Chapter 2

Arachidonic Acid Mediates Non-Capacitative Calcium Entry Evoked by CB₁-Cannabinoid Receptor Activation in DDT₁ MF-2 Smooth Muscle Cells


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Abstract

Cannabinoid CB₁-receptor stimulation in DDT₁ MF-2 smooth muscle cells induces a rise in [Ca²⁺]ᵢ which is dependent on extracellular Ca²⁺ and modulated by thapsigargin-sensitive stores, suggesting capacitative Ca²⁺ entry (CCE), and by MAP kinase. Non-capacitative calcium entry (NCCE) stimulated by arachidonic acid (AA) partly mediates histamine H₁-receptor-evoked increases in [Ca²⁺]ᵢ in DDT₁ MF-2 cells. In the current study both Ca²⁺ entry mechanisms and a possible link between MAP kinase activation and increasing [Ca²⁺]ᵢ were investigated. In the whole-cell patch clamp configuration, the CB-receptor agonist CP 55,940 evoked a transient, Ca²⁺-dependent K⁺ current, which was not blocked by the inhibitors of CCE, 2-APB and SKF 96365. 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 CB₁ antagonist SR 141716A. The non-selective Ca²⁺ channel blockers La³⁺ and Gd³⁺ inhibited the CP 55,940-induced current at concentrations that had no effect on thapsigargin-evoked CCE. La³⁺ also inhibited the AA-induced current. CP 55,940-induced AA release was abolished by Gd³⁺ and by phospholipase A₂ inhibition using quinacrine; this compound also inhibited the outward current. The CP 55,940-induced AA release was strongly reduced by the MAP kinase inhibitor PD 98059. The data suggest that in DDT₁ MF-2 cells AA is an integral component of the CB₁ receptor signalling pathway, upstream of NCCE and, via PLA₂, downstream of MAP kinase.
Introduction

Cannabinoids have been shown to stimulate specific $G_{i/o}$-protein coupled cannabinoid receptors (Howlett et al., 1986; Matsuda et al., 1990). To date only two receptors have been cloned, the cannabinoid CB$_1$ and CB$_2$ receptor. The CB$_1$ receptor is highly expressed within certain areas of the brain (Tsou et al., 1998) and in some peripheral tissues (Liu et al., 2000; Wagner et al., 2001), while the CB$_2$ receptor is localised mainly in the immune system (Munro et al., 1993; Schatz et al., 1997). Their activation is linked to the modulation of certain intracellular pathways including stimulation of MAP kinase (Bouaboula et al., 1995) and inhibition of adenylyl cyclase (Childers and Deadwyler, 1996). Stimulation of the CB$_1$ receptor has also been shown to inhibit voltage-operated Ca$^{2+}$ channels (L-, N- and P/Q-type) as well as to activate inwardly-rectifying K$^+$ channels (Gebremedhin et al., 1999; Mackie et al., 1995; Twitchell et al., 1997).

Cannabinoid-mediated changes in intracellular Ca$^{2+}$ concentration ([Ca$^{2+}]_i$) have been described in both neuronal and non-neuronal preparations. In the neuroblastoma-glioma cell hybrid, NG108-15, CB$_1$ receptor stimulation induces a rise in [Ca$^{2+}]_i$ through the activation of phospholipase C (PLC) and the subsequent release of Ca$^{2+}$ from inositol 1,4,5-trisphosphate (InsP$_3$)-sensitive intracellular Ca$^{2+}$ stores (Sugiura et al., 1996). DDT$_1$ MF-2 smooth muscle cells express CB$_1$ receptors, which can be stimulated by the herbal cannabinoid $\Delta^{9}$-tetrahydrocannabinol ($\Delta^{9}$-THC) to induce a rise in [Ca$^{2+}]_i$ (Filipeanu et al., 1997). Voltage-operated Ca$^{2+}$ channels are absent in these cells making them a good preparation to study receptor-mediated increases in [Ca$^{2+}]_i$ (Molleman et al., 1991). We have shown previously that the rise in [Ca$^{2+}]_i$ in response to the synthetic cannabinoid agonist, CP 55,940 is insensitive to PLC inhibition but was entirely dependent on Ca$^{2+}$ from the extracellular space (Begg et al., 2001). However, a release of Ca$^{2+}$ from thapsigargin-sensitive stores also played a role (Begg et al., 2001). These data supported a model for capacitative Ca$^{2+}$ entry (CCE), in which initiation of Ca$^{2+}$ influx is coupled to the filling state of the intracellular Ca$^{2+}$ stores.
(Putney, 1990). Furthermore the MAP kinase inhibitor PD 98059 reduced the rise in \([\text{Ca}^{2+}]_i\) (Begg et al., 2001).

