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Two distinct membrane currents activated by cyclopiazonic acid-induced calcium store depletion in single smooth muscle cells of the mouse anococcygeus

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1 By use of the whole-cell configuration of the patch-clamp technique, membrane currents induced by cyclopiazonic acid (CPA; an inhibitor of the sarcoplasmic reticulum (SR) calcium-ATPase) were investigated in single smooth muscle cells freshly dispersed from the mouse anococcygeus. Voltage-dependent calcium currents were blocked with extracellular nifedipine and caesium and tetraethylammonium chloride were used to block voltage-dependent potassium currents.

2 At a holding potential of −40 mV, CPA (10 μM) activated an inward current that consisted of two distinct components. The first was an initial transient current with an amplitude of 19.6±1.9 pA while the second was sustained and had an amplitude of 3.5±0.3 pA.

3 The current-voltage (*I-V*) relationship for the transient current showed marked outward rectification. The current had a reversal potential of 9.1±1.1 mV which was shifted to 29.0±4.2 mV when the extracellular chloride concentration was lowered from 148.4 to 58.4 mM. The sustained current had a near-linear *I-V* relationship and a reversal potential of 31.0±2.7 mV. Removal of extracellular calcium had no effect on the transient current, but shifted the reversal potential of the sustained current to 18.2±5.7 mV.

3 The initial transient current was abolished in cells bathed in extracellular solutions containing the chloride channel blockers, 4,4′ diisothiocyanato-stilbene-2,2′-disulphonic acid (DIDS; 1 mM) or anthracene-9-carboxylic acid (A-9-C; 1 mM), and was absent in cells containing the calcium buffers EGTA (1 to 5 mM) or BAPTA (10 mM). The second sustained current was unaffected by either the chloride channel blockers or the intracellular calcium buffers.

4 Treatment of the cells with caffeine (10 mM) produced similar inward currents to those produced by CPA. In the presence of caffeine, CPA (10 μM) induced no further inward current.

5 In organ bath studies, CPA (10 μM)-induced contractions of the mouse anococcygeus were inhibited by cadmium and nickel (both 50–400 μM) and the general calcium entry blocker, SKF 96365 (10 μM); lanthanum and gadolinium had no effect at concentrations up to 400 μM. The pharmacology of the CPA-induced non-selective cation current mirrored that of the CPA-induced whole muscle contraction being reversed by cadmium (100 μM) and SKF 96365 (10 μM), but unaffected by lanthanum (400 μM). The initial chloride conductance was unaffected by cadmium, SKF 96365 or lanthanum.

6 It is concluded that CPA activates a transient calcium-dependent chloride current as a consequence of calcium release from intracellular stores; this current would result in depolarization and opening of voltage-operated calcium channels, which mediate the nifedipine-sensitive component of muscle contraction. In addition, as a result of emptying the SR, CPA activates a non-selective cation conductance which may underlie the nifedipine-insensitive calcium entry process utilised during sustained contraction.

Keywords: Anococcygeus (mouse); caffeine; calcium-dependent chloride current; calcium store depletion; cyclopiazonic acid; non-selective cation current; whole-cell patch-clamp

Introduction

Recently we reported that cyclopiazonic acid (CPA), a mycotoxin isolated from *Aspergillus* and *Penicillium*, produces strong, well-sustained contractions of the mouse anococcygeus muscle (Gibson *et al.*, 1994). CPA has been shown to inhibit sarco-endoplasmic reticulum (SR) calcium-ATPases and in doing so depletes these intracellular stores of calcium (Seidler *et al.*, 1989; Imaizumi *et al.*, 1992). It is well documented that the release of calcium from intracellular stores, either by agonists activating receptors coupled to the inositol phosphate/diacyl glycerol signalling pathway, or by agents such as CPA, activates calcium entry into cells from the extracellular space *via* a pathway distinct from the well characterized voltage- and receptor-operated calcium channels (Putney, 1986; Demaurex *et al.*, 1992; Hoth & Penner 1992; 1993; Randriamampita & Tsien, 1993). Following initial observations in rat peritoneal mast cells (Hoth & Penner 1992; 1993), membrane calcium currents activated as a result of store depletion have been reported in a number of cell types (Fasolato *et al.*, 1994), although notably, not in smooth muscle cells. This is despite indirect evidence that store-regulated calcium entry occurs in these cells (van Breemen & Saida, 1989; Missiaen *et al.*, 1990; Pacuad *et al.*, 1993; Felder *et al.*, 1994; Somlyo & Somlyo, 1994; Amrani *et al.*, 1995). For example, in the mouse anococcygeus, the contraction to CPA, whilst being largely unaffected by nifedipine, is abolished in calcium-free extracellular medium and by the general calcium entry blocker, SKF 96365 (Gibson *et al.*, 1994).

