} channels utilised during NCCE. In A7r5 vascular smooth muscle cells AA-mediated NCCE was mimicked by NO and abolished by the NOS inhibitor L-NAME (Moneer *et al.*, 2003). In mouse parotid acini the NOS inhibitor 7-nitroindazole reduced AA-mediated Ca^{2+} influx (Watson *et al.*, 2004). Interestingly, previous reports have shown that the NO donors GEA3162 and sodium nitroprusside evoked an increase in $[Ca^{2+}]_i$ in DDT_1 MF-2 cells (Favre *et al.*, 1998). This increase was abolished in Ca^{2+}-free medium suggesting that NO induces Ca^{2+} influx from the extracellular space. From these collective results it is interesting to speculate that, in DDT_1 MF-2 cells, CB_1 or H_1 receptor stimulation may evoke a rise in $[Ca^{2+}]_i$ through an AA-mediated increase in NO, which in turn stimulates NCCE.

4.1.4 La^{3+} and Gd^{3+}-sensitive Ca^{2+} influx

The non-selective Ca^{2+} channel blocker La^{3+} abolished the CP 55,940-evoked outward current. At the same concentration La^{3+} has been shown to inhibit I_{ARC} in HEK293 cells (Mignen and Shuttleworth, 2000), bovine aortic endothelial cells (Fiorio Pla and Munaron, 2001) and DDT_1 MF-2 cells (Van der Zee *et al.*, 1995). La^{3+} was also able to inhibit outward currents evoked by AA, which suggests that AA production occurs upstream of Ca^{2+} entry and hence Ca^{2+} channel activation.

Complete abolition of the AA evoked response was not seen at a concentration that abolished the CP 55,940-induced response. This may be due to an AA-mediated release of Ca^{2+} from internal stores, which has been shown to occur at higher AA concentrations (Fioro Pla and Munaron, 2001; Watson *et al.*, 2004). AA has also been shown to activate $I_{K,Ca}$ directly in vascular smooth muscle cells at concentrations exceeding 5 μM (Kirber *et al.*, 1992). However, if this mechanism solely mediated the outward current evoked by AA then such a significant reduction would not have been observed in the presence of La^{3+}. La^{3+} can also inhibit CCE (Putney, 2001) so the effects of this inhibitor on thapsigargin-evoked CCE in DDT_1 MF-2 cells were investigated. Thapsigargin depletes Ca^{2+} stores by inhibiting the Ca^{2+} ATPase pumps present on the sarcoplasmic reticulum, thereby initiating CCE (Holda *et al.*, 1998). Ni^{2+} decreased Ca^{2+} entry evoked by thapsigargin in a concentration-dependent manner but interestingly La^{3+} had no effect on the Ca^{2+} influx in response to thapsigargin, at a concentration seen to abolish the CP 55,940-evoked current. This clearly implicates a Ca^{2+} influx pathway separate from CCE, utilised during CB_1 receptor signalling in DDT_1 MF-2 cells.

Gd^{3+}, another inhibitor of Ca^{2+} influx, is able to distinguish between CCE and NCCE in rat aortic smooth muscle cells (Broad *et al.*, 1999). At low concentrations (1 μM) Gd^{3+} inhibited CCE, while at higher concentrations (100 μM) both CCE and NCCE were inhibited. In DDT_1 MF-2 cells Gd^{3+} (1 μM) inhibited the outward current in response to CP 55,940 but had no effect on CCE evoked by thapsigargin. Although other authors have shown the CCE pathway to be potently inhibited by 1 μM Gd^{3+} (Luo *et al.*, 2001a; Putney, 2001), Gd^{3+}-insensitive SOCCs are also expressed in other experimental systems (Fernando and Barritt, 1994, 1995). The data obtained from the current study is consistent with previous work showing that low concentrations of

Gd^{3+} can inhibit currents mediated by I_{ARC} in HEK293 cells (Mignen *et al.*, 2003) and inhibit Ca^{2+} influx in response to AA application in rat astrocytes (Sergeeva *et al.*, 2003). This may suggest that the AA-sensitive channels in rat aortic smooth muscle cells are distinct from those described in HEK293 cells and DDT_1 MF-2 cells. In support of this, SKF 96365 (100 nM) inhibited NCCE in rat aortic cells (Moneer *et al.*, 2003) but at a 100 fold greater concentration has no effect on the CP 55,940-evoked outward current in DDT_1 MF-2 cells. Once again the Gd^{3+} results are consistent with the idea that the Ca^{2+} influx pathway initiated during CB_1 receptor stimulation is not capacitative.

4.1.5 Properties of the arachidonate-regulated Ca^{2+} (ARC) channel

Three key properties are exhibited by ARC channels: Ca^{2+} entry is (1) activated by low AA concentrations (< 5 μM), (2) directly triggered by AA and not by its metabolites, (3) independent from intracellular Ca^{2+} store depletion (Broad *et al.*, 1999; Fiorio Pla and Munaron, 2001; Luo *et al.*, 2001b). Consistent with these properties of ARC channels, low concentrations of AA evoke a significant outward current in DDT_1 MF-2 cells while inhibitors of AA metabolism have no effect on CP 55,940-evoked currents. However, it was previously shown that thapsigargin inhibited currents induced by CP 55,940 in DDT_1 MF-2 cells (Begg *et al.*, 2001). This suggests that CB_1 receptor-mediated Ca^{2+} influx is partly dependent on intracellular Ca^{2+} release, therefore opposing the third property exhibited by ARC channels. This could be explained by the reciprocal regulation of CCE and NCCE, described in cells exhibiting AA-mediated Ca^{2+} influx (Luo *et al.*, 2001a; Mignen *et al.*, 2001; Moneer and Taylor, 2002). It was suggested that these pathways are coupled to one another, in

an inverse manner, forming two non-overlapping Ca^{2+} entry pathways. Hence thapsigargin, by evoking CCE, may be inhibiting CB_1 receptor-mediated increases in $[Ca^{2+}]_i$ in DDT_1 MF-2 cells due to the antagonism of AA-mediated NCCE. This mutual antagonism may provide an important mechanism for the cell guarding against toxic Ca^{2+} overload, which might occur if both Ca^{2+} entry pathways were operational at the same time. Interestingly, further work by Mignen *et al.* (2003) showed that calcineurin, a serine/ threonine protein phosphatase regulated by Ca^{2+}-dependent calmodulin binding, was involved in inhibiting I_{ARC} in HEK293 cells. Inhibitors of calcineurin reversed the inhibitory effect on ARC channels. Calcineurin activation was shown to be dependent on the sustained elevation of cytosolic Ca^{2+} resulting from the activation of SOCCs only and hence was shown to mediate the CCE-evoked inhibition of ARC channels (Mignen *et al.*, 2003).

The biophysical properties of the AA-activated Ca^{2+} channels are as yet unknown although recently light-sensitive channels present in *Drosophila* photoreceptors, belonging to the transient receptor potential-family, have been shown to be directly activated by fatty acids, including AA (Chyb *et al.*, 1999).

4.1.6 AA production via phospholipase A_2 (PLA_2)

Another objective of the current study was to establish a link between MAP kinase activation and the resulting rise in $[Ca^{2+}]_i$. The results suggest that AA is involved in the signalling pathways induced during stimulation of the CB_1 receptor. The liberation of AA can occur through the direct action of PLA_2 on phospholipids. In particular the $cPLA_2$ enzyme is associated with AA liberation, regulated by Ca^{2+} and requiring phosphorylation by MAP kinase for maximal activation (Lin *et al.*, 1993;

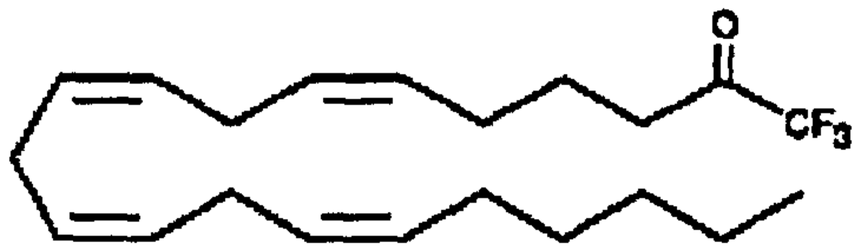
Kudo and Murakami, 2002). In Balb-C 3T3 mouse fibroblasts, exhibiting NCCE, AA production was shown to occur through the activation of MAP kinase and $cPLA_2$ (Munaron *et al.*, 1997). In addition, experiments in fetal lung fibroblasts have yielded results indicating that a cannabinoid-mediated increase in AA is also associated with an increased phosphorylation and hence activity of both MAP kinase and $cPLA_2$ (Wartmann *et al.*, 1995), although later work revealed this was mediated by the CB_2 receptor (Hunter and Burnstein, 1997). To establish if a PLA_2 enzyme was stimulated during CB_1 receptor stimulation in DDT_1 MF-2 cells, thus providing a link between MAP kinase and increasing $[Ca^{2+}]_i$, inhibitors of PLA_2 were used to try and block CP 55,940-induced currents.

The $cPLA_2$ inhibitor ATK (15 μM) significantly inhibited the CP 55,940-evoked outward current, although by itself it induced a transient outward current in DDT_1 MF-2 cells. In neutrophils ATK has been shown to evoke a significant rise in AA, although only at concentrations exceeding 15 μM (Susztak *et al.*, 1997). ATK might induce a rise in AA in DDT_1 MF-2 smooth muscle cells at concentrations lower than those seen to generate AA in neutrophils. ATK (10 μM) has also been shown to inhibit lipoxygenase in neutrophils (Fonteh *et al.*, 2002), which may contribute to a rise in AA if there is normally a continuous turnover of AA in DDT_1 MF-2 cells through this metabolic pathway. If the response to ATK is due to an increase in AA then the reduced cannabinoid response may be due to a desensitisation of the Ca^{2+} influx channels, similar to that seen with exogenous AA application prior to CP 55,940. ATK is an analogue of AA (Fig. 4.1) and is known to inhibit $cPLA_2$ by binding to the active site of the enzyme (Trimble *et al.*, 1993). Hence, ATK may be able to mimic the cellular actions of AA, including I_{ARC} activation, which would account for the outward current.

