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Cellular mechanisms underlying carbachol-induced oscillations of calcium-dependent membrane current in smooth muscle cells from mouse anococcygeus

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1 At a holding potential of −40 mV, carbachol (50 μM) produced a complex pattern of inward currents in single smooth muscle cells freshly isolated from the mouse anococcygeus. Membrane currents were monitored by the whole-cell configuration of the patch-clamp technique. Previous work has identified the first, transient component as a calcium-activated chloride current ($I_{Cl(Ca)}$) and the second sustained component as a store depletion-operated non-selective cation current (I_{DOC}). The object of the present study was to examine the cellular mechanisms underlying the third component, a series of inward current oscillations (I_{oscil}) superimposed on I_{DOC}.

2 Carbachol-induced I_{oscil} (amplitude 97±11 pA; frequency 0.26±0.02 Hz) was inhibited by the chloride channel blocker anthracene-9-carboxylic acid (A-9-C; 1 mM), and by inclusion of 1 mM EGTA in the patch-pipette filling solution.

3 In calcium-free extracellular medium (plus 1 mM EGTA), carbachol produced an initial burst of oscillatory current which lasted 94 s before decaying to zero; I_{oscil} could be restored by re-admission of calcium. The frequency, but not the amplitude, of I_{oscil} increased with increasing concentrations of extracellular calcium (0.5–10 mM).

4 Inclusion of the inositol triphosphate (IP_3) receptor antagonist heparin (5 mg ml^{-1}) in the patch-pipette filling solution, or pretreatment of cells with the sarcoplasmic reticulum (SR) calcium ATPase inhibitor cyclopiazonic acid (CPA; 10 μM), prevented the activation of I_{oscil} by carbachol. Caffeine (10 mM) activated both $I_{Cl(Ca)}$ and I_{DOC} and prevented the induction of I_{oscil} by carbachol. Caffeine and CPA also abolished I_{oscil} in the presence of carbachol, as did both a low (3 μM) and a high (30 μM) concentration of ryanodine.

5 Carbachol-induced I_{oscil} was abolished by the general calcium entry blocker SKF 96365 (10 μM) and by Cd^{2+} (100 μM), but was unaffected by La^{3+} (400 μM). As found previously, I_{DOC} was also blocked by SKF 96365 and Cd^{2+}, but not La^{3+}; the inhibition of I_{DOC} preceded the abolition of I_{oscil} by 27 s with SKF 96365 and by 30 s with Cd^{2+}. Nifedipine (1 μM) produced a partial inhibition of the carbachol-induced I_{oscil} frequency at holding potentials of −20 mV and −60 mV and, in addition, reduced I_{DOC} at −60 mV by 18%.

6 It is concluded that carbachol-induced inward current oscillations in mouse anococcygeus cells are due to a calcium-activated chloride current, and reflect oscillatory changes in cytoplasmic calcium ion concentration. These calcium oscillations are derived primarily from the SR stores, but entry of calcium into the cell is necessary for store replenishment and maintenance of the oscillations. Capacitative calcium entry (*via* I_{DOC}) appears to be important not only for sustained contraction of this tissue, but also as a route for re-filling of the SR and, therefore, represents an important target for the development of novel and selective drugs.

Keywords: Anococcygeus (mouse); calcium-activated chloride current; calcium oscillations; calcium stores; capacitative calcium entry; carbachol; cyclopiazonic acid; depletion-operated current; ryanodine; smooth muscle

Introduction

Calcium store depletion activates a biphasic inward current in mouse anococcygeus smooth muscle cells (Wayman *et al*., 1996a). An initial transient current results from the release of stored calcium from the sarcoplasmic reticulum (SR) activating a calcium-dependent chloride current ($I_{Cl(Ca)}$). As this first current decays, a smaller, sustained, non-selective cation current becomes apparent, which is directly activated by calcium store depletion. This depletion-operated current (I_{DOC}) is thought to underlie capacitative calcium entry in the anococcygeus and is the calcium entry pathway required for sustained contractions of the whole muscle (Gibson *et al*., 1994; Wayman *et al*., 1996a). These two currents are observed with agents which deplete SR calcium stores in either a receptor-dependent, or a receptor-independent, manner. However, when the muscarinic receptor agonist carbachol (50 μM) is applied a more complex response is obtained; superimposed on the sustained I_{DOC} are a series of regular inward current oscillations (I_{oscil}; Wayman *et al*., 1996b). Similar current oscillations have been described in a number of vascular (Desilets *et al*., 1989) and non-vascular (Pacaud & Bolton, 1991; Komori *et al*., 1993; 1996; Zholos *et al*., 1994; Liu & Farley, 1996a, b, c) smooth muscles cells and are thought to reflect directly oscillations in the intracellular concentration of free calcium ions. By use of fluorescence techniques, cytosolic calcium oscillations have been described in a variety of cell types, both excitable and non-excitable (Berridge & Galione, 1988; Berridge & Irvine, 1989; Rink & Jacob, 1989; Berridge, 1990). Preliminary investigations suggest that the carbachol-induced I_{oscil} in mouse anococcygeus cells might result from a calcium-activated chloride current (Wayman *et al*., 1996b). In other smooth muscles, the current oscillations are initiated by the release of calcium ions from the SR, while their maintenance is