Recently, a novel mechanism for \(\text{Ca}^{2+}\) entry has been described which is independent of store depletion and subsequent capacitative \(\text{Ca}^{2+}\) influx. This so called non-capacitative \(\text{Ca}^{2+}\) entry (NCCE) pathway, through which intracellular messengers such as arachidonic acid (AA) mediate \(\text{Ca}^{2+}\) influx, has been described in a variety of cell types (Broad et al., 1999; Fiorio Pla and Munaron, 2001; Mignen and Shuttleworth, 2000; Munaron et al., 1997). AA but not its metabolites, was shown to induce \(\text{Ca}^{2+}\) channel opening (Broad et al., 1999; Fiorio Pla and Munaron, 2001). This \(\text{Ca}^{2+}\) influx has since been designated \(I_{\text{ARC}}\) (arachidonate regulated \(\text{Ca}^{2+}\) current) (Mignen and Shuttleworth, 2000).

\(\text{DDT}_1\) MF-2 cells also express histamine-H\(_1\) receptors, activation of which results in an increase in \([\text{Ca}^{2+}]_i\), resulting primarily from an InsP\(_3\)-mediated release of \(\text{Ca}^{2+}\) from intracellular stores (Den Hertog et al., 1992). However, a small contribution to the overall increase in \([\text{Ca}^{2+}]_i\) is due to \(\text{Ca}^{2+}\) influx from the extracellular space; this influx pathway was activated by AA (Van der Zee et al., 1995). The latter pathway could also mediate the \(\text{Ca}^{2+}\) entry process seen during CB\(_1\) stimulation. The putative pathways leading to AA production have not fully been elucidated but a common mechanism for AA liberation involves direct action of phospholipase A\(_2\) (PLA\(_2\)) on phospholipids. In HEK 293 cells type IV cytosolic PLA\(_2\) has been shown to liberate AA, which in turn stimulates \(I_{\text{ARC}}\) (Osterhout and Shuttleworth, 2000). PLA\(_2\) activity can be regulated by MAP kinase (Kudo and Murakami, 2002) and could be the intermediate step between MAP kinase activation and the resulting rise in \([\text{Ca}^{2+}]_i\) during CB\(_1\) stimulation.

In the present study, we aimed to identify the \(\text{Ca}^{2+}\) influx mechanism responsible for the CB\(_1\) receptor-induced increase in \([\text{Ca}^{2+}]_i\), including the intracellular signalling pathways involved. Generation of free AA was observed in response to CB\(_1\) receptor activation, which may arise from the
action of PLA$_2$ on membrane phospholipids. Our data argue against the classical CCE model and support the possibility that in DDT$_1$ MF-2 cells, AA is a major component of the CB$_1$ signalling pathway activating I$_{ARC}$, resulting in NCCE.

**Materials and Methods**

*Cell Culture* - DDT$_1$ MF-2 smooth muscle cells were cultured under standard conditions as described previously (Molleman et al., 1989). Briefly, cells were grown at 37°C in Dulbecco’s Modified Eagle’s Medium (DMEM), supplemented with 10% fetal-calf serum, penicillin (50 μg/ ml), streptomycin (50 μg/ ml) and L-glutamine (2 mM). They were grown to confluency, in an atmosphere of 5% CO$_2$/ 95% O$_2$ and subsequently plated onto glass cover slips for electrophysiological studies or into 6 well plates for AA release studies. All culture media and supplements were obtained from Gibco (UK).

*Patch Clamp* - Micropipettes (Harvard Apparatus, GC150TF-10) were heat-polished (MF-830, Narishige) and back-filled with intracellular solution (ICS, in mM: NaCl, 5; KCl, 142; MgCl$_2$, 1.2; Hepes, 20; glucose, 11; K-ATP, 5; Na-GTP, 0.1; pH 7.2) to give a typical resistance of 2-7 MΩ. Membrane currents were amplified (Axon Instruments, Axopatch 1D) and recorded at room temperature using a digital interface (Axon Instruments, Digidata 1200) and then viewed in real-time, on a personal computer incorporating the pCLAMP 6 software (Axon Instruments). Monitoring of the movement of the micropipette towards the cell was performed by the application of an electrical pulse (5mV, 5ms, 100Hz), which allowed the analysis of resting leak current and compensation of the capacitive transient. Cells were clamped at a holding potential of –60mV, which is close to the resting membrane potential of these cells (Kudo and Murakami, 2002). Currents were measured at a holding potential of –30 mV and converted to pA per pF membrane capacitance to correct for cell surface area.

Electrophysiological measurements were carried out in extracellular salt (ECS) solution containing (mM): NaCl, 125; KCl, 6; MgCl$_2$, 2.5; NaH$_2$PO$_4$, 1.2;
Hepes, 20; glucose, 11; sucrose, 67; CaCl₂, 1.2; pH 7.4. 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 and distilled water to ensure complete washout of residual ligands.