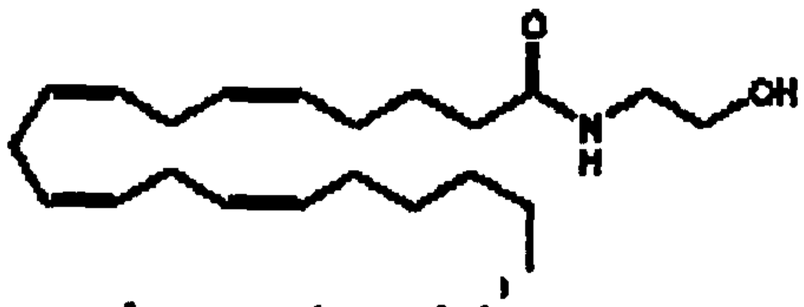
ATK has been shown to displace [^{3}H]CP 55,940 binding to the CB_1 receptor in intact neuroblastoma cells, with a K_i of 0.65 μM (Koutek *et al.*, 1994). The structural similarities to the endogenous cannabinoids may underlie the compound's ability to bind to the CB_1 receptor (Fig. 4.1). Hence, the reduction in the CP 55,940-evoked current may be due to an inability of the cannabinoid to bind to the CB_1 receptor in the presence of ATK. ATK has also been shown to inhibit the $iPLA_2$ isoform at concentrations used to inhibit $cPLA_2$ (Osterhout and Shuttleworth, 2000). Therefore it is impossible to determine if CB_1 receptor stimulation activates $cPLA_2$ using this inhibitor alone.

ATK has been shown to bind to the CB_1 receptor and in the present study also induces a response similar to that of CP 55,940 suggesting that it may also have efficacy at the receptor. To test this hypothesis ATK was applied in the presence of the CB_1 receptor antagonist SR 141716A to try and block the response. SR 141716A had no effect on the ATK-evoked current suggesting that the response is independent of CB_1 receptor activation. Another selective inhibitor of $cPLA_2$ (and analogue of AA) methyl arachidonyl fluorophosphonate (MAFP) (Lio *et al.*, 1996) was also shown to exhibit antagonist actions at the CB_1 receptor in the myenteric plexus longitudinal muscle (MPLM) preparation (Fernando and Pertwee, 1997). However, MAFP alone had no effect on electrically-evoked contractions suggesting it did not stimulate the CB_1 receptor (Fernando and Pertwee, 1997). Our data supports the idea that ATK may also be an antagonist at the CB_1 receptor, although its stimulant effects in DDT_1 MF-2 cells are likely to involve a direct action on intracellular signalling pathways.

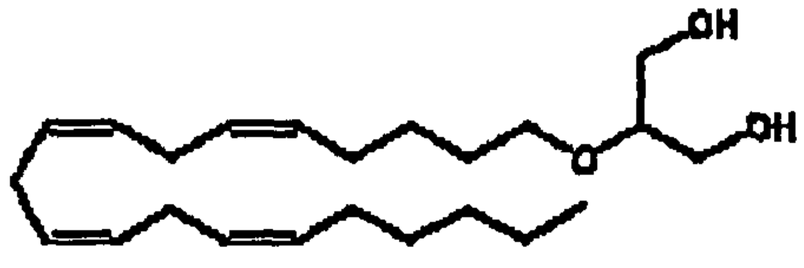
Subsequent experiments to establish the role of PLA_2 in CB_1 receptor signalling used non-specific PLA_2 inhibitors including 4-BPB and quinacrine. 4-BPB



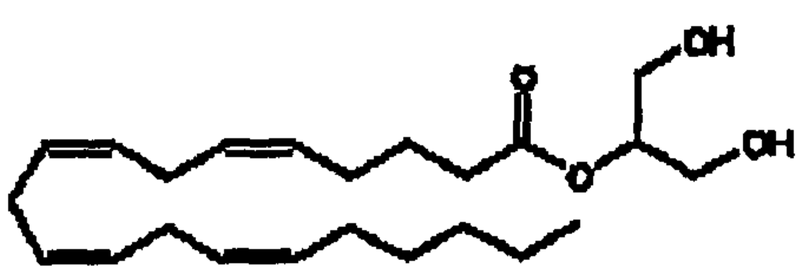
ATK



Anandamide



Noladin ether



2-Arachidonyl glycerol

Figure 4.1 Structural similarity of the cPLA$_2$ inhibitor ATK with the endogenous cannabinoids.

(10 μM) significantly inhibited the outward current evoked by CP 55,940 but alone evoked an outward current in DDT_1 MF-2 cells. Previous studies in human gingival fibroblasts showed that 4-BPB significantly increased $[Ca^{2+}]_i$ in a concentration-dependent manner (1-100 μM) (Ogata *et al.*, 2002). The 4-BPB-induced Ca^{2+} mobilisation was abolished by the elimination of extracellular Ca^{2+} suggesting an influx of Ca^{2+} from the external medium is part of the response. The effect of 4-BPB was thought not to occur through its ability to inhibit PLA_2 as ATK and quinacrine failed to evoke Ca^{2+} mobilisation (Ogata *et al.*, 2002). Hence, 4-BPB may evoke an outward current in DDT_1 MF-2 cells by inducing Ca^{2+} influx. Interestingly, prior application of CP 55,940 inhibited the subsequent response evoked by 4-BPB. This suggests that 4-BPB may exploit a number of intracellular signalling pathways utilised during CB_1 receptor stimulation, e.g. the activation of non-capacitative Ca^{2+} channels. Hence similar to ATK, the results obtained with 4-BPB and CP 55,940 cannot attribute AA production solely through the actions of PLA_2.

Quinacrine (10-30 μM) reduced the CP 55,940-evoked current in DDT_1 MF-2 cells but paradoxically at 100 μM had no effect. Application of quinacrine (100 μM) alone had no effect on membrane currents. Also noteworthy is that the level of inhibition was maximal at 10 μM, with no further significant decrease at 30 μM, which may suggest that CB_1 receptor stimulation in DDT_1 MF-2 cells induces a rise in Ca^{2+} via at least two pathways. Δ^9-THC induced a release of AA in mouse peritoneal cells, which involved the activation of both PLA_2 and PLD (Burnstein *et al.*, 1994). AA can be released from DAG by DAGL (Lee and Severson, 1994). The generation of DAG, a secondary product of PLD metabolism, was thought to lead to the generation of AA in mouse peritoneal cells (Burnstein *et al.*, 1994). In addition, quinacrine has been shown to directly interfere with ion channel function (Xiao *et al.*,

2000), including Ca^{2+}-dependent K^+ channels in rat arterial smooth muscle (Vanheel *et al.*, 1999). This may account for the effects of quinacrine on evoked $I_{K,Ca}$ measured in DDT_1 MF-2 cells. Therefore the effects of quinacrine on CP 55,940-evoked [^{3}H]AA release was determined, an assay independent of $I_{K,Ca}$ activation. Quinacrine (100 μM) alone had no effects on basal AA release and completely abolished the generation of AA induced by CP 55,940. The AA release data suggests that the primary pathway for AA production, during CB_1 receptor stimulation, involves the activation of PLA_2. However, as it is unclear what the non-specific actions of quinacrine are in DDT_1 MF-2 cells, which seem to interfere with evoked CB_1 receptor signalling pathways downstream of AA production, further work is needed to support a CB_1-mediated activation of PLA_2.

The intracellular signalling pathways that evoked a release of AA in DDT_1 MF-2 cells during H_1 receptor stimulation have not been identified, although stimulation of the receptor has been shown to induce phosphorylation of MAP kinase, with significant phosphorylation occurring after only a minute (Robinson and Dickenson, 2001). This suggests an immediate action of MAP kinase, which possibly implicates a PLA_2-induced release of AA during H_1 receptor stimulation in DDT_1 MF-2 cells. Previous work has shown that both histamine- and CP 55,940-induced currents in DDT_1 MF-2 cells were inhibited by the MAP kinase inhibitor PD 98059, although histamine was less sensitive to this inhibition (Begg *et al.*, 2001). This is consistent with the idea that the main increase in $[Ca^{2+}]_i$ of the histamine response is derived from $InsP_3$-sensitive stores and that PD 98059 could be inhibiting the NCCE component of the histamine response.

The activation of a PLA_2 pathway during CB_1 receptor stimulation could provide another explanation for a reduction in the CP 55,940-evoked outward current

seen with thapsigargin in DDT_1 MF-2 cells (Begg *et al.*, 2001). Ca^{2+} is required for $cPLA_2$ translocation to the nuclear envelope and also stabilises the association of $cPLA_2$ with the nuclear membrane (Kudo and Murakami, 2002). It could be proposed that a release of Ca^{2+} from internal thapsigargin-sensitive stores is required to initiate this process. However, it was shown that a Ca^{2+}-independent $cPLA_2$ underlies the receptor stimulation of AA-mediated NCCE in HEK293 cells (Osterhout and Shuttleworth, 2000). Until more specific $cPLA_2$ inhibitors are developed that do not interfere with cannabinoid signalling it will be difficult, pharmacologically, to ascertain an involvement of this subtype in CB_1 receptor-mediated responses in DDT_1 MF-2 cells.

4.1.7 Phosphorylation of p42/44 MAP kinase

The subtype of MAP kinase activated during CB_1 receptor stimulation in DDT_1 MF-2 cells along with the time course of its activation was investigated. Previous studies have shown that CB_1 receptor stimulation activates a p42/44 MAP kinase in CHO cells and cultured U373MG human astrocytoma cells (Bouaboula *et al.*, 1995a, b; Galve-Roperh *et al.*, 2002). In addition, Δ^9-THC activates p42/44 MAP kinase in the murine hippocampus (Derkinderin *et al.*, 2003), striatum and cerebellum (Rubino *et al.*, 2004), in an SR 141716A-sensitive manner. Hence, it seemed probable that CB_1 receptor stimulation in DDT_1 MF-2 smooth muscle cells would also activate a p42/44 MAP kinase.