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dependent on entry of extracellular calcium to re-fill the depleted stores. Entry through L-type voltage operated calcium channels (VOCCs) contributes partially to the maintenance of oscillations, but it is clear that another, non-VOCC, pathway is important (Komori *et al*., 1993; 1996; Khoda *et al*., 1996; Liu & Farley, 1996a). However, the precise nature of this entry mechanism has remained obscure.

The aim of the present study was to use the whole-cell configuration of the patch-clamp technique to investigate in more detail the cellular mechanisms underlying carbachol-induced I_{oscil} in mouse anococcygeus cells, with particular emphasis on the respective roles of extracellular and intracellular calcium ions in the initiation and maintenance of the oscillations. In addition, the possibility that the novel, depletion-operated cation current, I_{DOC}, may be the non-VOCC calcium entry pathway necessary for maintenance of current oscillations in this tissue was investigated. Some of the results have been published in abstract form (Wayman *et al*., 1996c).

Methods

The enzymatic dissociation protocol for the preparation of single smooth muscle cells isolated from the mouse anococcygeus is described by Wayman *et al*. (1996a). Electrophysiological recordings of muscle cell membrane currents were obtained by use of the whole-cell configuration of the patch-clamp technique. The extracellular physiological salt solution (PSS) bathing the cells contained (in mM): NaCl 120, KCl 6, $MgCl_2$, 1.2, glucose 11, HEPES 10, $CaCl_2$ 10, pH 7.20 (with NaOH). In experiments where the extracellular calcium concentration was reduced to zero, $CaCl_2$ was omitted and the calcium buffer ethylene glycol-*bis* (β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA, 1 mM) was added. Dihydropyridine-sensitive (L-type) voltage-dependent calcium channels (VOCCs) were blocked by the addition of nifedipine (1 μM) to the extracellular solution immediately before the start of an experiment, except in experiments where the influence of VOCCs was being investigated. The patch-pipette filling solution contained (mM; all from Sigma): CsCl 130, tetraethylammonium chloride (TEA) 20, HEPES 10, ATP (Na salt) 0.5, GTP (Na salt) 0.5, pH 7.20 (with CsOH), dissolved in 'Milli-Q' ultrapure water. When filled with this solution, patch-pipettes had d.c. resistances of around 6 MΩ. In a small number of experiments, nystatin (0.2 mg ml^{-1}) was included in the patch-pipette solution and allowed to 'perforate' the patch of membrane underlying the electrode (series resistance less than 50 MΩ), rather than the conventional whole-cell recording configuration being employed.

Membrane currents were recorded and filtered at 1 kHz (−3 dB) by an Axopatch 200A amplifier (Axon Instruments Inc., Burlingame, U.S.A.) and digitized (>3kHz) with a Digidata 1200 interface (Axon Instruments Inc.) in combination with a personal computer (Mesh 486DX) running pClamp software (Axon Instruments Inc.). Acquired data were stored directly on to the hard disk of the computer. Additionally, two continuous records of membrane current were obtained, one filtered at 200 Hz and played onto a thermal chart recorder (Gould TA550) and the other filtered at 1 kHz and recorded by a digital tape recorder (DTR-1200; Biologic, France). 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 continuously perfused with either drug-free or drug-containing solutions, and switching between drug solutions was achieved by means of solenoid valves. The delay between changing reservoirs and ejection of the drug was approximately 5 s.