In the current series of experiments we have used the whole-cell configuration of the patch clamp technique to study CPA-induced currents in single cells isolated from the mouse ano-

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coccygeus. The experiments had two main aims; first to confirm that CPA releases calcium from intracellular stores in these cells and second, to obtain direct, electrophysiological evidence for the presence of a store-regulated calcium entry pathway. We measured changes in intracellular calcium indirectly by monitoring the calcium-dependent chloride current found in these cells. A preliminary account of this work has been published (Wayman *et al.*, 1995).

Methods

Electrophysiological recordings

Male mice (LACA strain, 25–35 g) were killed by stunning and exsanguination and the anococcygeus muscles dissected. Single smooth muscle cells were dissociated enzymatically by a method based on that described for the rat anococcygeus (McFadzean & England, 1992). The muscles were incubated for 10 min at 37°C in a physiological salt solution (PSS) containing zero added calcium plus, in mM: NaCl 120, KCl 6, $MgCl_2$ 1.2, Na_2HPO_4 1.2, glucose 11, HEPES 10, pH 7.2. Following this, the tissues were incubated for 12.5 min at 37°C in PSS to which had been added (all from Sigma) bovine serum albumin (fatty acid free, 2.6 mg ml^{-1}), papain (0.8 mg ml^{-1}), collagenase (Type 1A; 0.5 mg ml^{-1}) and dithioerythritol (1.2 mM). The tissues were then washed twice in enzyme-free PSS, and the single cells isolated by passing the muscle pieces through a wide-bore Pasteur pipette several times. The resultant cell suspension was centrifuged at approximately 180 *g* for 1 min, and the cell-free supernatant discarded. The pellet was resuspended in PSS containing 0.75 mM calcium, and droplets of the cell-rich suspension were placed on poly-lysine (5%)-coated glass coverslips and stored at 4°C for at least 2 h prior to making electrophysiological recordings. Membrane currents were recorded by the whole-cell variant of the patch-clamp technique. The extracellular solution bathing the cells contained (mM); NaCl 90, tetraethylammonium chloride (TEA) 30, KCl 6, $MgCl_2$ 1.2, glucose 11, HEPES 10, $CaCl_2$ 10, pH 7.20 (with NaOH). In experiments where a 'low chloride' solution was used, 90 mM NaCl was replaced with 90 mM Na benzenesulphonate. The patch-pipette filling solution contained (mM): CsCl 130, TEA 20, HEPES 10, ATP 0.5, GTP 0.5, pH 7.20 (with CsOH). When filled with this solution, patch-pipettes had d.c. resistances of around 6 MΩ. The combination of intracellular and extracellular TEA plus intracellular CsCl minimized current flow through voltage-dependent potassium channels. Dihydropyridine-sensitive (L-type) voltage-dependent calcium currents were blocked by the addition of 1 μM nifedipine to the extracellular solution immediately before starting an experiment. 'Calcium-free' extracellular solution contained zero added calcium plus 1 mM EGTA.

Currents were recorded and filtered at 1 kHz (−3 dB) with an Axopatch 200A amplifier (Axon Instruments Inc., Burlingame, U.S.A.) and digitised (>2 kHz) with a Digidata 1200 interface (Axon Instruments Inc.) in combination with a personal computer (Mesh 486DX) running pClamp acquisition and analysis software (Axon Instruments Inc.) and stored directly onto the hard disk of the computer. A continuous, filtered (200 Hz) record of membrane current was obtained with a thermal chart recorder (Gould TA550). All electrophysiological experiments were performed at room temperature (20–24°C).

Drugs were applied extracellularly by a gravity-fed system incorporating a fine bore (0.28 mm internal diameter) catheter tube placed within 100 μm of the cell under test. Cells were perfused continuously with either drug-free or drug-containing solutions, and switching between drug solutions was achieved by means of solenoid valves. Whilst this constant perfusion system minimized pressure changes in the delivery tube, control protocols were carried out at regular intervals. During such protocols, all drug-delivery reservoirs were filled with either drug-free solution or PSS containing 0.1% dimethyl sulphoxide (DMSO), the solvent for CPA, and the solenoid valves were switched at random whilst recording from a cell. The delay between changing reservoirs and ejection of the drug was approximately 5 s.