[^3H]Arachidonic Acid (AA) Release - AA release was measured as described previously (23). Cells were grown to confluency in 9.6 cm² six well plates and labelled with 0.25 μCi of[^3H]AA (Specific Activity: 60-100Ci (2.22-3.70TBq)/mmol) in serum-free DMEM (1 ml), for 3 h at 37°C. Cells were washed once in ECS, twice with ECS containing 1% BSA (fatty-acid free) and once again with ECS to eliminate any unincorporated radioactivity. They were then allowed to equilibrate for 15 minutes during which time inhibitors of the signalling pathway under investigation could be added. Following this the cells were incubated with an agonist for 5 minutes. Experiments ended when the ECS was removed and[^3H]AA release was determined by liquid-scintillation counting. Inhibitors were also applied alone to determine any effects they may have on AA efflux by their own.

Intracellular Ca²⁺ Measurements - [Ca²⁺]ᵢ was measured using fura-2 fluorometry as reported previously (Filipeanu et al., 1997). Cells in 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 using 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 the data obtained.

Drugs - All drugs were obtained from Sigma (UK), unless stated otherwise. CP 55,940 ((-) cis-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxy-propyl) cyclohexanol) and SR 141716A (N-Piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide) were kind gifts from Pfizer (UK) and Sanofi (France) respectively. Stock solutions of CP 55,940 and AA were prepared in ethanol when they were used for
electrophysiological experiments, shielded from light and stored at -20°C. When measuring [³H]AA (Perkin Elmer Life Sciences, NL) efflux, CP 55,940 and SR 141716A were dissolved in DMSO. Histamine was dissolved in distilled water and kept at 4°C. Lanthanum (III) chloride, gadolinium (III) chloride and nickel (II) chloride solutions were freshly made each day and were dissolved in distilled water. 2-APB (2-aminoethoxydiphenyl borate), SKF 96365 (1-[2-(4-methoxyphenyl)-2-[3-(4-methoxyphenyl)propoxy]ethyl-1H-imidazole hydrochloride) and PD 98059 (2-(2-amino-3-methoxyphenyl)-4H-1benzopyran-4-one) were obtained from Tocris-Cookson (UK). 2-APB and PD 98059 were dissolved in DMSO and stored at -20°C. SKF 96365 was dissolved in distilled water. Indomethacin and phenidone solutions were made fresh each day and were dissolved in ethanol and DMSO respectively. Quinacrine was dissolved in DMSO and diluted in distilled water. Thapsigargin was dissolved in DMSO and stored at -20°C.

Data Analysis - Values are expressed as means ± standard error of the mean (S.E.M.). Comparison of pairs of treatments was made using unpaired Student’s t-test. A one-way 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 significant.

Results

CP 55,940-Evoked Increases in [Ca²⁺]: Activate An Outward Current in DDT₁ MF-2 Cells: Role of Extracellular Calcium - An increase in [Ca²⁺], in DDT₁ MF-2 cells activates a Ca²⁺-dependent K⁺ current (I_{K,Ca}), which can be measured using the whole cell version of the patch clamp technique (Begg et al., 2001). The synthetic cannabinoid receptor agonist CP 55,940 (10 μM) evoked a transient, outward current, with a peak amplitude of 32.4 ±1.3 pA/ pF after 208 ±54 seconds of administration (Fig. 1A, n=6). Histamine (10 μM), which releases Ca²⁺ from InsP₃-sensitive stores and induces calcium influx (Van der Zee et al., 1995), 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. 1B, n=8).
Figure 1. The cannabinoid receptor agonist CP 55,940 and histamine evoke outward currents in DDT1 MF-2 cells. A, sample trace of the transient outward current evoked by 10 μM CP 55,940. B, sample trace of the transient outward current evoked by 10 μM histamine. Bars indicate the presence of ligands.

CP 55,940 induced a concentration-dependent (10^-7-10^-4 M) increase in [Ca^{2+}]_i, which was abolished with the removal of extracellular calcium (Fig. 2). Moreover, in Ca^{2+}-free medium, higher concentrations (>10^-6 M) of CP 55,940 decreased [Ca^{2+}]_i.