The amplitude of I_{DOC} was measured between 60 and 90 s after the application of carbachol, when $I_{Cl(Ca)}$ had decayed and between the superimposed oscillations. Measurements of oscillation amplitude and frequency were obtained by taking the mean of at least 5 successive oscillations. Results are expressed as mean ± s.e.mean. Statistical analysis was carried out by use of Student's *t* test (paired or unpaired where appropriate); $P<0.05$ was taken as significant.

The following drugs were used: anthracene-9-carboxylic acid (A-9-C; Sigma); cadmium chloride (Sigma); caffeine (Sigma); carbachol (Aldrich); cyclopiazonic acid (CPA; Sigma); ethylene glycol-*bis* (β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA; Sigma); heparin (Sigma); lanthanum chloride (Sigma); nifedipine (Sigma); nystatin (Sigma); ryanodine (High purity; Calbiochem) and SKF 96365 (1-{β-[3-(4-methoxyphenyl) propoxyl]-4-methoxyphenethyl}-1H-imadazole HCl; Affiniti Research Products Ltd.). All salts used were of reagent grade or better. Drugs were prepared as stock solutions in de-ionised water with the exception of CPA (10 mM stock in dimethylsulphoxide), nifedipine (10 mM stock in ethanol), nystatin (50 mg ml^{-1} stock in dimethylsulphoxide) and A-9-C (100 mM stock in ethanol).

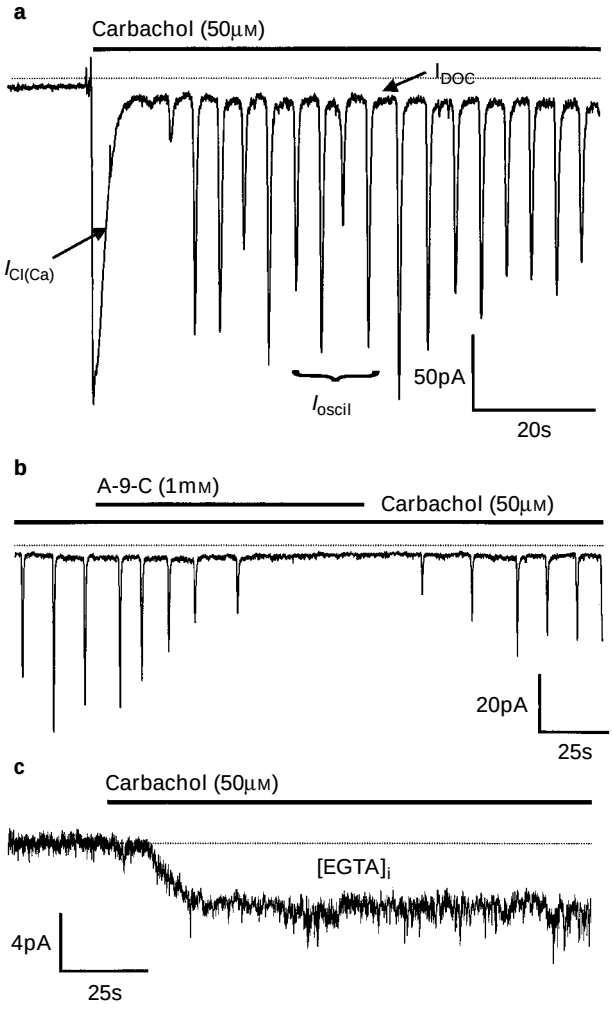

Figure 1 The carbachol-induced oscillations in membrane current result from activation of a calcium-dependent chloride current in mouse anococcygeus cells. (a) The whole-cell current response evoked by application of carbachol (50 μM) consisted of three distinguishable components of inward current; an initial transient current due to activation of a calcium-dependent chloride current ($I_{Cl(Ca)}$), a sustained, depletion-operated cation current (I_{DOC}) and regular inward current oscillations (I_{oscil}). (b) The carbachol (50 μM)-induced oscillations were abolished by application of the chloride channel blocker anthracene-9-carboxylic acid (A-9-C; 1 mM). (c) Inclusion of the calcium buffer EGTA (1 mM) in the patch-pipette filling solution prevented activation of either $I_{Cl(Ca)}$ or I_{oscil} by carbachol (50 μM), although the agonist was able to activate I_{DOC}. Cells were held at a membrane potential of −40 mV and zero current is indicated by the dotted lines. Drugs were applied for the duration of the solid bars.