DDT_1 MF-2 cells were treated with CP 55,940 (+ ethanol) for time periods ranging between 30 seconds and 30 minutes, before proteins were separated by SDS-PAGE and immunoblotted with a specific anti-phospho p42/44 MAP kinase antibody.

The results hinted at a possible time-dependent activation of p42/44 MAP kinase, although densitometry data showed that this was not statistically significant, probably due to the limited n numbers. Hence, further western blot analysis (and densitometry data) is needed to increase the n values and thus establish if CP 55,940 produces a significant increase in p42/44 MAP kinase phosphorylation.

Ethanol (0.1%) alone increased the phosphorylation of p42/44 MAP kinase. The phosphorylation was as intense, if not more intense, when compared to CP 55,940 at corresponding time points. This suggests that the effects observed with CP 55,940 could be due to the activity of the ethanol. In contrast, the electrophysiological data showed that 0.1% ethanol had no effect on membrane currents in DDT_1 MF-2 cells. Previous studies have also demonstrated that ethanol is able to activate a p42/44 MAP kinase, albeit at greater concentrations. In vascular smooth muscle cells 0.3% ethanol was able to induce a significant phosphorylation of MAP kinase compared to control, although at 0.1% ethanol no effect was observed (Sachinidis *et al.*, 1999). In rat pancreatic cells ethanol also induced a marked phosphorylation of p42/44 MAP kinase, but the concentration of ethanol used was again three times higher than that used in the present study (Masamune *et al.*, 2002).

Paradoxically the results obtained with CP 55,940 in ethanol do not mirror those of ethanol alone. This could be explained if ethanol evoked an increase in MAP kinase phosphorylation, which was in fact reduced by CP 55,940. Recently such an effect of the cannabinoid WIN 55,212-2 has been demonstrated in mouse splenocytes (Kaplan and Kaminski, 2003). However, this contradicts the electrophysiological results demonstrating that MAP kinase activation is required to evoke a CB_1 receptor-mediated increase in $[Ca^{2+}]_i$ in DDT_1 MF-2 cells (Begg *et al.*, 2001). Non-specific effects on intracellular signalling molecules including protein kinases have been

reported with PD 98059 (Davies *et al.*, 2001). Therefore the electrophysiological data, suggesting that MAP kinase activation is required to evoke an increase in $[Ca^{2+}]_i$ during CB_1 receptor stimulation, needs further support from results using other MAP kinase inhibitors.

To further establish if CB_1 receptor stimulation induces the phosphorylation of a p42/44 MAP kinase, SR 141716A was used to try and inhibit the effects of CP 55,940. However, the results proved difficult to interpret and were inconclusive.

4.1.8 Problems associated with CB_1 receptor signalling in DDT_1 MF-2 cells

In the current study there would be times when, at the beginning of a new passage, cells would stop responding to CP 55,940 but the application of histamine would still produce an outward current comparable to that of controls. The reason for this sudden abolishment, in what seemed only cannabinoid signalling, was unknown. Normally a new batch of cells (with a lower passage number) was unfrozen and experiments could continue. Hence, at regular intervals, especially in experiments involving the complete abolition of the cannabinoid response, DDT_1 MF-2 cells were frequently tested to see if they still responded to CP 55,940. However, this phenomenon became irreversible, even in newly unfrozen cells, which meant that further cannabinoid experiments in the DDT_1 MF-2 cell line became impossible to perform. Approximately 3-4 months was spent trying to establish and rectify the problem but to no avail. Numerous batches of DDT_1 MF-2 cells were unfrozen and tested. Morphologically they looked no different from cells used at the beginning of the study. The same batch of CP 55,940 was able to inhibit electrically-evoked contractions of the MPLM suggesting that the agonist was still active. All culture

media was changed, including the suppliers from which they were obtained but the problem still persisted. DDT_1 MF-2 cells, shown to respond to CP 55,940, were also obtained from our collaborators in Holland. These cells would work for a week or so but then, again, stop responding to CP 55,940 on the next passage. New cells were even obtained from the European Collection of Cell Cultures but although they responded to histamine, CP 55,940 still had no effect. Due to time constraints experiments concerned with DDT_1 MF-2 signalling had to cease.

The abolition of the normal response to CP 55,940 suggests either an effect on the signalling pathways attributable solely to CB_1 receptor stimulation or a direct effect on CB_1 receptors. Previous studies have shown that CB_1 receptors undergo agonist-induced desensitisation and internalisation in transfected HEK293 and neuroblastoma N18TG2 cells (Keren and Sarne, 2003). However, this would mean that cannabinoid agonists would have to be present before experiments i.e. during the cell culture stage. Valk *et al.* (1997) demonstrated that the IL-3-induced proliferation of myeloid 32D cells was enhanced by anandamide, in serum-free medium. When the cells were cultured in FCS, anandamide had no effect suggesting that anandamide or another cannabinoid ligand was present in FCS. Hence, although speculative, it may be possible that the FCS used to culture the DDT_1 MF-2 cells during the later stages of the study contained a high enough concentration of cannabinoid ligands to irreversibly desensitise and internalise CB_1 receptors. As yet, no further explanations can be presented to explain the loss of CP 55,940-evoked currents.

4.1.9 Summary

In summary the results obtained in DDT_1 MF-2 smooth muscle cells have shown for the first time that stimulation of cannabinoid CB_1 receptors can lead to Ca^{2+} influx mediated by AA (Fig. 4.2). This influx pathway is distinct from CCE and instead may involve the activation of I_{ARC} and hence NCCE. The CCE pathway is operational in DDT_1 MF-2 cells and can refill depleted intracellular stores between responses, as shown with histamine H_1 receptor stimulation. The current study also provides evidence for a CB_1 receptor-mediated activation of PLA_2, upstream of AA production, and likely downstream of MAP kinase. Western blot analysis hinted at a possible CP 55,940-induced phosphorylation of the p42/44 MAP kinase. However, due to the effects of vehicle (ethanol) alone on MAP kinase phosphorylation these results are difficult to interpret and thus further work is clearly required to convincingly associate CB_1 receptor stimulation with p42/44 MAP kinase activation, in DDT_1 MF-2 cells.

AA-mediated NCCE may underlie $[Ca^{2+}]_i$ oscillations as demonstrated in cells derived from the exocrine avian nasal gland (Shuttleworth, 1996) and in HEK293 cells (Shuttleworth and Thompson, 1998). Such signals are probably key determinants in the control of critical cell activities such as targeted regulation of kinases and phosphatases, ion channels, energy metabolism and secretions (Shuttleworth, 1999). Further work is required to establish if a similar role can be attributed to the activation of AA-mediated NCCE by CB_1 receptors in DDT_1 MF-2 cells.