Figure 2. CP 55,940 increases [Ca^{2+}], which is dependent on Ca^{2+} from the extracellular space. CP 55,940 increases [Ca^{2+}] in a concentration-dependent manner (n=4). This increase is abolished in the absence of extracellular Ca^{2+} (n=4). In Ca^{2+}-free medium higher concentrations of CP 55,940 reduce [Ca^{2+}].
Capacitative Ca\textsuperscript{2+} Entry (CCE): Role of Store-Operated Ca\textsuperscript{2+} Channels (SOCCs) - To examine the involvement of InsP\textsubscript{3} receptors in the cannabinoid-induced response, the membrane permeable InsP\textsubscript{3} receptor blocker, 2-APB, was used (Maruyama et al., 1997). In addition to its effects on InsP\textsubscript{3} receptors, 2-APB (10 μM) has been shown to directly block SOCCs in human platelets as well (Dobrydneva and Blackmore, 2001) which would make it the most suitable to establish the contribution of CCE in DDT\textsubscript{1} MF-2 cells. However, 2-APB (10 μM) had no effect on the outward current evoked by 10 μM CP 55,940 (110 ± 43% of control, Fig. 3A and C,) while at the same concentration 2-APB strongly reduced the histamine response to 8.6 ±7.3 % of control (Fig. 3B and C, P<0.01). The effect of another inhibitor of CCE, SKF 96365 (Merritt et al., 1990), on the CP 55,940-induced current was also investigated. At a concentration of 10 μM, SKF 96365 had no effect on the cannabinoid-induced outward current (106±19 % of control, n=6).
Figure 3. 2-APB has no effect on CP 55,940-evoked currents but inhibits the currents evoked by histamine. A, sample trace of the effect of 2-APB on the CP 55,940 (10 μM)-mediated current. B, sample trace of the effect of 2-APB on the histamine-evoked current. Bars indicate the presence of ligands. C, 2-APB had no effect on the outward current evoked by 10 μM CP 55,940 (n=6) at a concentration that inhibited histamine-evoked currents (n=5).

Significant difference from histamine control: * P<0.01.
Figure 4. AA induces an outward current, which inhibits the response to CP 55,940. A, sample trace of the transient, outward current evoked by 10 μM AA and the effect of this on the CP 55,940 (10 μM)-induced current. B, sample trace of the outward current evoked by 50 μM AA and the effect of this on the CP 55,940 (10 μM)-evoked current. Bars indicate the presence of ligands. C, AA produces a concentration-dependent increase in membrane current (n≥4). 10 μM AA reduced the outward current evoked by 10 μM CP 55,940 (n=6) and at a concentration of 50 μM completely abolished the response (n=6). Significant difference from AA (1 μM): * P<0.01, † P<0.0001. **
Non-Capacitative Ca$^{2+}$ Entry (NCCE): Role of Arachidonic Acid (AA) - AA has been shown to produce a concentration-dependent rise in [Ca$^{2+}$] in DDT$_1$ MF-2 cells, with a maximal release of Ca$^{2+}$ obtained at 1 mM AA (Mackie et al., 1995). This increase in Ca$^{2+}$ could therefore evoke an outward K$^+$ current. In the present study, AA evoked a concentration-dependent, transient outward current indeed (Fig. 4).

If pre-exposure to AA had no effect on the CP 55,940-evoked outward current it would suggest that the CB$_1$ signalling pathways do not involve the generation of AA. To examine this, DDT$_1$ MF-2 cells were exposed to AA to induce a transient response and as soon as membrane currents had returned to baseline, CP 55,940 was administered. Pre-exposure to 10 μM AA significantly inhibited the outward current induced by 10 μM CP 55,940 (30 ± 2% of control, Fig. 4A and C, P<0.0001) and at a 5 times higher concentration (50 μM) the cannabinoid-evoked response was completely abolished (Fig. 4B and C, P<0.0001).

Figure 5. CP 55,940 evokes a release of [$^3$H]AA. [$^3$H]AA-prelabelled DDT$_1$ MF-2 cells were stimulated with CP 55,940 and the release of radiation measured and presented as increase in the release as % of basal level. Data represent 8 experiments in triplicate. Basal AA release: 16.6 ±2.6 dps/well.

To further test the hypothesis that AA is generated in response to CB$_1$ stimulation, AA release was measured in [$^3$H]AA-prelabelled DDT$_1$ MF-2 cells. CP 55,940 produced a concentration-dependent efflux of AA (EC$_{50}$ = 0.10 ±
0.04 μM, Fig. 5). To establish whether the production of AA was receptor-mediated, the effect of the CB₁ antagonist SR 141716A (1 μM) was studied. CP 55,940 (1 μM) -mediated release of AA was completely prevented in the presence of SR 141716A (Table 1).

Indomethacin, a non-selective cyclooxygenase inhibitor (Bakalova et al., 2002) and phenidone, a dual inhibitor of both cyclooxygenase and lipoxygenase (Kim et al., 2000) were used to establish if the outward current in response to CB₁ stimulation requires the production of AA metabolites. Pretreatment of cells with indomethacin (10 μM) had no significant effect on the outward current evoked by 10 μM CP 55,940 (93 ± 10% of control, n=7). Phenidone (100 μM) also had no effect on this response (114 ± 13% of control, n=5).