Results

Characterization of the carbachol-induced inward current oscillations

Under our normal experimental conditions (10 mM extracellular calcium and 1 μM nifedipine), carbachol (50 μM) activated a biphasic inward current when applied to mouse anococcygeus muscle cells held at a membrane potential of -40 mV ($n=87$). Additionally, 79 out of the 87 cells displayed carbachol-induced inward current oscillations (I_{oscil}). The biphasic current consisted of the transient calcium-activated chloride current ($I_{Cl(Ca)}$; amplitude 148 ± 18 pA, $n=42$) followed by the sustained, non-selective cation current (I_{DOC}; amplitude 5.3 ± 0.3 pA, $n=58$). A series of inward current oscillations (I_{oscil}), superimposed on I_{DOC}, had an amplitude of 97 ± 11 pA, $n=58$ and a frequency of 0.26 ± 0.02 Hz, $n=58$ (Figure 1a).

We have previously shown that the carbachol-induced I_{oscil} may result from the activation of a calcium-dependent chloride conductance (Wayman *et al*., 1996b). Pretreatment of the cells with the chloride channel blocker A-9-C inhibited the carbachol-induced I_{oscil} in all cells tested (1 mM, 9 cells), as did application of A-9-C to cells in which carbachol had already activated I_{oscil} (4 cells; Figure 1b). Inclusion of the calcium buffer EGTA (1 mM) in the intracellular pipette solution (Figure 1c) abolished the carbachol-induced I_{oscil} in 5 out of 5 cells, although a clear I_{DOC} was still obtained. These results, in combination with preliminary data showing a reversal potential close to zero (Wayman *et al*., 1996b), confirm that the carbachol-induced I_{oscil} results from activation of a calcium-activated chloride current.

Extracellular calcium in the initiation and/or the maintenance of I_{oscil}

To determine whether extracellular calcium was required for the initiation or maintenance of I_{oscil}, carbachol was applied when the extracellular calcium concentration ($[Ca^{2+}]_o$) had been reduced to zero (plus 1 mM EGTA). Under these conditions, application of carbachol produced an initial burst of oscillatory current, lasting 94 ± 16 s, which then decayed (Figure 2b). When calcium was added back ($[Ca^{2+}]_o=10$ mM) there was partial recovery of I_{oscil}. This result suggested that extracellular calcium was necessary for the maintenance, rather than the initiation of I_{oscil}. This was supported by experiments in which $[Ca^{2+}]_o$ was reduced to zero (plus 1 mM EGTA) once carbachol-induced I_{oscil} had been established in medium containing 10 mM $[Ca^{2+}]_o$; again, after a lag period (93 ± 17 s), I_{oscil} decayed and could be re-activated by restoration of $[Ca^{2+}]_o$ (Figure 2c). In a further series of experiments (Figure 2a), the calcium concentration-dependence of I_{oscil} was investigated (PSS containing 0.5 mM, 2.5 mM or 10 mM Ca^{2+}). Increasing or decreasing $[Ca^{2+}]_o$ resulted in significant changes in the mean frequency of the carbachol-induced I_{oscil}; these frequency effects were not dependent on the order in which $[Ca^{2+}]_o$ changes were made (going from 0.5, to 2.5, to 10 mM; 0.06 ± 0.02, 0.11 ± 0.01, 0.19 ± 0.03 Hz, respectively, $n=4$: going from 10, to 2.5, to 0.5 mM; 0.19 ± 0.02, 0.13 ± 0.01, 0.06 ± 0.01 Hz, respectively, $n=6$). There were no significant changes in the amplitude of carbachol-induced I_{oscil} or I_{DOC} (data not given).

Carbachol-induced oscillations were also present in recordings obtained by use of the nystatin 'perforated-patch' configuration and an extracellular calcium concentration of