Tension recordings

The anococcygeus muscles were dissected and set up in 1 ml glass organ baths containing Krebs-bicarbonate buffer (mM: NaCl 118.1, KCl 4.7, $MgCl_2$ 1.0, KH_2PO_4 1.0, $NaHCO_3$ 25.0, glucose 11.1, $CaCl_2$ 2.5) maintained at 37°C and saturated with 95% O_2/5% CO_2. A resting tension of 200–400 mg was placed on the tissue and changes in tension recorded with a Grass FT03 force-displacement transducer attached to a Graphtec pen recorder (WR3101). The Krebs solution also contained 1 μM phentolamine, 50 μM N^G-nitro-L-arginine (L-NOARG) and 1 μM nifedipine. Muscles were allowed to equilibrate for at least 45 min before beginning the experiment, including a 10 min pre-incubation in 30 μM guanethidine. The phentolamine, L-NOARG and guanethidine were used to prevent any effects due to the release of transmitter from either the motor noradrenergic or inhibitory nitrergic nerves within the tissue, whilst the nifedipine was used to block calcium entry *via* voltage-dependent calcium channels.

The effects of various cations on the contraction produced by CPA were studied by construction of cumulative concentration-response curves for the cations against the increase in tone produced by 10 μM CPA (Gibson *et al.*, 1994). The cations were not added to the organ baths until the response to CPA had reached a plateau. pIC_{50} values (negative log of the molar concentration of each ion reducing CPA-induced tone by 50%) were calculated by regression analysis of individual concentration-response curves.

Results are expressed as mean ± s.e.mean. Statistical analysis was carried out with Student's *t* test (paired or unpaired where appropriate); $P<0.05$ was taken as significant.

The following drugs were used: A-9-C (anthracene-9-carboxylic acid; Sigma); cyclopiazonic acid (Sigma); BAPTA (1,2-*bis*(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; Sigma); caffeine (Sigma); DIDS (4,4′ diisothiocyanato-stilbene-2,2′-disulphonic acid; Sigma); EGTA (ethylene glycol-*bis*(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid; Sigma); nifedipine (Sigma); N^G-nitro-L-arginine (Sigma); phentolamine mesylate (Ciba); guanethidine monosulphate (Sigma); SKF 96365 (1-{β-[3-(4-methoxyphenyl) propoxyl]-4-methoxyphenethyl}-1H-imadazole HCl; Smithkline Beecham). All salts used were of reagent grade or better. All drugs were prepared as stock solutions in de-ionised water with the exception of CPA (10 mM stock in DMSO), nifedipine (10 mM stock in ethanol), DIDS and A-9-C (both 100 mM stock in ethanol).

Results

During the course of this study, CPA (10 μM) was applied to 142 mouse anococcygeus cells. The cells were held at a potential of −40 mV, and at this potential, CPA evoked an inward current in 113 cells. In the majority of cells which responded to CPA, the inward current was composed of two distinct phases; an initial, transient current followed by a smaller, sustained current (Figure 1a). The initial current was present in 81 of the 113 cells. It had an amplitude of 19.6 ± 1.9 pA (measured at the peak of the response) and a duration (measured at 70% amplitude) of 14.5 ± 0.9 s. The second current was present in 110 out of 113 cells that responded to CPA and had an amplitude of 3.5 ± 0.3 pA and was sustained for up to 5 min following the application of CPA; the amplitude of this current was measured at a time, usually between 90 and 120 s after the application of CPA, when the transient current had decayed, and the sustained current had stabilized.Two cells showed a transient current without a sustained component, whilst 32 had no transient

current, but did show a sustained component. There appeared to be no correlation between the amplitudes of the first and second components (linear regression analysis; $r=0.46$).

Current-voltage (*I-V*) relationships for the cells were constructed in the absence and in the presence of CPA (10 μM) by ramping the membrane potential from −100 mV to +60 mV over 1 s. Under control conditions, the *I-V* relationship for the CPA-induced transient current (obtained by subtracting the control *I-V* curve from that obtained near the peak of the response) showed marked outward rectification and reversed at 9.1±1.1 mV ($n=51$; Figure 1b). This was in contrast to the second current which had a near-linear current-voltage relationship, and a reversal potential of 31.0±2.7 mV ($n=39$; Figure 1b), suggesting that the two components of the CPA-induced current were carried by distinct conductances.

The reversal potential and current-voltage relationship of the transient current suggested that it might result from activation of a calcium-dependent chloride current such as that initially described in cells isolated from the rat anococcygeus (Byrne & Large, 1987). We therefore carried out a series of experiments to test this hypothesis.