### Table 1. The effect of various inhibitors on [³H] arachidonic acid release (dps/well)

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Control</th>
<th>+ CP 55,940 (1μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (DMSO, 0.6 %)</td>
<td>16.5±1.2 (20)</td>
<td>32.2±3.7 (30) *</td>
</tr>
<tr>
<td>SR 141716A (1μM)</td>
<td>21.9±2.9 (7)</td>
<td>22.6 ±2.1 (14)</td>
</tr>
<tr>
<td>Quinacrine (100μM)</td>
<td>18.9±3.0 (10)</td>
<td>17.4±1.6 (11)</td>
</tr>
<tr>
<td>Gd³⁺ (1μM)</td>
<td>19.1±2.0 (8)</td>
<td>17.4±2.0 (13)</td>
</tr>
<tr>
<td>PD 98059 (30μM)</td>
<td>21.3±2.0 (13)</td>
<td>24.5±1.3 (12)</td>
</tr>
</tbody>
</table>

Data are presented as mean ± sem (number of experiments). Significance level: *, P< 0.002 vs. control

**Lanthanum (La³⁺)-Sensitive and Gadolinium (Gd³⁺)-Sensitive Ca²⁺ Influx** - In the presence of the non-specific Ca²⁺ channel blocker La³⁺ (50 μM), the histamine-evoked Ca²⁺ influx in DDT₁ MF-2 cells has been shown to be abolished, leading to a reduction of the outward current (Van der Zee et al., 1995). This Ca²⁺ influx may represent a NCCE pathway as observed in other cell lines, which is also sensitive to inhibition by La³⁺ (Fiorio Pla and Munaron, 2001; Mignen and Shuttleworth, 2000). Indeed La³⁺-sensitive Ca²⁺ channels
are activated during CB₁ stimulation, as application of 50 μM La³⁺, prior to CP 55,940 (10 μM) application, abolished the CP 55,940-evoked outward current (Fig. 6, P<0.0001). To further explore this activation of NCCE, the effect of La³⁺ on AA-induced Ca²⁺ entry was established since AA has also been shown to activate Iₖ,Ca (Kirber et al., 1992). These results would help to ascertain if AA production does indeed occur upstream of the Ca²⁺ entry process. Indeed La³⁺ (50 μM) was able to reduce the response to 10 μM AA to 17 ± 13% of control (Fig. 6, P<0.001).

Figure 6. La³⁺ inhibits the outward current evoked by CP 55,940 and AA. 50 μM La³⁺ inhibited the AA (10 μM)-evoked outward current (n=6) and completely abolished the response to 10 μM CP 55,940 (n=7). Significant difference from CP 55,940 and AA control: * P<0.001, ** P<0.0001.

CCE in DDT₁ MF-2 cells can be evoked with the sarcoplasmic-endoplasmic reticulum Ca²⁺-ATPase (SERCA) inhibitor thapsigargin. Nickel (Ni²⁺), which has been shown to inhibit CCE in other cell systems (Kerschbaum and Cahalan, 1999), inhibited the Ca²⁺ influx component of thapsigargin in a concentration-dependent manner (EC₅₀: 0.8 mM, n=3, Fig. 7A.). At concentrations ranging from 0.01 to 100 μM, neither La³⁺ nor Gd³⁺ had any effect on the thapsigargin-induced elevation in [Ca²⁺]ᵢ (Fig. 7B and C), while Ni²⁺ clearly inhibited the Ca²⁺ influx. This suggests that separate Ca²⁺ influx pathways are initiated by CP 55,940 (NCCE) and thapsigargin (CCE).
In rat aortic smooth muscle cells low concentrations (1 μM) of Gd³⁺ inhibited CCE, while higher concentrations (100 μM) were shown to inhibit both CCE and NCCE (Broad et al., 1999). Therefore, the effects of Gd³⁺ on CP 55,950 evoked membrane current and AA efflux were also investigated. Gd³⁺ (1 μM) inhibited the outward current produced by 10 μM CP 55,940 to 23 ± 11% of control (Fig 8, P<0.0001). However, the observation that 100 μM, Gd³⁺ had no effect on thapsigargin induced rise in [Ca²⁺]ᵢ implies that CP 55,940 activates...
a Ca$^{2+}$ influx pathway distinct from CCE. The Ca$^{2+}$ influx generated through the NCCE pathway in response to CP 55,940 appears to be essential for PLA$_2$ activation in view of the abolition of AA release in the presence of Gd$^{3+}$ (1 μM, Table 1).

Figure 8. Gd$^{3+}$ inhibits the outward current evoked by CP 55,940. A, sample trace of the effect of Gd$^{3+}$ (1 μM) on the CP 55,940 (10 μM)-evoked outward current. Bars indicate the presence of ligands. B, Gd$^{3+}$ significantly reduces the outward current evoked by 10 μM CP 55,940 (n=9). Significant difference from CP 55,940 control: * P<0.0001.

AA Production: Role of Phospholipase A$_2$ (PLA$_2$) and MAP kinase - Cannabinoid-induced mobilisation of AA was shown to involve the activities of the cytoplasmic PLA$_2$ isoenzyme in WI-38 lung fibroblasts (Wartmann et al., 1995). To explore the possibility that AA is being generated by PLA$_2$ in DDT$_1$ MF-2 cells, the effects of a non-selective inhibitor of PLA$_2$, quinacrine, were investigated (Lu et al., 2001). At concentrations of 10 μM and 30 μM quinacrine inhibited the outward current evoked by 10 μM CP 55,940 to 64
±8% of control (Fig. 9B, P<0.001) and 63 ±6% of control (Fig. 9A and B, P<0.01) respectively.