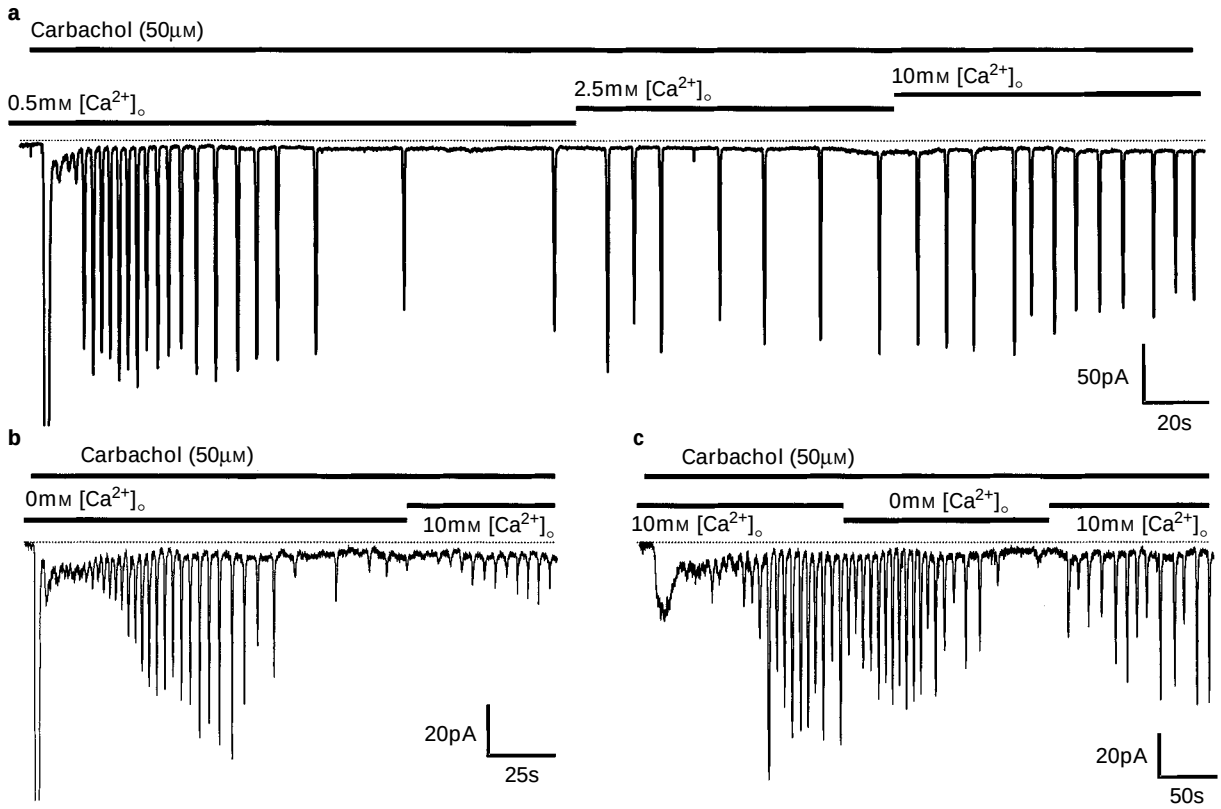

Figure 2 Maintenance of the oscillations required extracellular calcium. (a) Increasing the extracellular calcium concentration increased the frequency of the oscillations evoked by carbachol (50 μM). Note the initial 'burst' of oscillations seen in the presence of 0.5 mM calcium. (b) Application of carbachol (50 μM) to a cell bathed in an extracellular physiological salt solution (PSS) containing zero $[Ca^{2+}]_o$ plus EGTA (1 mM) initiated a transient burst of oscillatory activity. The oscillations were partly reactivated on addition of calcium (10 mM) to the PSS. (c) The oscillations evoked by carbachol (50 μM) were reversibly abolished on the removal of extracellular calcium. Cells were held at a membrane potential of -40 mV, and zero current is indicated by the dotted lines. Drugs were applied as indicated by the solid bars.

2.5 mM (frequency 0.15 ± 0.02 Hz; amplitude 44 ± 14 pA; $n = 8$).

The role of SR calcium stores in the generation of I_{oscil}

The observation of a transient burst of I_{oscil} under calcium-free conditions (see earlier) suggested the involvement of intracellular calcium stores in the initiation of the current oscillations. Experiments were carried out to investigate the nature of the stores involved.

Inclusion of the IP_3 receptor antagonist heparin (5 mg ml^{-1}) in the intracellular pipette solution (Figure 3a) abolished the carbachol-induced biphasic current, and carbachol-induced I_{oscil}, in all cells tested at -40 mV ($n = 5$). To determine the viability of the cells, caffeine (10 mM) was then applied and this activated the biphasic inward current, but not I_{oscil}, in all cells ($n = 5$).