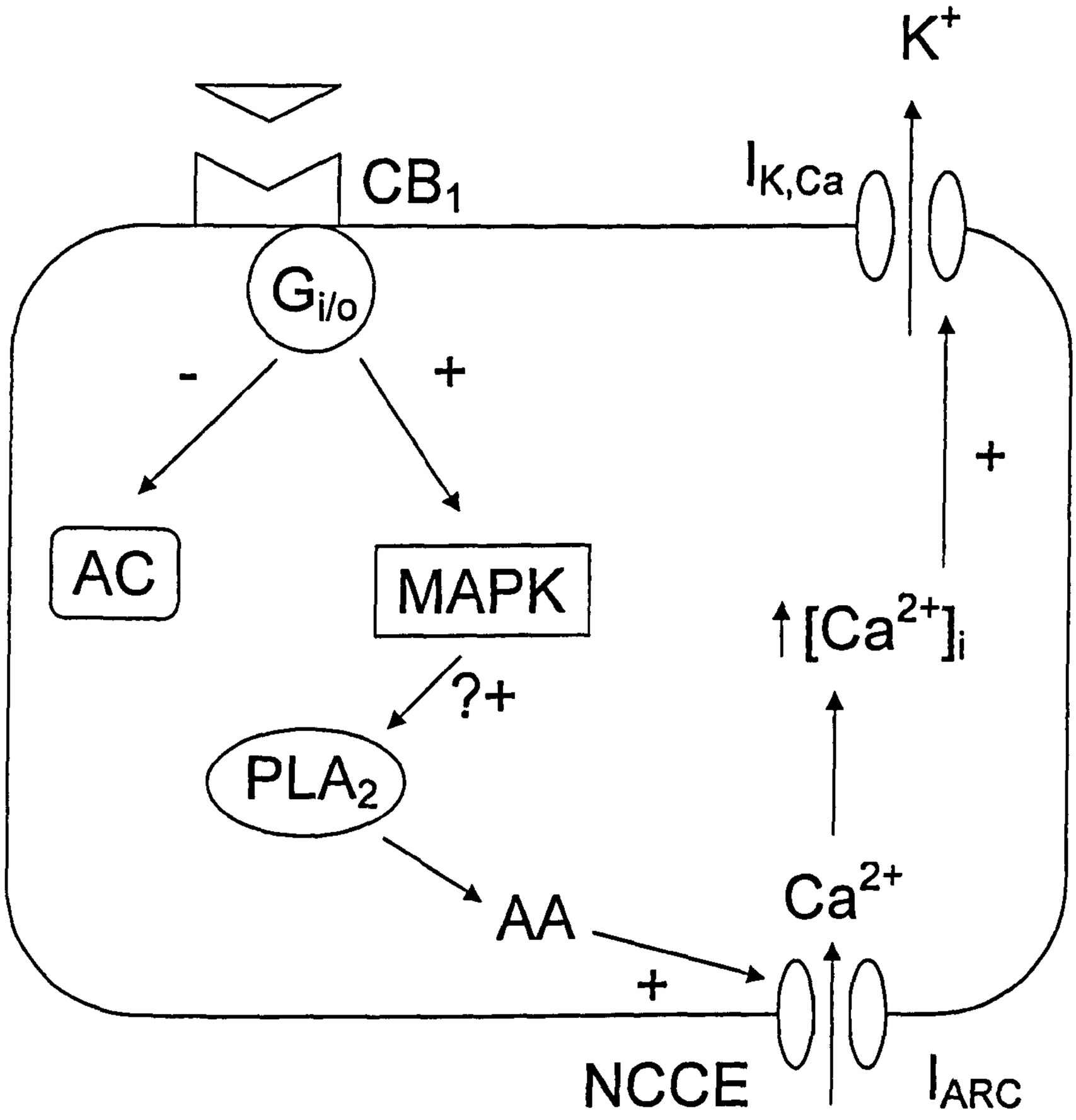


Figure 4.2 Possible intracellular signalling pathways evoked in DDT_1 MF-2 cells during CB_1 receptor stimulation, which lead to an increase in $[Ca^{2+}]_i$.
+ suggests activation, - suggests inhibition, ? suggests a possible mechanism. CB_1 receptor stimulation activates MAPK and inhibits AC, through $G_{i/o}$ proteins. MAPK may subsequently phosphorylate PLA_2, which in turn generates AA. AA induces an increase in $[Ca^{2+}]_i$ via the activation of ARC-like channels, present on the cell membrane, and thus initiates NCCE.

4.2 Immunohistochemical study of cultured myenteric neurons

Cannabinoids can inhibit gastrointestinal motility, which involves the stimulation of presynaptic CB_1 receptors in the myenteric plexus that reduce ACh release (Pertwee *et al.*, 1996a; Coutts and Pertwee, 1997). The signalling pathways associated with this inhibition are poorly understood so it was of great interest to establish the electrophysiological effects of cannabinoids in isolated myenteric neurons maintained in primary culture. However, before these neurons could be used they had to be validated as a model for myenteric neurons *in situ*. For instance, it had to be established that cultured myenteric neurons express CB_1 receptors. Myenteric neurons also form a heterogeneous population (Costa *et al.*, 1996), although 82% of myenteric neurons in the guinea-pig ileum are cholinergic (Coutts *et al.*, 2002). Therefore it was also necessary to know the most prevalent class of myenteric neuron, present in culture, which expressed the CB_1 receptor. Any effect of cannabinoids could then be associated with a specific neuron that had been patched.

4.2.1 CB_1 receptor expression on cholinergic neurons

Immunohistochemistry, performed in collaboration with Dr. Angela Coutts at Aberdeen University, was used to characterise the neurons. Detection of cholinergic neurons was achieved with antibodies raised against ChAT, while CB_1 receptors were detected with antibody raised against the C-terminus of the receptor. 97% of cholinergic neurons in culture expressed the CB_1 receptor but more importantly all the neurons that labelled positively for CB_1 were cholinergic. This indicates that any CB_1 receptor-mediated effect in these cultures is on a cholinergic neuron. The data is

consistent with that of Coutts *et al.* (2002) who found that 98.5% of CB_1-positive myenteric neurones, *in situ*, were cholinergic.

The CB_1 receptor antibody densely labelled both the neuronal cell body and neuronal processes. It is generally assumed that CB_1 receptors are localised to the presynaptic terminals where they inhibit the release of neurotransmitter. The cultured cells were initially permeabilised before being incubated with the antibody, which means intracellular epitopes are targeted. Therefore the dense labelling of the soma may be consistent with the production of CB_1 receptor protein in the cell body and its subsequent transport to the processes.

In the intact guinea-pig ileum, immunohistochemical studies identified CB_1 receptors on myenteric primary afferent, interneuronal and motor neuronal cell bodies and nerve fibres (Coutts *et al.*, 2002). In the ascending excitatory pathway, which mediates smooth muscle contraction, these neuronal types are predominantly cholinergic (Brookes *et* al., 1997; LePard and Galligan, 2000; Furness and Sanger, 2002). As all CB_1 receptor-positive neurons were cholinergic in the myenteric cultures this implies that any effects obtained with cannabinoids could represent an action on any of these three main neuronal types.

4.2.2 Neurofilament (NF) immunostaining

Cultured cells were also incubated with antibody (NFP-200) raised against NF proteins as they show the cell morphology much more clearly than antibodies for CB_1 receptors or ChAT. The NF antibody labelled neurons of different sizes and shapes and differing intensity of labelling of the soma suggesting different types of cells are present in culture. This is consistent with the findings of Brehmer *et al.* (2002) who

used NF immunohistochemistry to look at pig myenteric neurones *in situ*. The great variation in morphology of the cultured neurons suggests, at least, the presence of Dogiel type I and II neurons and thus motor and/ or interneurons and IPANs. However, due to the clumping of cells in culture it was impossible to see the morphology of every cell in order to be able to classify it, so it is unknown if some cell types survive the culturing process better than others. Moreover, the proportion of Dogiel types would inevitably vary even without clumping.

Some neurons in culture were shown not to label with NFP-200 at all, which again has also been demonstrated in guinea-pig myenteric neurons *in situ* (Coutts *et al.*, 2002). Only a subset of CB_1-positive cells expressed NF protein, which unfortunately was not quantified. In the whole guinea-pig myenteric plexus 58% of neurons that were positive for the CB_1 receptor also labelled for NF protein (Coutts *et al.*, 2002).

4.2.3 Summary

In summary, the immunohistochemistry has shown that cultured myenteric neurons express CB_1 receptors, where they are localised on cholinergic neurons. Furthermore, the great variation in morphology of these cultured myenteric neurons is consistent with the variability in morphology of myenteric neurons *in situ*. This suggests the presence of different types of neuron (motor neuron, interneuron and IPANs) in culture, although an accurate proportion of Dogiel types could not be established. Hence, cultured myenteric neurons seem to be a valid model for establishing the mechanisms underlying a cannabinoid-mediated inhibition of cholinergic neurotransmission *in situ*.

4.3 Cannabinoid signalling in the myenteric plexus: effects on K^+ and Ca^{2+} conductance

The immunohistochemical data validates cultured myenteric neurons as a model for those *in situ*. Hence, myenteric cultures can be used to help identify the CB_1 receptor-mediated signalling events that may lead to a reduction in neurotransmitter release in the intact myenteric plexus. A CB_1 receptor-mediated activation and inhibition of K^+ channels and Ca^{2+} channels respectively has been described previously (Mackie *et al.*, 1995; Twitchell *et al.*, 1997; McAllister *et al.*, 1999; Mu *et al.*, 1999) and has been shown to underlie the inhibitory actions of cannabinoids on neurotransmitter release in areas such as the mouse nucleus accumbens, rat striatum and hippocampus (Lenz *et al.*, 1998; Huang *et al.*, 2001; Robbe *et al.*, 2001). Indeed, the inhibitory actions of WIN 55,212-2 on electrically-evoked contractions of the MPLM were attenuated by forskolin and augmented by reducing extracellular Ca^{2+} (Coutts and Pertwee, 1998). This implies a cannabinoid-mediated inhibition of Ca^{2+} channels and possibly, via the inhibition of the cAMP/ PKA pathway, activation of I_A. Hence, electrophysiological experiments were used to establish the effects of cannabinoids on ion channels present in cultured myenteric neurons.

4.3.1 Identification of voltage-operated ion channels

A voltage step protocol was used to activate any voltage-operated ion channels present in myenteric cultures. Voltage steps evoked fast inward currents followed by a slower more prolonged inward current and finally an outward current. The fast inward

current was blocked by the Na^+ channel inhibitor TTX and the outward current was blocked by the combination of Cs^+ and TEA suggesting that voltage steps also activate voltage-dependent K^+ channels in the cultures. This unmasked inward currents associated with the activation of voltage-operated Ca^{2+} channels (VOCCs), as the non-specific Ca^{2+} channel antagonist Cd^{2+} significantly inhibited them. Threshold for the activation of Ca^{2+} currents was –30 mV and the peak inward current was recorded at a test potential of 0 mV. This is consistent with previous reports investigating Ca^{2+} conductance in cultured myenteric neurons, derived from the guinea-pig ileum (Ren *et al.*, 2001; Bian *et al.*, 2004).

4.3.2 Action potential propagation

Action potentials were evoked in cultured neurons held under current clamp. Even at large membrane depolarisations all the neurons tested only generated a single action potential. S-neurons, which generate fEPSPs (Galligan, 2002), would be expected to evoke more than a single action potential during the period of current injection. After each action potential a long AHP was observed which was still evident at the end of current injection. Long AHPs in myenteric neurons have been associated with AH-neurons/ IPANs and are due to the activation of Ca^{2+}-dependent K^+ channels (Furness *et al.*, 1998). The AHP can last from 1-20 seconds and explains why AH-neurons fire significantly less action potentials compared to S-type neurons in a given time period. However, the action potential of AH-neurons also exhibits a prominent Ca^{2+} hump (Furness *et al.*, 1998), which was not observed in action potentials evoked in the myenteric cultures. Hence, the predominant neuronal type (S- or AH-neuron) present in culture cannot be identified using the electrophysiological

data alone. Furthermore, the results also hang a large question mark over the current electrophysiological classification of myenteric neurons, obtained using intracellular recordings, and therefore needs further clarification.

4.3.3 The effect of CP 55,940 on peak positive and negative membrane currents

Both CP 55,940 and anandamide were initially applied to naïve neurons but were shown to exert no change in membrane currents. Previous reports have mostly described CB_1 receptor-mediated effects on ion channels when the channel itself is active, such as the enhancement of currents through GIRK channels (McAllister *et al.*, 1999), the enhancement of I_A (Hampson *et al.*, 1995; Mu *et al.*, 2000) and the inhibition of N- and P/Q-type Ca^{2+} currents (Mackie *et al.*, 1995; Twitchell *et al.*, 1997). Therefore the effects of CP 55,940 were determined on peak positive and negative membrane currents evoked by voltage steps. As the time taken for CP 55,940 to induce an effect in cultured myenteric neurons was unknown, currents in response to voltage steps were recorded every 30 seconds for a period of 5 minutes, in the presence of the cannabinoid. A slight decrease in both peak positive and negative current was demonstrated over time, although this was not significant.