**Figure 9.** Quinacrine inhibits the CP 55,940-induced outward current and release of [³H]AA. Cells were pre-treated with quinacrine (10-30 μM) for 10 minutes before agonist application. A, sample trace of the effect of 30 μM quinacrine on the outward current evoked by 10 μM CP 55,940. B, Quinacrine at 10 (n=5) and 30 μM (n=9) reduced the CP 55,940 (10 μM)-induced current. Significant difference from CP 55,940 control: * P<0.01, ** P<0.001.

Quinacrine (100 μM) alone had no effect on membrane currents in DDT₁ MF-2 cells (results not shown, n=4). To add further support to the involvement of CB₁-mediated activation of PLA₂, the effect of quinacrine (100 μM) on CP 55,940-evoked [³H]AA release was established. Quinacrine alone had no effect on basal [³H]AA efflux, but the CP 55,940 (1 μM)-evoked AA release was abolished (Table 1). CP 55,940-induced AA release is also inhibited strongly by the MAP kinase pathway inhibitor PD 98059 (30 μM, Table 1), supporting the hypothesis that PLA₂ activation by CP 55,940 is regulated by MAP kinase.
Discussion

In the present study we further explored 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, which is completely abolished by the removal of Ca$^{2+}$ from the bathing solution (Begg et al., 2001). Similarly, we showed a concentration-dependent increase in [Ca$^{2+}$]$_i$ by CP 55,940, which was abolished by the removal of extracellular Ca$^{2+}$. This suggested that the CB$_1$-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 was also shown to play a role (Begg et al., 2001). This would imply a model for CCE, where the depletion of intracellular Ca$^{2+}$ stores activates membrane-bound SOCCs, resulting in Ca$^{2+}$ influx (Putney, 1990). Interestingly, in the current study we show that in the absence of extracellular Ca$^{2+}$, higher concentrations of CP 55,940 even decreased [Ca$^{2+}$]$_i$. This suggests that at least part of the Ca$^{2+}$ transport mechanism is still operational under these conditions, but reversed due to the inverted driving force for Ca$^{2+}$.

DDT$_1$ MF-2 cells have been used previously to study changes in [Ca$^{2+}$]$_i$ caused by activation of histamine H$_1$ and purine P$_2$ receptors (Molleman et al., 1991). It was demonstrated that activation of these receptors elevates [Ca$^{2+}$]$_i$ through an InsP$_3$-mediated release from internal Ca$^{2+}$ stores. The production of InsP$_3$ involves the activation of PLC. The inhibitory effect of thapsigargin on the cannabinoid response (Begg et al., 2001) would suggest that a release of Ca$^{2+}$ from internal stores is also required to mediate this response. However, inhibition of PLC has no effect on the outward current evoked by the cannabinoid receptor agonist CP 55,940 (Begg et al., 2001). Similarly we found in the current study that the InsP$_3$ receptor antagonist, 2-APB had no effect at all on the CP 55,940 response at a concentration that was shown to inhibit histamine-evoked currents. In addition to its effects on the InsP$_3$ receptors 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 $[\text{Ca}^{2+}]_{i}$ in DDT$_1$ MF-2 cells. We also used the SOCC inhibitor SKF 96365 to support our data obtained with 2-APB. The inhibitor was found to have no effect on the CP 55,950-evoked response, at a concentration shown to inhibit CCE in other systems (Merritt et al., 1990). Thus, taken together, the SKF 96365 and 2-APB data strongly argue against CCE as a mechanism for CB$_1$ evoked increases in $[\text{Ca}^{2+}]_{i}$.

Recently an NCCE pathway has been described to operate independently of intracellular store depletion. In this pathway the rise in $[\text{Ca}^{2+}]_{i}$ occurs via $\text{Ca}^{2+}$ influx activated by intracellular messengers, including AA. AA-evoked $\text{Ca}^{2+}$ 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 (Shivachar et al., 1996). In HEK 293 cells the channels responsible for the AA-mediated $\text{Ca}^{2+}$ influx were investigated and the resulting membrane current was designated as $I_{\text{ARC}}$ (Mignen and Shuttleworth, 2000). In addition, AA has been shown to initiate $\text{Ca}^{2+}$ influx during H$_1$ stimulation in DDT$_1$ MF-2 cells (Van der Zee et al., 1995), suggesting that a NCCE pathway is operational in this cell line. This lends support to the possibility that CB$_1$ stimulation induces a rise in $[\text{Ca}^{2+}]_{i}$ through a similar non-capacitative pathway.