Cyclopiazonic acid (CPA) inhibits calcium uptake into intracellular calcium stores by inhibition of the sarcoendoplasmic reticulum calcium ATPase (SERCA), and this ultimately leads to calcium store depletion (Seidler *et al*., 1989) with consequent activation of I_{DOC} (Wayman *et al*., 1996a). The carbachol (50 μM)-induced I_{oscil} were absent in all cells pretreated with CPA (10 μM; $n = 4$); CPA-induced I_{DOC} was unaffected (Figure 3b). Concomitant application of CPA (10 μM), after carbachol (50 μM) had induced the oscillations (Figure 3c), led to a slow inhibition of the I_{oscil} (48 ± 4 s; $n = 4$); again the carbachol-induced I_{DOC} was unaffected. The inhibitory effect of CPA on I_{oscil} was not reversible, at least up to 5 min after washout; longer periods of washout were not recorded with CPA.

Application of the ryanodine receptor agonist caffeine to mouse anococcygeus muscle cells held at -40 mV activates the biphasic inward current (Wayman *et al*., 1996b). Concomitant addition of caffeine (10 mM), after carbachol (50 μM) had induced the oscillations (Figure 4a), led to a rapid (8 ± 1 s; $n = 5$) and complete inhibition of I_{oscil}, which was readily reversible on wash out of caffeine. Pretreatment of mouse anococcygeus muscle cells with caffeine (10 mM) abolished the carbachol-induced biphasic current and the carbachol-induced I_{oscil} in all cells tested (Figure 4b; $n = 4$).

Ryanodine itself has been shown to have differential effects on the release of calcium from ryanodine-sensitive calcium stores depending on the concentration at which it is applied. At low concentrations, ryanodine acts as an agonist resulting in the release of calcium, whereas at concentrations > 10 μM ryanodine acts as an antagonist, inhibiting calcium release (Ehrlich *et al*., 1994). In a further series of experiments, we investigated the effects of ryanodine at 3 μM and 30 μM on the carbachol-induced I_{oscil}. Concomitant application of ryanodine (3 μM), after carbachol (50 μM) had induced the oscillations (Figure 4c), resulted in a slow inhibition of I_{oscil} (71 ± 11 s; $n = 4$). During this inhibition, there was no effect on carbachol-induced I_{DOC}. Concomitant application of ryanodine (30 μM; Figure 4d), produced a more rapid abolition of I_{oscil} (9 ± 4 s; $n = 4$) and, again, carbachol-induced I_{DOC} was unaffected by ryanodine at this concentration.

The role of L-type VOCCs in the maintenance of I_{oscil}

The results obtained under calcium-free conditions (see earlier) indicated that entry of extracellular calcium was necessary for the maintenance of I_{oscil}. Two main pathways of calcium entry have been described in the anococcygeus, L-type VOCCs (Gibson *et al*., 1994; England & McFadzean, 1995) and I_{DOC} (Wayman *et al*., 1996a).

In order to investigate the role of L-type VOCCs in maintaining I_{oscil} we studied the actions of nifedipine (1 μM) on the response to carbachol. In cells held at -20 mV nifedipine clearly had inhibitory effects on the frequency of I_{oscil} (Figure 5). This inhibitory action was also apparent at a holding potential of -60 mV (Figure 5). Further, it was interesting to note that nifedipine produced a small inhibitory effect on I_{DOC} ($18 \pm 5\%$ inhibition; $n = 6$) at -60 mV. These results suggested that, as in other smooth muscles, some of the calcium necessary for maintenance of current oscillations enters through L-type VOCCs, but that some other non-VOCC pathway is also involved.