4.3.4 CP 55,940-mediated activation of GIRK channels

Stimulation of CB_1 receptors has been shown to activate GIRK channels (McAllister *et al.*, 1999; Guo and Ikeda, 2004), which would contribute to a reduction in neurotransmitter release. K_{ir} channels are present in myenteric neurons (Zholos *et al.*, 1999; Ren *et al.*, 2001). Therefore CP 55,940 was used to determine if

cannabinoids could activate GIRK channels in cultured myenteric neurons. Within a 5 minute period of application, CP 55,940 had no effect on the average current evoked by ramp responses, designed to identify the activation of GIRK channels. An extremely important consideration when interpreting the results obtained in cultured myenteric neurons is that the patch is made on the soma. However, CB_1 receptors are thought to be located on the presynaptic terminals (Pertwee *et al.*, 1996a; Coutts and Pertwee, 1997), which are too small to be patched. Indeed, immunohistochemistry has shown that antibody for the CB_1 receptor was closely associated with antibody for synpasin I, which labels protein related to the cytoplasmic surface of synaptic vesicles (Coutts *et al.*, 2002). The current results suggest, at least, that cannabinoids do not activate somatodendritic GIRK channels. The possibility that CP 55,940 may be stimulating GIRK channels closer to presynaptic sites, which we are unable to record due to space clamp problems, cannot be ruled out. In neurons, the cytoplasm of narrow dendrites or axons can be considered as accumulating resistance along the length of the branch. As a result, areas of membrane distant from the pipette electrode (i.e. near the synapse) are poorly clamped. This phenomenon of poor voltage clamp due to significant cytoplasmic resistance is known as space clamp.

4.3.5 CP 55,940-mediated inhibition of VOCCs

The inhibitory effect of cannabinoids on Ca^{2+} channels, mediated by the CB_1 receptor, has been well documented and includes N-, L- and P/Q-type Ca^{2+} channels (Mackie *et al.*, 1995; Twitchell *et al.*, 1997; Hampson *et al.*, 1998; Gebremedhin *et al.*, 1999). The activation of N-type Ca^{2+} channels has been shown to mediate the presynaptic release of ACh in the myenteric plexus (Takahashi *et al.*, 1992; Tran and

Boot, 1997). At higher neuronal firing frequencies P/Q-type Ca^{2+} channels evoke NANC contractions in the guinea-pig ileum (Tran and Boot, 1997), which may result from a release of TKs from motor neurons that occurs at higher levels of stimulation (Bornstein *et al.*, 2004). Hence, a CB_1 receptor-mediated inhibition of N- and P/Q-type Ca^{2+} channels would reduce the presynaptic release of neurotransmitter.

Evoked inward Ca^{2+} currents were isolated in cultured myenteric neurons. The effect of CP 55,940 on these Ca^{2+} currents was established and compared to time-matched vehicle controls. It was found that the cannabinoid significantly inhibited Ca^{2+} currents compared to currents evoked in the presence of ethanol. The inhibitory effect of CP 55,940 was significant after 3.5 minutes and was maximal at 5 minutes. The maximal inhibitory effect of WIN 55,212-2 on electrically-evoked contractions of the guinea-pig MPLM lasted for 20 minutes, before being reversed by SR 141716A (Pertwee *et al.*, 1996a). The maximal inhibitory effect of Δ^9-THC on evoked contractions of the MPLM lasted at least 60 minutes (Pertwee *et al.*, 1992). Collectively, these studies suggest a continual inhibition of evoked contractions of the MPLM for as long as cannabinoids are present. This is consistent with the results of the current study, where the inhibitory effect on Ca^{2+} currents did not diminish after time. A further reduction in Ca^{2+} currents by CP 55,940 may have been observed if recordings had continued for longer. Further studies are now required to establish if the inhibitory effects of CP 55,940 are mediated by the CB_1 receptor.

Bian *et al.* (2004) investigated the subtypes of VOCCs that constitute the Ca^{2+} currents evoked in guinea-pig myenteric neurons maintained in primary culture. The Ca^{2+} currents carried by N-type and P/Q-type channels represented approximately 25% and 20% respectively of the total Ca^{2+} current. In the present study maximal Ca^{2+} currents, recorded at 5 minutes, were inhibited by approximately 37% in the presence

of CP 55,940, which is in agreement with the combined percentage of the Ca^{2+} current carried by N- and P/Q-type channels in myenteric cultures (~45%) (Bian *et al.*, 2004). This supports the possibility that CP 55,940 inhibits N- and P/Q-type Ca^{2+} currents in cultured myenteric neurons. GABA release from the myenteric plexus was shown to couple to N- and P/Q-type Ca^{2+} channels (Reis *et al.*, 2002). Hence, the CB_1 receptor-mediated inhibition of GABA release, described in the MPLM (Begg *et al.*, 2002b), may also result from an inhibition of these Ca^{2+} channels.

R-type Ca^{2+} channels carry around 50% of the total Ca^{2+} current evoked in myenteric neurons (Bian *et al.*, 2004). Although there have been no reports of a cannabinoid-mediated inhibition of R-type Ca^{2+} channels, they may regulate neurotransmitter release in the myenteric plexus (Bian *et al.*, 2004) and thus their inhibition by CP 55,940 cannot be ruled out. Clearly, further studies are required to establish the effect of cannabinoids on isolated Ca^{2+} currents carried by specific channel subtypes.

Once again the neuronal region in which this inhibition was recorded should be considered. The current data suggests that CP 55,940 inhibits somatodendritic Ca^{2+} currents, which may contribute to other physiological effects. For instance, N-type channels contribute to Ca^{2+} influx during the action potential in IPANs (Furness *et al.*, 1998) and therefore may participate in the regulation of cell excitability. Hence, a cannabinoid-induced inhibition of somatodendritic N-type channels might reduce neurotransmitter release through a decrease in neuronal excitability.

4.3.6 Summary

In summary, cultured myenteric neurons express voltage-dependent Na^+, K^+ and Ca^{2+} channels, whose activation are involved in the generation of action potentials. On the basis of these action potentials alone it is difficult to classify the neurons according to the current S- or AH-type electrophysiological classification scheme. However, it is assumed that both S- and AH-neurons are present in culture due to the presence of both Dogiel type I and type II neurons as demonstrated by immunohistochemistry.

CP 55,940 did not activate GIRK channels in the myenteric cultures, at least on somatodendritic sites. Cannabinoids may activate GIRK channels in regions closer to the synaptic terminal, which we were possibly unable to record.

Inhibition of Na^+ and K^+ currents unmasks inward Ca^{2+} currents, which were inhibited by CP 55,940. Although it seems that cannabinoids are inhibiting somatodendritic VOCCs the current data supports the possibility that cannabinoids may inhibit neurotransmitter release in the myenteric plexus by interfering with Ca^{2+} conductance. Further work is required to establish the involvement of the CB_1 receptor in the cannabinoid-induced inhibition and the Ca^{2+} channel subtype(s) that is(are) targeted.

4.4 The interaction between cannabinoids and nACh receptors in cultured myenteric neurons

Cannabinoids have been shown to modulate ligand-gated ion channels (Hampson *et al.*, 1998; Barann *et al.*, 2002; Oz *et al.*, 2002) including an inhibition of

currents mediated by nACh receptors (Oz *et al.*, 2003), although only in expression systems. A cannabinoid-induced inhibition of myenteric nACh currents would present a novel physiological mechanism by which cannabinoids regulate neurotransmission in the myenteric plexus. Therefore the effect of cannabinoids on nicotine-evoked currents in cultured myenteric neurons was investigated.

4.4.1 Cannabinoid-mediated inhibition of nACh currents

Previous studies using cultured myenteric neurons have demonstrated that stimulation of nACh receptors, with either ACh or nicotine, evokes a concentration-dependent desensitising inward current, which can be abolished by the nACh receptor antagonist hexamethonium (Zhou and Galligan, 1998; Zhou *et al.*, 2002). Similarly, we showed that nicotine evokes a transient inward current in cultured myenteric neurons that was abolished in the presence of hexamethonium. This suggests the presence of somatodendritic nACh receptors, which is consistent with previous reports in the myenteric plexus localising nACh receptors to the somatodendritic region (Toroscik *et al.*, 1991).

Prior application of either CP 55,940 or the endogenous cannabinoid anandamide significantly reduced the nicotine-evoked inward current. At higher anandamide concentrations (10 μM) the inward current was virtually abolished. In *Xenopus* oocytes, transfected with α7-containing nACh receptors, anandamide inhibited nicotine-evoked inward currents, which were virtually abolished at 10 μM (Oz *et al.*, 2003). CP 55,940 also significantly inhibited nicotinic currents, albeit at higher concentrations (≥ 3 μM) suggesting a greater potency of anandamide in this preparation (Oz *et al.*, 2004). The effects of anandamide could not be reversed by

SR 141716A or SR 144528 suggesting it was independent of CB_1/ CB_2 receptor activation (Oz *et al.*, 2003). The anandamide-mediated inhibition was also insensitive to PTX treatment, the cAMP analogue 8-Br-cAMP and the AMT inhibitor AM404 and anandamide caused no shift in the concentration-response curves for nicotine (Oz *et al.*, 2003). Together these results suggest that anandamide and CP 55,940 act directly on nACh receptors in a non-competitive manner. Consistent with previous studies by Zhou *et al.* (2002), functional α7 nACh receptors are not present in cultured myenteric neurons, as the α7-specific nACh receptor antagonist MLA had no effect on nicotine-evoked currents.