Application of AA to DDT$_1$ MF-2 cells evoked a transient outward current, similar to that caused by application of CP 55,940. Exogenous application of AA has been shown to induce $\text{Ca}^{2+}$ influx in cells utilising NCCE (Broad et al., 1999; Fiorio Pla and Munaron, 2001; Mignen and Shuttleworth, 2000; Munaron et al., 1997), including DDT$_1$ MF-2 cells (Van der Zee et al., 1995). Application of AA prior to histamine resulted in inhibition of the histamine-evoked NCCE-mediated $\text{Ca}^{2+}$ influx, as reflected by abolition of outward current (Van der Zee et al., 1995). This suggests that AA activates the same $\text{Ca}^{2+}$ channels that mediate $\text{Ca}^{2+}$ influx during CB$_1$ as well as H$_1$ receptor stimulation. In order to determine whether CB$_1$-induced increases in $[\text{Ca}^{2+}]_{i}$
occur through the activation of an I_{ARC}-like channel, the effects of AA on the CP 55,940-evoked current were established. AA was found to inhibit concentration-dependently the CP 55,940-evoked response.

In support of an NCCE signalling pathway mediated by AA we observed an increase in $[^3H]AA$ release in response to CP 55,940 application, indicating that CB$_1$ stimulation results in AA production. This was confirmed by using the CB$_1$ antagonist SR 141716A, which was able to block the CP 55,940-evoked AA release completely. Previous work using DDT$_1$ MF-2 cells has shown that SR 141716A reduces the outward currents evoked by the herbal cannabinoid $\Delta^9$-THC and by CP 55,940 (Filipeanu et al., 1997; Begg et al., 2001). Together with the present 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 (Shivachar et al., 1996) $\Delta^9$-THC has been found 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 is thought to activate Ca$^{2+}$ channels directly in DDT$_1$ MF-2 cells, as inhibitors of the cyclooxygenase and lipoxygenase pathway did not affect the characteristics of the histamine-induced $[^3H]AA$ 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. Comparable observations showing that AA rather than its metabolites mediate NCCE have also been made in other preparations (Putney, 1990; Munaron et al., 1997; Mignen and Shuttleworth, 2000).

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 HEK 293 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 almost completely, which suggests that AA production occurs upstream of Ca$^{2+}$ entry and hence Ca$^{2+}$ channel activation. The small remaining part of the outward current induced by AA in the presence of the same La$^{3+}$ concentration that abolished the CP 55,940-induced response, may be attributed to AA-mediated release of Ca$^{2+}$ from internal stores (Watson et al., 2004; Van der Zee et al., 1995; Fiorio Pla and Munaron, 2001). La$^{3+}$ can also inhibit CCE (Putney, 2001), so we investigated the effects of this inhibitor on thapsigargin-evoked CCE in DDT$_1$ MF-2 cells.

Thapsigargin depletes Ca$^{2+}$ stores by inhibiting the SERCA pumps on the sarcoplasmic reticulum, thereby initiating Ca$^{2+}$ influx (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 this Ca$^{2+}$ influx, at a concentration that abolished the CP 55,940-evoked current. This clearly implicates a Ca$^{2+}$ influx pathway utilised during CB$_1$ signalling in DDT$_1$ MF-2 cells distinct from CCE.

Gd$^{3+}$, another inhibitor of Ca$^{2+}$ influx, is able to distinguish between AA-sensitive CCE and NCCE in rat aortic smooth muscle cells; at low concentrations (1 μM) Gd$^{3+}$ inhibited CCE, while at higher concentrations (100 μM) both CCE and NCCE were inhibited (Broad et al., 1999). Other authors have shown the CCE pathway to be potently inhibited by 1 μM Gd$^{3+}$ (Luo et al., 2001; Putney, 2001), but Gd$^{3+}$-insensitive SOCCs are also expressed in some experimental systems (Fernando and Barritt, 1994; Fernando and Barritt, 1995). In DDT$_1$ MF-2 cells Gd$^{3+}$ (1 μM) reduced the outward current in response to CP 55,940, but had no effect on CCE evoked by thapsigargin. This may suggest that the AA-sensitive channels in DDT$_1$ MF-2 cells are distinct from those described in rat aortic smooth muscle cells, which is also supported by the finding that SKF 96365 which at 100 nM inhibited NCCE in rat aortic cells (Moneer et al., 2003), at a 100 fold greater concentration has no effect on the CP 55,940-evoked outward current in DDT$_1$ MF-2 cells. Our data agree with previous work showing that low concentrations of Gd$^{3+}$ can inhibit currents mediated by I$_{ARC}$ and Ca$^{2+}$ influx in response to AA application.
in HEK 293 cells (Mignen et al., 2003) and rat astrocytes (Sergeeva et al., 2003), respectively, indicating that the present Gd$^{3+}$ results are consistent with the idea that the Ca$^{2+}$ influx pathway initiated during CB$_1$ stimulation is not capacitative.