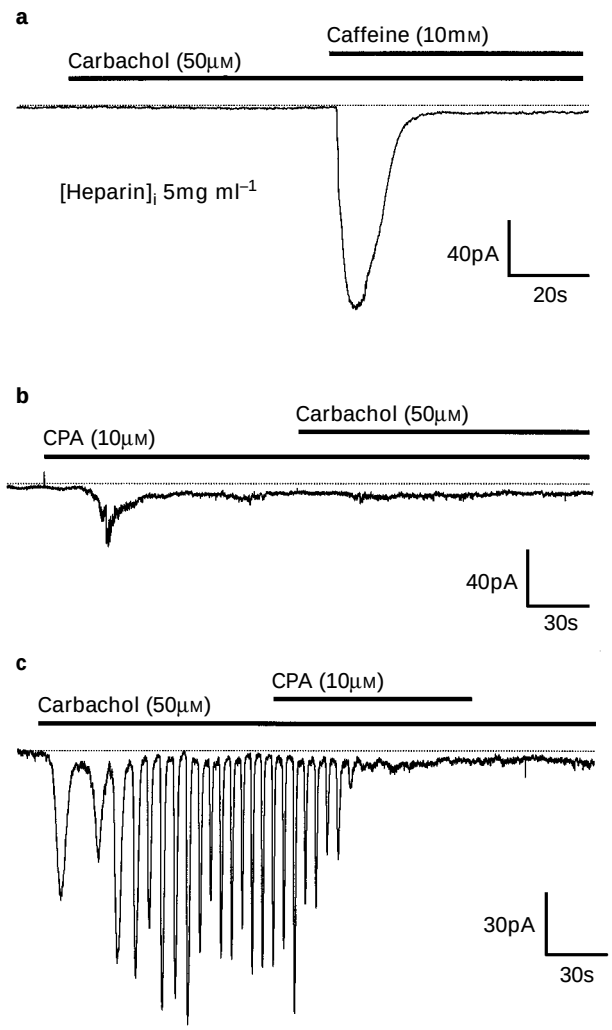

Figure 3 Carbachol-induced oscillations require a functional IP_3-sensitive calcium store for their initiation and maintenance. (a) Inclusion of the IP_3 receptor antagonist heparin (5 mg ml^{-1}) in the patch-pipette filling solution abolished all three components of the carbachol response. However, caffeine was still able to activate $I_{Cl(Ca)}$ and I_{DOC}. (b) Pretreatment of cells with the sarcoplasmic reticulum ATPase inhibitor cyclopiazonic acid (CPA; 10 μM) activated a small $I_{Cl(Ca)}$ plus I_{DOC} and abolished all three components of the carbachol response. In (c) concomitant application of CPA (10 μM) led to a slow inhibition of the carbachol-induced oscillations without effecting I_{DOC}. Cells were held at a membrane potential of -40 mV, and zero current is indicated by the dotted lines. Drugs were applied for the duration of the solid bars.

The role of I_{DOC} *in the maintenance of* I_{oscil}

I_{DOC} is a depletion-operated, non-selective cation current, found in mouse anococcygeus cells, which is believed to be an important route of calcium entry during sustained contraction of the whole muscle; the current has a distinctive pharmacology, being inhibited by the general calcium entry blocker SKF 96365 and cadmium ions (Cd^{2+}) but resistant to lanthanum ions (La^{3+}; Wayman *et al*., 1996a). Importantly, neither SKF 96365 nor Cd^{2+} had any effect on $I_{Cl(Ca)}$ (Wayman *et al*., 1996a). These compounds were used to investigate the invol-