As mentioned above, the reversal potential of the transient current under control conditions was 9.1±1.1 mV ($n=51$) which corresponded reasonably to the chloride equilibrium potential (0 mV). Lowering the extracellular chloride concentration from 148.4 to 58.4 mM (E_{Cl} 22 mV) significantly moved the reversal potential of the transient current to 29.0±4.2 mV ($n=6$) compared to the control value, whilst having no effect on the reversal potential of the sustained current which was 26.2±5.8 mV ($n=6$) in low extracellular chloride. The transient current was abolished by the chloride channel blockers DIDS (1 mM; $n=6$; Figure 2a) and A-9-C (1 mM; $n=6$; Figure 4b) and was absent in cells containing either EGTA (1–5 mM, $n=8$; Figure 2b) or BAPTA (10 mM, $n=13$). The second, sustained current was observed both in the presence of the chloride channel blockers (Figures 2a, 4b), and in cells loaded with EGTA (Figure 2b) or BAPTA.

Taken together, these data suggest that the transient current activated by CPA is a calcium-dependent chloride current. To determine whether this occurred as a result of CPA releasing calcium from intracellular stores, we pretreated cells with caffeine (10 mM) to deplete the stores, and studied the effect of this on the response to CPA. Caffeine itself produced a response similar to that produced by CPA; a large transient current (153.1±48.0 pA; $n=9$) followed by a second, smaller (8.2±2.0 pA; $n=9$) sustained component (Figure 3a) which was reversed on washout of caffeine (Figure 3b). Both components of current activated by caffeine were significantly larger than those seen in response to CPA. In the presence of caffeine, CPA produced neither a transient nor a sustained current (Figure 3b), but it was noticeable that in cells treated with CPA, the response to caffeine did not reverse on washing, presumably because the intracellular stores were unable to refill due to inhibition of the calcium-ATPase by the mycotoxin (Figure 3b).

A number of cells (50 out of 142 cells) exhibited spontaneous, transient inward currents (STICS) under control conditions. These STICS had a reversal potential close to 0 mV (Figure 4a) and were abolished by DIDS (Figure 2a) and A-9-C (Figure 4b) and absent in cells containing EGTA (1–5 mM) or BAPTA (10 mM). Similar currents have been described in cells isolated from a number of smooth muscles, and are thought to arise as a result of spontaneous discharges of cal-

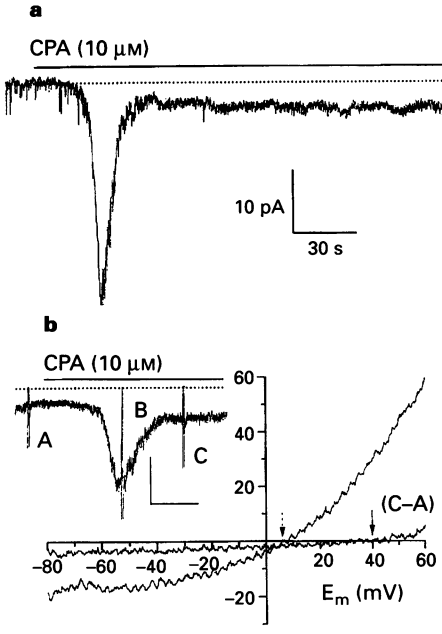

Figure 1 CPA activates two distinct components of inward current in mouse anococcygeus cells. (a) The response to CPA (10 μM) in a mouse anococcygeus cell held at a membrane potential of −40 mV. The drug was applied as indicated by the solid bar and produced an initial, transient inward current followed by a smaller sustained inward current. Zero current is indicated by the dotted line. (b) Current-voltage (*I-V*) relationships for the transient and sustained components of CPA-induced current. The inset shows a response to CPA (10 μM). Three *I-V* relationships (denoted A, B and C) were constructed by ramping the membrane potential from −100 to +60 mV over 1 s, before and during the response to CPA. The main part of the figure shows the *I-V* relationships for the CPA induced currents. These were constructed by subtracting the control *I-V* relationship (A in the inset) from those recorded during the transient (B in the inset) and sustained (C in the inset) components of the CPA response. In this cell, the reversal potential for the transient current (broken arrow) was +6 mV whilst that for the sustained current (solid arrow) was +40 mV.