A major property of I$_{ARC}$ activation is that it occurs independently from intracellular Ca$^{2+}$ store depletion (Broad et al., 1999; Fiorio Pla and Munaron, 2001; Mignen and Shuttleworth, 2000). However, it was previously shown that thapsigargin inhibited the outward current induced by CP 55,940 in DDT$_1$ MF-2 cells, suggesting that CB$_1$-mediated Ca$^{2+}$ entry is partly dependent on intracellular Ca$^{2+}$ release (Begg et al., 2001). Our present results, indicating a NCCE evoked response after CP 55,940 stimulation, offer an explanation for this apparent inhibition, since emptying Ca$^{2+}$ stores by thapsigargin pretreatment will already activate the outward current (Begg et al., 2001), thereby antagonising the CP 55,940-induced NCCE signal to these channels.

Reciprocal regulation of CCE and NCCE has been described in other cells exhibiting AA-mediated Ca$^{2+}$ influx (Moneer and Taylor, 2002; Moneer et al., 2003; Mignen et al., 2001; Luo et al., 2001). In accordance with the proposition of I$_{ARC}$ activation after CB$_1$ receptor stimulation, such a reciprocal mechanism seems not to be active in DDT$_1$ MF-2 cells since inhibition of CCE by SKF 96365 was without effect on the CP 55,940-induced outward current.

Another objective of the current study was to examine the putative link between MAP kinase activation and the rise in [Ca$^{2+}$]$_i$ following the generation of AA by stimulation of the CB$_1$ receptor. Liberation of AA can occur through the direct action of PLA$_2$ on phospholipids. In particular cytosolic PLA$_2$ has been shown to mediate AA liberation, regulated by Ca$^{2+}$ and requiring phosphorylation by MAP kinase for maximal activation (Kudo and Murakami, 2002).

Experiments in fetal lung fibroblasts have suggested that a cannabinoid-mediated increase in AA is associated with an increased phosphorylation and activity of both MAP kinase and cytosolic PLA$_2$ (Wartmann et al., 1995). The non-selective PLA$_2$ inhibitor quinacrine reduced the CP 55,940-evoked
current in DDT₁ MF-2 cells and abolished the [³H]AA release. Inhibition of AA release was also observed in the presence of the MAP kinase inhibitor PD 98059. Together, these AA release data suggest that the primary pathway for AA production, during CB₁ stimulation, involves the activation of both PLA₂ and MAP kinase. Ca²⁺ influx through the NCCE pathway is essential for this activation in view of the complete inhibition of AA release observed in the presence of Gd³⁺.

The intracellular signalling pathways that evoked a release of AA in DDT₁ MF-2 cells during H₁-receptor stimulation, have not been identified, although stimulation of the receptor has been shown to induce phosphorylation of MAP kinase, with considerable phosphorylation occurring already after one minute (Robinson and Dickenson, 2001). This suggests an immediate action of MAP kinase, presumably resulting in PLA₂-induced release of AA, during H₁ stimulation in DDT₁ MF-2 cells. Previous work has shown that both the histamine- and CP 55,940-induced currents in DDT₁ MF-2 cells were inhibited by the MAP kinase inhibitor PD 98059, but that histamine was less sensitive to this inhibition (Begg et al., 2001). This is consistent with the idea that the main increase in [Ca²⁺]ᵢ of the histamine response is derived from InsP₃-sensitive stores and that PD 98059 is only inhibiting the NCCE component of the histamine response.
Figure 10. Model for cannabinoid-induced NCCE in DDT₁ MF-2 cells. In this scheme all experimental data presented are depicted. The pathway elicited by CB₁ receptor stimulation with CP 55,940 (bold lines) generates arachidonic acid (AA) mediated by MAP kinase-sensitive cytosolic PLA₂ activity. Release of AA induces non capacitative Ca²⁺ entry (NCCE) via I_{ARC} and subsequent activation of Ca²⁺-dependent K⁺ channels. NCCE is essential for further AA release. Relevant parts of the CCE pathway are also depicted, showing Ca²⁺ entry in response to depletion of 2-APB-sensitive Ca²⁺ pools by thapsigargin. + indicates activation and − inhibition.

The simplified scheme as depicted in Fig.10 summarizes the main features of the results obtained, which are consistent with the proposition that cannabinoid signalling generates AA-induced NCCE by MAP kinase regulated activation of cPLA₂. The resulting Ca²⁺ influx via I_{ARC} further stimulates cPLA₂ activity and AA release, and simultaneously elicits outward current.
In conclusion, we have shown for the first time that stimulation of CB$_1$ receptors can lead to Ca$^{2+}$ influx mediated by AA. This influx pathway is distinct from CCE and instead involves the activation of I$_{ARC}$ and hence NCCE. Our results also provide evidence for a CB$_1$ receptor-mediated activation of PLA$_2$, upstream of AA production and presumably downstream of MAP kinase activation.

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References


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