4.4.2 CB_1 receptor-independent inhibition of nACh currents

With previous reports demonstrating a direct inhibitory effect of anandamide on nACh receptors, the effect of SR 141716A on the cannabinoid-mediated inhibition of nicotinic currents was established. Interestingly, SR 141716A did not significantly reverse the inhibition seen to either CP 55,940 or anandamide. In fact, SR 141716A alone induced a significant inhibition of the inward current evoked by nicotine. This suggests an inhibitory effect of CP 55,940 and anandamide, which is independent of the CB_1 receptor. The results obtained with SR 141716A alone may also indicate that this(ese) novel site(s) of action for the cannabinoids does not distinguish between established agonists or antagonists. Barann *et al.* (2002) showed that cannabinoids (specific to both the CB_1 and CB_2 receptor) directly inhibit the $5\text{-}HT_{3A}$ receptor transfected in HEK293 cells, including the CB_1 receptor antagonist LY 320135, although SR 141716A had no effect. Given that SR 141716A alone inhibits nicotinic currents in myenteric neurons it would seem feasible that the application of both

SR 141716A and CP 55,940 would produce a greater inhibitory effect. However, although insignificant, SR 141716A showed a slight reversal of the CP 55,940-evoked inhibition. This reversal was not observed with SR 141716A and anandamide suggesting that anandamide may act at a different site to that of CP 55,940. Anandamide can act as an agonist at vanilloid VR_1 receptors (Smart *et al.*, 2000), which are expressed in myenteric neurons (Kulkarni-Narla and Brown, 2001). Activation of the receptor induces an influx of Ca^{2+}, which produced a significant inward current in transfected HEK293 cells and rat DRG neurons (Smart *et al.*, 2000). However, application of anandamide alone in cultured myenteric neurons produced no change in membrane currents suggesting that VR_1 receptors are not activated.

In frog saccular hair cells the opioid agonists endomorphin-1 and dynorphin B and the non-specific opioid antagonist naloxone were all shown to directly modulate α9/ α10-containing nACh receptors (Lioudyno *et al.*, 2002). Endomorphin-1 and dynorphin B inhibited ACh-evoked currents that were only slightly reversed by naloxone. Naloxone produced a significant inhibition of the ACh-evoked currents by itself, although this inhibition was considerably less than that of the opioid agonists. From this the authors hypothesised that naloxone may compete with the opioid agonists in their interactions with the nACh receptor so the opioid effects are partially reduced. The same mechanism cannot be attributed to the effects of SR 141716A and CP 55,940 because, at the concentrations used in the present study, the inhibitory effects of SR 141716A alone on nicotinic currents were similar to that of CP 55,940.

To further support a CB_1 receptor-independent action of cannabinoids on currents mediated by nACh receptors, the effects of the cannabinoid ligand PEA were established. Unlike CP 55,940 and anandamide, PEA does not bind efficiently to CB_1 and CB_2 receptors (Lambert and Di Marzo, 1999) but does mimic the effect of

cannabinoids in several assays. As such PEA decreases spontaneous activity in mice (Adams *et al.*, 1995), possesses analgesic (Calignano *et al.*, 1998) and anti-inflammatory activity (Berdyshev *et al.*, 1998) and relaxes the rat isolated mesenteric artery (White and Hiley, 1998). PEA also significantly inhibited the nicotine-evoked currents in cultured myenteric neurons.

The actions of the $G_{i/o}$ inhibitor PTX on the cannabinoid-mediated inhibition were also determined. PTX also showed no reversal of the inhibitory effect evoked by either CP 55,940 or anandamide, at concentrations shown to significantly disrupt CB_1 receptor signalling in DDT_1 MF-2 cells (Begg *et al.*, 2001). Hence, collectively the results strongly suggest that currents mediated by nACh receptors are inhibited by cannabinoids independently of CB_1/ CB_2 receptor activation in myenteric neurons.

Cannabinoids were shown to directly interact with NMDA (Hampson *et al.*, 1998), 5-HT_3 (Barann *et al.*, 2002; Oz *et al.*, 2002) and nACh receptors (Oz *et al.*, 2003, 2004), so the effect of CP 55,940 and anandamide on single channel recordings obtained from myenteric nACh receptors was investigated. However, due to the amplification of noise seen at gains that were required to visualise the opening of single nicotinic channels it was impossible to distinguish between the two. The noise problems could not be rectified within the time period of the current study but the work is essential to support a cannabinoid-mediated inhibition of nicotinic currents through a direct interaction with myenteric nACh receptors.

Cannabinoids were thought to inhibit the opening of 5-HT_{3A} channels in HEK293 cells through a possible binding to an allosteric modulatory site on the receptor (Barann *et al.*, 2002). nACh receptors express binding sites for numerous exogenous and endogenous non-competitive molecules, including those for fatty acids such as AA, which inhibit channel function (Arias, 1998). This might suggest at least

why anandamide, an analogue of AA, is able to inhibit currents mediated by nACh receptors. Oz *et al.* (2004) showed that AA dose-dependently inhibited currents evoked by α7 nACh receptors in *Xenopus* oocytes. In the same study 2-AG and the metabolically stable analogue methanandamide showed a higher potency than AA for inhibiting nicotinic currents suggesting that it was the intact endocannabinoid and not the metabolite that altered receptor function. Hence, nACh receptors may express specific cannabinoid modulatory sites. These sites may exhibit a greater affinity or efficacy (or both) for the endocannabinoids compared to the synthetic cannabinoids. This could account for the greater potency of anandamide compared to CP 55,940 in inhibiting α7 nACh currents (Oz *et al.*, 2004). Alternatively, there may be multiple sites that distinguish between endocannabinoids and synthetic cannabinoids, which could explain the effects of SR 141716A on the CP 55,940- but not anandamide-evoked inhibition of nicotinic currents observed in cultured myenteric neurons.

4.4.3 Cannabinoid-mediated inhibition of 5-HT-evoked currents

Zhou and Galligan (1999) have previously demonstrated that 5-HT application concentration-dependently evokes an inward current in cultured myenteric neurons. The inward current is biphasic, characterised by a rapidly desensitising current followed by a small, sustained current. Consistent with these observations we showed that 5-HT application also induced a biphasic inward current in cultured myenteric neurons. The 5-HT_3 receptor antagonist ondansetron inhibited the rapidly developing and desensitising 5-HT-induced current leaving the smaller sustained current (Zhou and Galligan, 1999). Pharmacologically, the 5-HT receptor mediating the sustained current was never identified, but from the time course of the response the authors

believed it was consistent with it being mediated by 5-HT_{1P} receptors (Zhou and Galligan, 1999).

With the observed effects of cannabinoids on myenteric nACh currents and previous reports into a direct modulatory role of cannabinoids on 5-HT_3 receptors (Barann *et al.*, 2002; Oz *et al.*, 2002), preliminary experiments were used to establish the effect, if any, of CP 55,940 on the 5-HT-evoked currents in myenteric neurons. CP 55,940 had no effect on the initial rapidly developing inward current but significantly inhibited the sustained current. This suggests that cannabinoids do not modulate 5-HT_3 receptor-mediated currents in cultured myenteric neurons but may inhibit currents evoked by the activation of the 5-HT_{1P} receptor. Further work is required to establish if other cannabinoids, including anandamide, mimic the effects of CP 55,940 and if so determine the mechanism by which they mediate this effect e.g. through the activation of CB_1 receptors or via another CB_1-independent mechanism.

4.4.4 Functional implications: cannabinoid-mediated inhibition of nACh receptors

The current study provides evidence for a novel mechanism by which cannabinoids may modulate cholinergic neurotransmission in the myenteric plexus. As well as the inhibitory effect of cannabinoids on presynaptic ACh release (Coutts and Pertwee, 1997), they may be able to reduce gut motility through the inhibition of currents mediated by nACh receptors. Indeed, ACh acting at nACh receptors is the principal mechanism of excitatory neurotransmission in the myenteric plexus, eliciting fEPSPs in both S- and AH-neurons (Nishi and North, 1973; Hirst *et al.*, 1974). As S-neurons are likely to be interneurons and motor neurons (Galligan, 2002)

while AH-neurons are IPANS (Furness *et al.*, 1998; Galligan, 2000), cannabinoids may be able to modulate cholinergic neurotransmission in all three types of neuron present in the myenteric plexus. Lopez-Redondo *et al.* (1997) demonstrated that electrically-evoked fEPSPs could be depressed in myenteric S-type neurons by CP 55,940 and WIN 55,212-2. The inhibitory effect of WIN 55,212-2 was only reversed in 38% of neurons with SR 141716A suggesting a predominantly CB_1 receptor-independent mechanism for the cannabinoid-induced reduction. In addition, SR 141716A alone caused a significant reduction in the amplitude of fEPSPs. These results would be consistent with the findings of the present study, where WIN 55,212-2 and SR 141716A could inhibit nACh receptor-mediated currents in a CB_1 receptor-independent manner. The inhibition of cholinergic neurotransmission would in turn lead to a reduction in the amplitude of fEPSPs recorded in S-neurons.