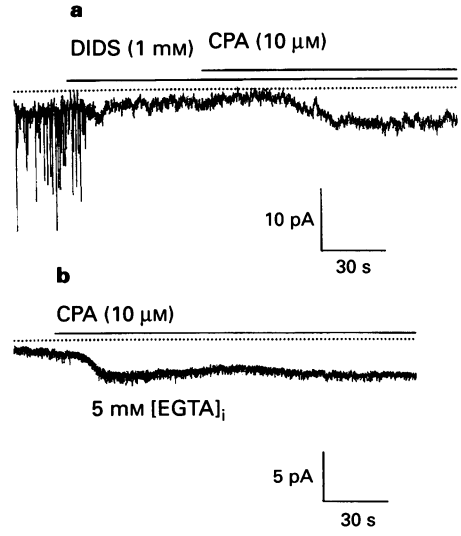

Figure 2 The transient current activated by CPA is a calcium-dependent chloride current. (a) The initial transient current activated by CPA (10 μM) was blocked by the chloride channel blocker, 4,4′ diisothiocyanato-stilbene-2,2′-disulphonic acid (DIDS; 1 mM), whilst the sustained current was unaffected. Note that the chloride channel blocker also abolished the spontaneous transient inward currents. (b) The transient current activated by CPA (10 μM) under control conditions was absent when recordings were made using patch-pipettes containing 5 mM EGTA. In (a) and (b), the holding potential was −40 mV and the dotted lines represent zero current. Drugs were applied where indicated by the solid bars.

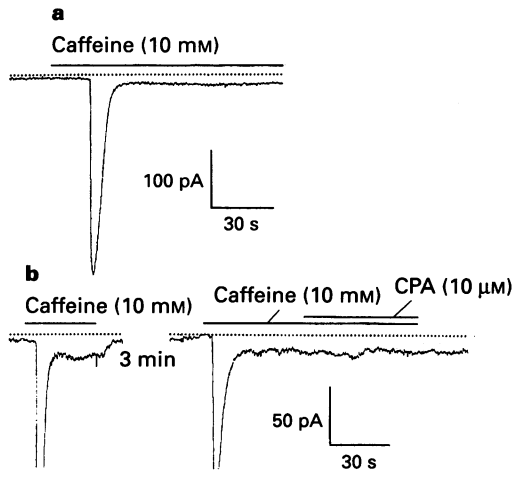

Figure 3 Caffeine activates both the transient and sustained components of current and prevents the response to CPA: (a) and (b) show response to caffeine (10 μM) in different cells held at a membrane potential of −40 mV. As shown in (b), the response to caffeine reversed readily on washing with drug-free extracellular solution, and was repeatable as shown by the second response when caffeine was reapplied after 3 min. A concomitant application of CPA (10 μM) failed to produce a response, although that to caffeine no longer reversed on washing. Note that a high recorder gain was used in these experiments to facilitate clear representation of the sustained current. This resulted in loss of the peak of the initial transient current from the trace record. Drugs were applied where indicated by the solid bars. The dotted line shows zero current.

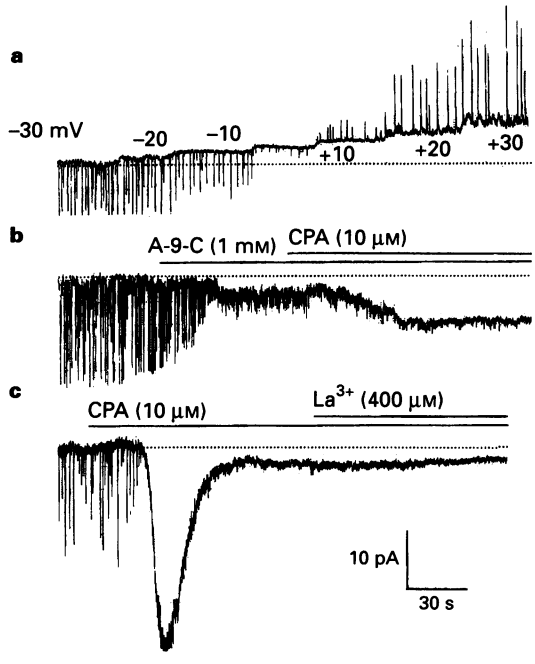

Figure 4 Spontaneous transient inward currents (STICs) in mouse anococcygeus cells: (a) spontaneous currents recorded in a single cell at the indicated membrane potentials. The spontaneous currents reversed at a potential between 0 and +10 mV. (b) At a membrane potential of −40 mV, the STICs were abolished by the chloride channel blocker anthracene-9-carboxylic acid (A-9-C; 1 mM). Note that in this cell, A-9-C prevented activation of the transient current by CPA (10 μM), although the sustained current was still present. (c) CPA (10 μM), in addition to activating the two components of inward current, also abolished STIC activity in a cell held at −40 mV. Note that in this cell, a concomitant application of lanthanum (La^{3+}; 400 μM) had no effect on the sustained current activated by CPA. In (a), (b) and (c), zero current is indicated by the dotted line. In (b) and (c) drugs were applied where indicated by the solid bars.

cium from the SR activating calcium-dependent chloride channels in the plasma membrane (Wang *et al.*, 1992). Both caffeine and CPA (Figure 4c) abolished the STICS, presumably by depleting the SR of calcium.

As outlined above, the first and second components of the CPA-induced current could be separated both electrophysiologically and pharmacologically. We considered the possibility that the second current might underlie the calcium entry pathway activated as a result of store depletion. To investigate this further we looked at the pharmacology of the second current in greater detail. In particular we compared its pharmacology with that of the CPA-induced contraction produced in the whole muscle, which is dependent upon calcium entry *via* a non-voltage-dependent pathway (see introduction).

We have previously shown that CPA-induced contractions of the whole muscle, whilst being largely unaffected by nifedipine (1 μM), are inhibited by SKF 96365, (pIC_{50} approximately 6.4; Gibson *et al.*, 1994). In the present series of experiments, we extended these observations by testing the ability of several divalent and trivalent cations to inhibit the CPA-induced contractions. As shown in Figure 5, the contractile response to CPA (10 μM) was inhibited in a concentration-dependent manner by both cadmium (pIC_{50} 4.17±0.05; $n=6$) and nickel (pIC_{50} 4.01±0.11; $n=9$) but was unaffected by either lanthanum or gadolinium (50–400 μM; $n>5$ at each concentration).

The sustained inward current activated by CPA (10 μM) at a holding potential of −40 mV was inhibited by 10 μM SKF 96365 (90±7% inhibition; $n=19$; Figure 6a) and by 100 μM cadmium (92±4% inhibition; $n=18$; Figure 6c), whilst being unaffected by 400 μM lanthanum (9.1±3% inhibition; $n=10$; Figure 4c). In cells pretreated with either SKF 96365 (10 μM; Figure 6b) or cadmium (100 μM; Figure 6d), CPA was still able to activate the transient inward current.

The pharmacology of the sustained current is consistent with it underlying calcium entry activated in response to store depletion. The current however was still present in zero extracellular calcium (Figure 7), although the reversal potential shifted significantly more negative, to 18.2±5.7 mV ($n=10$), compared to the control value.

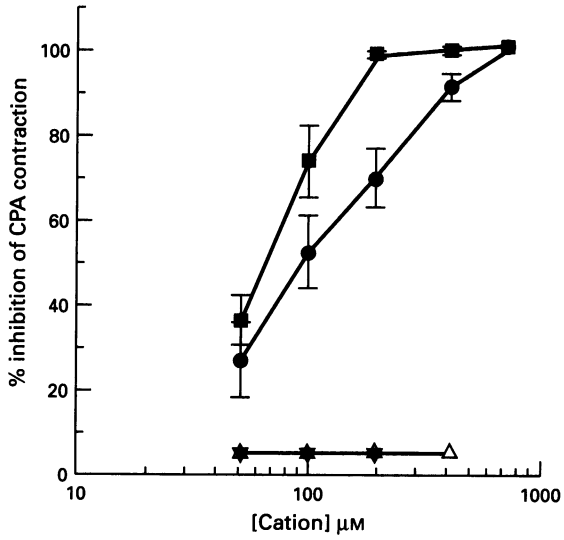

Figure 5 Concentration-response curves for the inhibition of the CPA (10 μM)-induced contraction of the mouse anococcygeus muscle by cadmium (Cd^{2+}; ■), nickel (N^{2+}; ●), lanthanum (La^{3+}; △) and gadolinium (Gd^{3+}; ▼). Each point represents the mean±s.e.mean from at least 6 different muscle preparations.

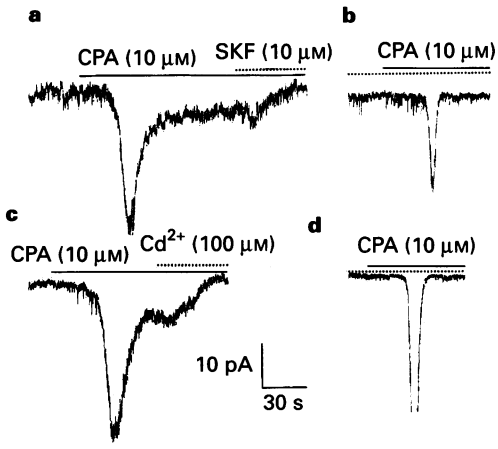

Figure 6 The sustained, but not the transient, components of the CPA-induced current were blocked by SKF 96365 and cadmium. In (a) and (c) the sustained currents activated by CPA (10 μM) were blocked by concomitant applications of SKF 96365 (10 μM) and cadmium (Cd^{2+}; 100 μM) respectively. In (b) and (d) CPA activated the transient component of inward current, but not the sustained component, in cells bathed in SKF 96365 (b) and cadmium (d) throughout. Note that in (b) and (c) the presence of the blockers is indicated by the dotted lines. In (d) the peak of the response to CPA was lost due to the high recorder gain.