The activation of nACh receptors is the predominant mechanism for ascending excitatory neurotransmission in the myenteric plexus (LePard and Galligan, 1999), thus regulating smooth muscle contraction. Approximately 27% of neurons in the guinea-pig myenteric plexus are motor neurons that innervate the longitudinal muscle; 25% are excitatory while only 2% are inhibitory (Furness, 2000). This implies that longitudinal muscle contraction is predominantly mediated by ascending excitatory cholinergic pathways (Brookes *et al.*, 1992). Thus, the MPLM preparation, which consists of only the myenteric plexus and longitudinal muscle, is a good preparation to study cholinergic neurotransmission and subsequent smooth muscle contraction. Further studies in our group investigated the effects of CP 55,940 on nicotine-evoked contractions in the MPLM (Demuth *et al.*, 2004). Nicotine predominantly evokes longitudinal muscle contraction by stimulating somatodendritic nACh receptors (Toroscik *et al.*, 1991), which induces neuronal depolarisation and

subsequent neurotransmitter release (Galligan and Bertrand, 1994). Although nACh receptors are expressed on each of the three types of neuron present in the ascending excitatory pathway, ultimately nicotine application results in the depolarisation of motor neurons innervating the longitudinal muscle. Both ACh and TKs acting at muscarinic and NK receptors respectively evoke a contraction of the longitudinal muscle (Galligan, 1999; Schneider *et al.*, 2000; Furness and Sanger, 2002). Nicotine induced a contraction in the MPLM, which was inhibited by CP 55,940 in a dose-dependent manner (Demuth *et al.*, 2004). SR 141716A did not reverse the inhibitory effect of CP 55,940 on nicotine-evoked contractions, at a concentration shown to significantly block the CP 55,940-induced inhibition of electrically-evoked contractions in the MPLM. This suggests a CB_1 receptor-independent inhibitory action of CP 55,940 on contractions mediated by nACh receptor activation and thus supports the physiological significance of a cannabinoid-mediated modulation of nACh receptor currents, as demonstrated in cultured myenteric neurons. As 12% of myenteric neurons in the guinea-pig small intestine are also excitatory ascending cholinergic neurons that innervate the circular muscle (Furness, 2000), it seems feasible that cannabinoids may, in part, be able to modulate the contraction of circular muscle through the same mechanism. Interestingly, PEA inhibited gastrointestinal motility in mice *in vivo* in an SR 141716A- and SR 144528-insensitive manner (Capasso *et al.*, 2001). PEA was also applied in combination with hexamethonium (a ganglion blocker) but the PEA-evoked inhibition still persisted suggesting a peripheral site of action. When administered alone hexamethonium had no effect on gastrointestinal transit, which suggests cholinergic neurotransmission in the mouse myenteric plexus was not affected (Capasso *et al.*, 2001). The effects of PEA on myenteric nicotinic currents, observed in the current study, might suggest a

mechanism by which the cannabinoid inhibits gastrointestinal motility in mice independently of CB_1/ CB_2 receptor activation.

Presynaptic nACh receptors are also present on myenteric motor neurons that innervate both longitudinal and circular muscle (Galligan, 1999; Schneider *et al.*, 2000). During periods of high frequency stimulation, resulting in higher concentrations of ACh at the neuromuscular junction, activation of these presynaptic receptors is thought to further facilitate the release of neurotransmitter (Galligan, 1999; Schneider *et al.*, 2000; Mandl *et al.*, 2003). Hence, if cannabinoids could also inhibit the presynaptic action of nACh receptors, this would present another mechanism by which cannabinoids modulate the release of neurotransmitter and thus gastrointestinal motility. Our research group investigated this concept using the MPLM preparation (Demuth *et al.*, 2004). In the presence of the Na^+ channel blocker TTX, effectively isolating the presynaptic nACh receptors, nicotine still evoked a contraction, although significantly less when compared to contractions in the absence of TTX. Both CP 55,940 and SR 141716A significantly inhibited the TTX-insensitive contraction evoked by nicotine suggesting that cannabinoids can also inhibit the opening of presynaptic nACh receptors. Interestingly, application of both CP 55,940 and SR 141716A had an additive effect producing a significantly greater inhibition than either CP 55,940 or SR 141716A alone. This additive effect of CP 55,940 and SR 141716A was not seen in cultured myenteric neurons suggesting that somatodendritic and presynaptic nACh receptors may exhibit different properties, with regard to cannabinoid modulation at least.

In the submucosal plexus of the guinea-pig ileum, immunohistochemistry demonstrated the expression of CB_1 receptors on secretomotor neurons (MacNaughton *et al.*, 2004). In addition, WIN 55,212-2 was shown to reduce

electrolyte transport in the ileum in response to electrical field stimulation, in an SR 141716A-sensitive manner (MacNaughton *et al.*, 2004) suggesting a CB_1 receptor-mediated inhibition of gastrointestinal secretions. fEPSPs are completely blocked by nACh receptor antagonists in the submucosal plexus (Evans and Surprenant, 1992). Hence, the current study may suggest another mechanism by which cannabinoids could inhibit gastrointestinal secretions i.e. an inhibition of currents mediated by nACh receptors expressed in the submucosal plexus. Indeed, our research group has shown that CP 55,940 inhibits nicotine-evoked ion transport in the guinea-pig ileum, in an SR 141716A-insensitive manner (unpublished work).

4.4.5 Functional implications: cannabinoid-mediated inhibition of $5\text{-}HT_{1P}$ receptors

Preliminary studies showed that CP 55,940 inhibited the 5-HT-mediated sustained inward current in cultured myenteric neurons. This sustained current may arise from the activation of $5\text{-}HT_{1P}$ receptors (Zhou and Galligan, 1999), although further studies are required to support the involvement of this receptor subtype.

$5\text{-}HT_{1P}$ receptors are expressed on myenteric AH-neurons (IPANs), where stimulation of the receptor evokes a long-lasting membrane depolarisation associated with the generation of sEPSPs (Mawe *et al.*, 1986, 1989). *In vivo* studies in mice have demonstrated that the $5\text{-}HT_{1P}$ receptor antagonists BRL 24924 and 5-HTP-DP increase the gastric emptying of a liquid meal (Mawe *et al.*, 1989) suggesting a tonic $5\text{-}HT_{1P}$ receptor-mediated activation of myenteric inhibitory neurons through the production of sEPSPs. This is consistent with the findings of Michael *et al.* (1997) who demonstrated that, of the 11.3% of guinea-pig myenteric neurons that express $5\text{-}HT_{1P}$ receptors, 74% are found in the descending projections. In addition, the same

study showed that the 5-HT_{1P} receptor agonist 5-OHIP reduced electrically-evoked contractions of the circular muscle, supporting the idea that 5-HT_{1P} receptors mediate transmission through the inhibitory pathways. In the current study 5-HT application also evokes a rapidly developing current mediated by the 5-HT_3 receptor (Zhou and Galligan, 1999) suggesting that myenteric neurons in primary culture express both 5-HT_3 and 5-HT_{1P} receptors. However, myenteric neurons that express both receptors have been shown to exhibit no preferential projection (Michael *et al.*, 1997).

An inhibitory effect of cannabinoids on currents mediated by 5-HT_{1P} receptors may suggest at least a reduction of sEPSPs in the myenteric plexus. If cannabinoids were able to reduce 5-HT_{1P} receptor-mediated neurotransmission in the descending inhibitory pathways this would imply that cannabinoids might also be able to facilitate gastrointestinal transit. However, this is in complete contrast to reports by Heinemann *et al.* (1999) who demonstrated that cannabinoids, in part, depress peristalsis through the potentiation of inhibitory pathways. Clearly more work is required to define the functional implications of a cannabinoid-mediated inhibition of 5-HT_{1P} receptors, if cannabinoids are indeed inhibiting currents mediated by this receptor subtype.

4.4.6 Summary

In summary CP 55,940 and anandamide have been shown to inhibit currents mediated by nACh receptors in cultured myenteric neurons, in a CB_1 receptor-independent manner. The data presents a novel mechanism by which cannabinoids regulate gastrointestinal motility through a possible modulation of excitatory cholinergic neurotransmission in the myenteric plexus. Hence, this is also the first report of its kind demonstrating the physiological relevance of such a cannabinoid-

mediated modulation of nACh receptors. Cannabinoids may exert their effects at both somatodendritic and presynaptic nACh receptors, leading to an inhibition of neuronal depolarisation and ultimately a reduction in ACh release at the neuromuscular junction.

Preliminary experiments have also demonstrated that CP 55,940 inhibits the sustained inward currents mediated by 5-HT application in cultured myenteric neurons. This may indicate an inhibitory effect of cannabinoids on currents mediated by 5-HT_{1P} receptors in the myenteric plexus but the functional significance of this observation needs to be investigated further.

FUTURE WORK

5.1 DDT_1 MF-2 cells

The current study has shown that AA forms an integral part of the signalling pathways associated with the stimulation of the CB_1 receptor in DDT_1 MF-2 smooth muscle cells. The results obtained with the compounds La^{3+} and Gd^{3+} helped us to identify this signalling pathway. However, where La^{3+} was shown to inhibit both the CP 55,940- and AA-evoked outward current, Gd^{3+} was only shown to inhibit CP 55,940-mediated currents. Therefore in order to further support a CB_1 receptor-mediated activation of NCCE, the effect of Gd^{3+} on AA-induced currents should also be established, using the same concentration that was shown to inhibit the CP 55,940-evoked response.

CP 55,940 was shown to increase $[Ca^{2+}]_i$, although this was only significant at high concentrations. To support the involvement of the CB_1-receptor, in response to increases in $[Ca^{2+}]_i$ mediated by high CP 55,940 concentrations, further experiments are needed to ascertain the effects of SR 141716A in this assay.

It is not known if AA directly activates Ca^{2+} influx or some intermediate molecule(s) is(are) involved. Previous reports in other preparations have shown that AA may evoke NCCE via the production of NO (Moneer *et al.*, 2003; Watson *et al.*, 2004), which is interesting in the light of observations demonstrating that NO donors can induce Ca^{2+} influx in DDT_1 MF-2 cells (Favre *et al.*, 1998). Future work could determine the effects of NOS inhibitors on both CP 55,940- and AA-evoked currents. Additionally, in the same manner where AA application was shown to inhibit subsequent responses to CP 55,940, the effect of NO donors on subsequent CP 55,940- or AA-evoked currents could be determined. Furthermore, the effects of La^{3+} and Gd^{3+} on NO-mediated Ca^{2+} influx could also be established.