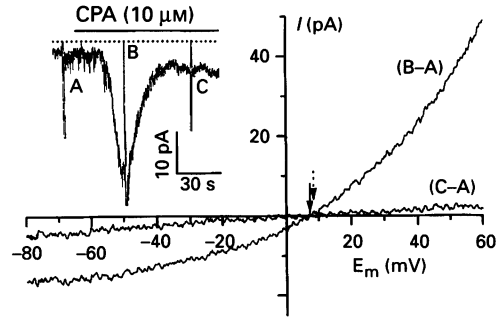

Figure 7 The reversal potential for the sustained current is shifted in zero extracellular calcium. Current-voltage relationships for the two components of CPA-induced current were constructed as outlined in the legend to Figure 1. Cells were bathed in physiological salt solution containing zero added calcium plus 1 mM EGTA. Under these conditions, CPA (10 μM) still evoked an inward current consisting of two components as shown in the inset. The reversal potential for the transient current (indicated by the broken arrow) was +8 mV whilst that for the sustained current (solid arrow) was +6 mV. In the inset, CPA was applied where indicated by the solid bar and the dotted line shows zero current.

Discussion

In the mouse anococcygeus muscle, CPA produces strong, well-sustained contractions that are dependent upon the entry of calcium from the extracellular space. This mycotoxin has been shown to inhibit calcium-ATPase in the SR of smooth muscle (Seidler *et al.*, 1989; Imaizumi *et al.*, 1992), and it is likely that this cellular action is responsible for the contractile response. Thus, inhibition of the calcium-ATPase empties the SR of calcium, which, in accordance with the capacatitive model is the trigger for calcium entry (Putney, 1986). In the present series of experiments we have studied the electrophysiological response to CPA in single cells isolated from the mouse anococcygeus, and have shown that the drug activates both a calcium-dependent chloride current, consistent with it releasing calcium from intracellular stores, and a smaller, sustained inward current with properties which suggest that it might underlie the calcium entry pathway activated in response to store depletion.

At a holding potential of −40 mV, CPA activated an inward current which consisted of two distinct components; an initial transient current followed by a smaller, sustained current. A number of pieces of evidence point to the initial current being a calcium-dependent chloride current activated as a result of CPA increasing the free intracellular calcium concentration. First, the current was absent in cells in which the intracellular free calcium concentration was buffered with EGTA or BAPTA. Second the current was blocked by the chloride channel blockers, DIDS and A-9-C, both of which have been shown to block calcium-dependent chloride currents in other smooth muscles (Baron *et al.*, 1991; Wang *et al.*, 1992). Third, the current-voltage relationship for the current showed marked outward rectification similar to that of the calcium-dependent chloride current first described in smooth muscle cells isolated from the rat anococcygeus (Byrne & Large, 1987). Finally, the reversal potential of the current corresponded closely with the chloride equilibrium potential under control conditions, and shifted accordingly when the extracellular chloride concentration was lowered. The reversal potential of the transient current was a few mV more positive than the chloride equilibrium potential. This can be explained if the initial current was contaminated by the second sustained current, which had a reversal potential of around +30 mV and which was unchanged in low extracellular chloride.

CPA did not activate the calcium-dependent chloride current in cells pretreated with caffeine. The most likely explanation for this is that CPA releases calcium from a caffeine-sensitive store. Like CPA, caffeine produced a transient activation of the calcium-dependent chloride current, although the response to caffeine was significantly larger than that to CPA. This may suggest that caffeine is able to deplete a larger store of calcium than CPA. Alternatively, the kinetics of the release produced by the two agents might differ. Caffeine, which acts to open the calcium release channels on the SR would be expected to produce a rapid depletion in much the same way as agonists which stimulate inositol trisphosphate (IP_3) production. CPA on the other hand, by inhibiting the calcium-ATPase, prevents the active uptake of calcium into the SR, and allows it to diffuse from the stores by way of background leak.

Caffeine, like CPA, activated a second sustained component of inward current. The current activated by CPA was present in cells containing EGTA or BAPTA, and was not blocked by DIDS or 9-A-C, demonstrating that it was not a calcium-dependent chloride current. It had a linear current-voltage relationship, and a reversal potential of around +30 mV which suggested that it was most likely due to activation of a non-selective cation conductance. The reversal potential of the current was shifted in the negative direction on the removal of extracellular calcium, suggesting that the underlying channels have significant permeability to calcium. Whilst this latter finding supports the idea that the non-selective cation conductance might underlie the calcium entry pathway activated by CPA, more conclusive evidence comes from a comparison of the pharmacology of the current with that of the CPA-induced contraction in the whole muscle. The contractions were inhibited by the cations cadmium and nickel, and by the calcium entry blocker SKF 96365. Lanthanum and gadolinium however had no effect on the contractile response to CPA. The non-selective cation current activated by CPA was blocked by cadmium and SKF 96365, each at concentrations similar to those which were effective in the organ bath, but not lanthanum. Taken together, these data suggest that the non-selective cation current activated by CPA in the single cells may underlie the entry pathway for calcium utilised during sustained contractions to CPA, and also to receptor antagonists such as carbachol and U46619, which have a similar dependence on extracellular calcium, are blocked by SKF 96365, and are largely unaffected by nifedipine (Gibson *et al.*, 1994).

Whilst calcium currents activated in response to store depletion have been recorded in several cell types (see below) no such current has been detected in smooth muscle cells. The first report of a store-regulated current arose following experiments on rat peritoneal mast cells (Hoth & Penner, 1992; 1993). This so-called calcium-release-activated calcium current (I_{CRAC}) was highly selective for calcium over other monovalent cations, showed marked inward rectification, and was blocked by a range of divalent cations, plus lanthanum. Each of these factors clearly distinguishes I_{CRAC} from the CPA-induced current in the mouse anococcygeus. I_{CRAC}-like currents, with properties similar to that in mast cells, have been characterized in a number of cell types (Fasalato *et al.*, 1994; Felder *et al.*, 1994). In addition, voltage-independent, non-selective cation currents activated in response to store depletion have been recorded in both bovine (Mendelowitz *et al.*, 1992) and human (Gericke *et al.*, 1993; Zhang *et al.*, 1994) endothelial cells, and more recently, in rat megakaryocytes (Somasundaram & Mahaut-Smith, 1994). Whilst there is relatively little information on the pharmacology of these cation currents, the current recorded from bovine aortic endothelial cells was blocked by lanthanum suggesting that in this respect at least, it differs from that in the mouse anococcygeus cells.

In a number of smooth muscles, agonists acting at G-protein coupled receptors activate a non-selective cation current. The best studied example of such a current is that activated by muscarinic receptor agonists in guinea-pig intestinal smooth muscle cells (Inoue & Isenberg, 1990a, b; Inoue, 1991; Pacaud & Bolton, 1991; Komori *et al.*, 1992). The current shows some calcium-dependence, being augmented by a rise in free intracellular calcium (Inoue & Isenberg, 1990b; Pacaud & Bolton, 1991), and we considered the possibility that a rise in the intracellular calcium concentration in response to CPA might be activating such a current in anococcygeus cells. However, several pieces of evidence argue against this hypothesis. First, the CPA-induced cation current is present in cells containing EGTA and BAPTA at concentrations sufficient to prevent activation of the calcium-dependent chloride current. Whilst it is possible that EGTA in particular is not fast enough to buffer the initial rise in calcium, the calcium concentration should eventually equilibrate at a low level, and under these conditions the response to CPA would not be sustained. These findings also argue against the CPA-induced current resulting from the activation of calcium-dependent cation channels which have been described in a number of cell types (Partridge & Swandulla, 1988) although not anococcygeus smooth muscle cells. Second, the receptor-operated current shows marked inward rectification, with relatively little outward current being recorded at positive membrane potentials (see for example Figure 2b of Inoue & Isenberg, 1990a); the CPA-dependent current had a near linear *I-V* relationship. Thirdly, the receptor-operated current appears to be dependent upon the presence of a receptor agonist, absent in our experiments. Agents which cause a rise in intracellular calcium in a receptor-independent manner, in particular caffeine, have been reported not to activate the current despite being able to reduce the response to muscarinic receptor activation, presumably by depleting the SR (Inoue & Isenberg, 1990b; Pacaud & Bolton, 1991). A cation conductance activated by noradrenaline has been reported in rabbit portal vein cells (Wang & Large, 1991) but again this current was not activated upon store depletion by caffeine.

In conclusion, both caffeine and CPA activated a calcium-dependent chloride current in mouse anococcygeus cells as a result of releasing calcium from intracellular calcium stores. In addition, both agents activated a non-selective cation current, the pharmacology of which mirrors that of the CPA-induced contraction of the tissue. We propose that the calcium-dependent chloride current results in depolarization and opening of voltage-operated calcium channels which mediate the nifedipine-sensitive component of the contractile response to CPA and receptor-agonists in this tissue (Gibson *et al.*, 1994). The non-selective cation current observed in this tissue might underlie the nifedipine-resistant calcium entry process utilised during sustained contraction produced by these agents as a result of emptying the SR.

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