The reciprocal regulation of CCE and NCCE (Luo *et al.*, 2001a; Mignen *et al.*, 2001) may be one reason why thapsigargin inhibited the CP 55,940-evoked current in DDT_1 MF-2 cells (Begg *et al.*, 2001). Calcineurin, stimulated during CCE, was shown to mediate the inhibition of NCCE in HEK293 cells (Mignen *et al.*, 2003). It would therefore be interesting to investigate the effects, if any, of calcineurin inhibitors, such as cyclosporin and ascomyscin (Mignen *et al.*, 2003), on the thapsigargin-mediated inhibition of CP 55,940 evoked responses in DDT_1 MF-2 cells.

The supposed specific $cPLA_2$ inhibitor ATK exhibited a cannabinoid-like action in this cell line, evoking an outward current alone. This is of great interest because the compound is known to bind to CB_1 receptors (Koutek *et al.*, 1994). The ATK-induced current was insensitive to CB_1 receptor blockade suggesting another mechanism of action that could be explored in more detail. Interestingly, ATK can inhibit electrically-evoked contractions of the guinea-pig ileum, which is again similar to the actions exhibited by cannabinoids (unpublished data). The CB_1 receptor antagonist SR 141716A did not reverse the inhibition. Future work could explore the actions of ATK in the MPLM preparation, establishing the compounds mechanism of action as a cannabinoid-like agonist or cannabinoid antagonist.

The results obtained from western blot experiments, to determine a time course of p42/44 MAP kinase phosphorylation, have been difficult to interpret, especially as ethanol may also be inducing phosphorylation. Clearly more work is required to obtain blots showing a CB_1 receptor-mediated phosphorylation of the p42/44 MAP kinase in DDT_1 MF-2 cells. To enable us to determine if the pattern of phosphorylation already seen is due to CP 55,940 or ethanol treatment, SR 141716 was used to try and block the effects of cannabinoid stimulation. At present these

results are inconclusive, however this work is critical to interpreting the western blots already produced using CP 55,940.

The western blot results could be interpreted as a CP 55,940-mediated inhibition of MAP kinase, although the MAP kinase inhibitor PD 98059 was shown to reduce CP 55,940-evoked outward currents (Begg *et al.*, 2001). Non-specific effects of PD 98059 on intracellular signalling molecules such as protein kinases have been described (Davies *et al.*, 2000). Therefore it would be a good idea to support the electrophysiological data obtained with PD 98059 with other MAP kinase inhibitors, such as U0126, which exhibits 100-fold higher affinity for MEK than PD 98059 (Favata *et al.*, 1998).

Western blots could also be used to establish the phosphorylation and hence activation of $cPLA_2$ in response to CB_1 receptor stimulation, where specific pharmacological tools have failed. Alternatively, a molecular approach could be used to determine the specific actions of $cPLA_2$. Hunter and Burnstein (1997) used CB_1 antisense probes to reduce receptor expression levels in N18 mouse neuroblastoma cells and help identify a CB_1 receptor-mediated increase in AA generation by Δ^9-THC. In the same manner an overall reduction in $cPLA_2$ in DDT_1 MF-2 cells could reveal that CP 55,940 evokes an increase in AA via this enzyme.

5.2 Myenteric cultures

Immunohistochemical studies were used to identify the presence of CB_1 receptors and their localisation, specifically to cholinergic myenteric neurons in primary culture. However, although it is believed that the cultures make up a hetrogenous population, the proportion of Dogiel types could not be established. It

was also difficult to classify the neurons based on the present electrophysiological data that was obtained. Now that cannabinoids have been shown to have effects in these cultures it would be of great interest to determine which neuronal type(s) (motor, interneuron or IPAN) exhibit(s) these cannabinoid-mediated effects. Hence, further immunohistochemistry could be used to identify the predominant neuronal type in culture and also its expression of CB_1 receptors. For instance, guinea-pig myenteric neurons can be classified on the basis of immunoreactivity to the Ca^{2+}-binding proteins calbindin and calretinin. *In situ* calbindin is a predominant marker for IPANs but not motor neurons (Coutts *et al.*, 2002). Conversely, calretinin is primarily immunoreactive for motor neurons and interneurons. In addition, AH-neurons, which are IPANs in the myenteric plexus (Furness *et al.*, 1998), can be distinguished from S-neurons on the basis of their sensitivity to TTX. The action potentials recorded from S-neurons are completely blocked by TTX whereas, due to the Ca^{2+} component, the action potential evoked in AH-neurons persists in the presence of the Na^+ channel blocker (Hirst *et al.*, 1974; Furness *et al.*, 1998). Hence, the effect of TTX on the action potentials evoked in cultured myenteric neurons may help identify the predominant neuronal type present in culture, along with the neuronal type(s) affected by cannabinoids.

CP 55,940 was shown to reduce both the peak positive and negative currents evoked in myenteric neurons. However, although this effect is probably due to a degradation of the patch, time-matched ethanol controls need to be performed and statistically compared to those results obtained in the presence of CP 55,940.

CP 55,940 was shown to inhibit inward Ca^{2+} currents evoked in myenteric cultures. Initially, it should be confirmed if this effect is mediated by CB_1 receptors. Thus the effects of CP 55,940 on evoked Ca^{2+} currents, in the presence of SR

141716A, should be determined. The inward Ca^{2+} current is associated with the activation of particular subtypes of VOCCs including N- and P/Q-type channels (Bian *et al.*, 2004), which are know to be inhibited by cannabinoids in some preparations (Mackie *et al.*, 1995; Twitchell *et al.*, 1997; Hampson *et al.*, 1998; Gebremedhin *et al.*, 1999). In addition, about 50% of the Ca^{2+} current in cultured myenteric neurons is carried by R-type channels (Bian *et al.*, 2004). Future work could isolate these specific currents, allowing the subtype of Ca^{2+} channel(s) inhibited by CP 55,940 to be identified.

A-type K^+ channels are expressed in myenteric neurons (Starodub and Wood, 2000; Ren *et al.*, 2001) and can be activated in a CB_1 receptor-dependent manner (Deadwyler *et al.*, 1995; Mu *et al.*, 2000). In addition, a reduction in cAMP is involved in the pathways leading to a CB_1 receptor-mediated decrease in neurotransmitter release in the myenteric plexus (Coutts and Pertwee, 1998), consistent with the activation of I_A. Therefore the effects of CP 55,940 on currents mediated by the activation of A-type K^+ channels should be investigated in the cultures.

Cannabinoids inhibit myenteric nACh currents in a CB_1 receptor-independent manner. The CB_1 receptor antagonist SR 141716 was also shown to inhibit nicotine-evoked currents. It would be interesting to establish the effect of other cannabinoid antagonists including O-2050, which acted as a 'silent' CB_1 receptor antagonist in the mouse vas deferens (Martin *et al.*, 2002). Future work should also try to resolve the 'noise' problems associated with single channel recording and thus establish if cannabinoids have a direct modulatory effect on the nACh receptors.

CP 55,940 also inhibited the sustained current evoked by 5-HT application in cultured myenteric cultures. The effects of vehicle (ethanol) on the 5-HT-induced

current should initially be determined. Subsequently, further work is needed to identify the 5-HT receptor mediating this sustained current. Previous work has suggested that it may be the 5-HT_{1P} receptor (Zhou and Galligan, 1999). 5-HT_{1P} receptor agonists such as 5-OHIP (Michael *et al.*, 1997) could be used to see if they induce the same sustained inward current similar to that observed during 5-HT application. If so, the actions of CP 55,940 on this evoked current could be verified. In addition, the effects of 5-HT_{1P} receptor antagonists, such as BRL 24924 (Mawe *et al.*, 1989), on the 5-HT-evoked sustained current could also be determined. Further work could then follow the same experimental design as those studies investigating the effects of cannabinoids on nicotinic currents in cultured myenteric neurons. Electrophysiological data could then be matched with functional 5-HT data obtained from the intact guinea-pig ileum.

Cultured neurons could be used to ascertain the effects of cannabinoids on cholinergic neurotransmission, in either a CB_1 receptor-dependent or independent manner. Although difficult, recordings could be made between neuronal synaptic connections, with the use of at least two electrodes. One electrode could be used to evoke neuronal excitation while the other electrode(s) could be used to measure the response to this, in postsynaptic neurons e.g. the generation of action potentials. Cannabinoid effects on synaptic transmission, through connecting myenteric neurons, could then be studied in more detail, including any effects mediated at the presynaptic terminals.

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APPENDIX

CONFERENCE PROCEEDINGS

Demuth, D.G., Parsons, M.E. and Molleman, A. (2003). Arachidonic acid is involved in the CB_1 receptor signalling pathway in a smooth muscle cell line. *13th Annual Symposium of the International Cannabinoid Research Society*, Burlington, Vermont. **Accepted for poster presentation.**

Coutts, A.A., **Demuth, D.G.** and Molleman, A. (2003). The immunohistochemistry of cannabinoid CB_1 receptors in guinea-pig cultured myenteric neurones. *International Symposium on Gastrointestinal Motility*, Barcelona, Spain. **Accepted for poster presentation.**

Demuth, D.G., Parsons M.E. and Molleman, A. (2004). Cannabinoid-mediated inhibition of nicotinic ACh currents in myenteric neurons. *14th Annual Symposium of the International Cannabinoid Research Society*, Peastum, Italy. **Accepted for oral presentation.**

Sones, W.R., **Demuth, D.G.,** Makwana, R., Parsons, M.E. and Molleman, A. (2004). Cannabinoid modulation of nicotine responses in guinea-pig ileum myenteric neurons. *British Pharmacological Society*, Winter Meeting, Newcastle. **Accepted for oral presentation.**

PAPERS ARISING FROM THIS THESIS

Demuth, D.G., Gkoumassi, E., Droge, M.J., Dekkers, B.G.J., Esselink, H.J., Ree, R.M.V., Parsons, M.E., Zaagsma, J., Molleman, A. and Nelemans, S.A. (2004). Arachidonic acid mediates non-capacitative calcium entry evoked by CB_1-cannabinoid receptor activation in DDT_1 MF-2 smooth muscle cells. *Journal of Cellular Physiology* **In Press.**