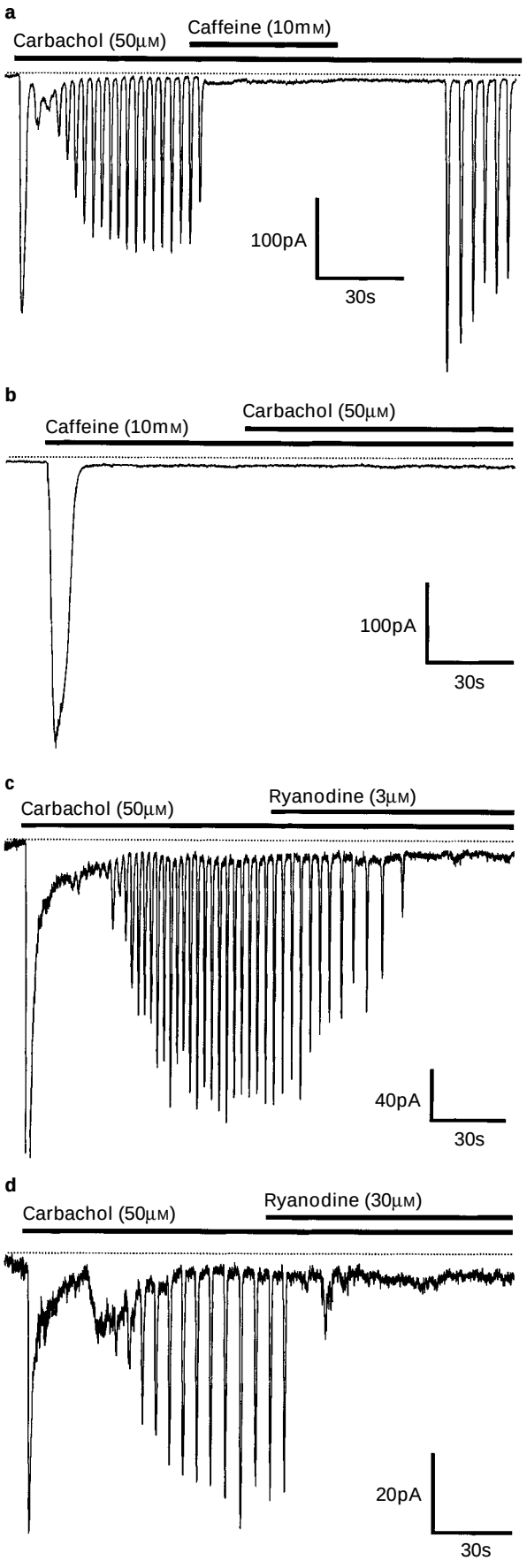

Figure 4 Carbachol-induced oscillations require a functional ryanodine (caffeine)-sensitive calcium store. In (a) the carbachol (50 μM)-induced oscillations were abolished by a concomitant application of caffeine (10 mM). The effect of caffeine was reversed on washing with caffeine-free solution. (b) Pretreatment of cells with caffeine (10 mM) activated $I_{Cl(Ca)}$ and I_{DOC} and abolished the response to carbachol (50 μM). In (c) the application of a low concentration of ryanodine (3 μM) led to a slow inhibition of the carbachol (50 μM)-induced oscillations. This can be compared to the response shown in (d) where a high concentration of ryanodine (30 μM) produced a rapid abolition of the oscillations. Cells were held at a membrane potential of −40 mV, and zero current is indicated by the dotted lines. Drugs were applied for the duration of the solid bars.

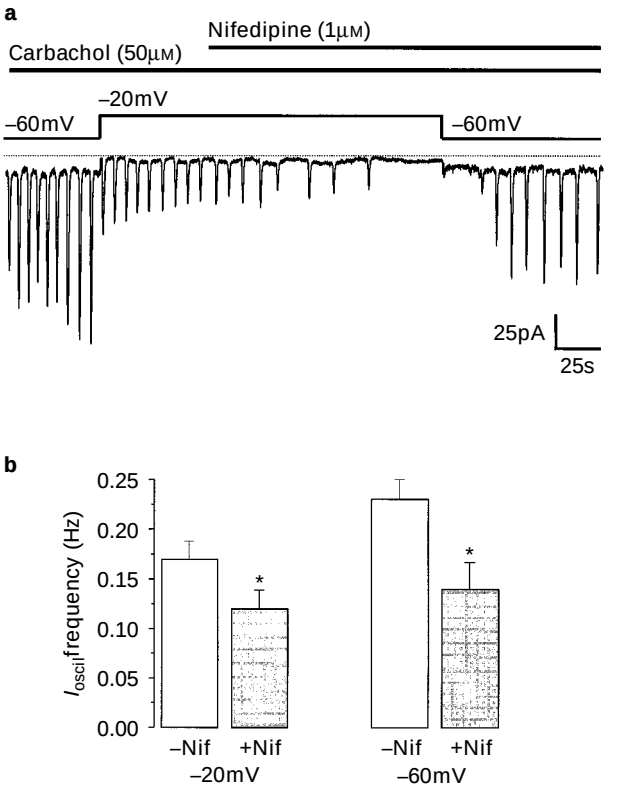

Figure 5 Nifedipine (Nif) reduced the frequency of the carbachol-induced oscillations recorded at both −20 and −60 mV. (a) The effect of nifedipine (1 μM) on carbachol (50 μM)-induced oscillations recorded at holding potentials of −60 mV and −20 mV. (b) Combined data obtained from 6 cells during experiments similar to those in (a). Vertical lines represent s.e.mean; *indicates significant difference ($P<0.05$; paired Student's t test).

vement of calcium ions entering through I_{DOC} in the maintenance of I_{oscil} (Figure 6).

Carbachol (50 μM)-induced I_{oscil} and I_{DOC} were both completely abolished by SKF 96365 (10 μM; $n=5$; Figure 6a) and Cd^{2+} (100 μM; $n=5$; Figure 6b). Interestingly, in both cases, it took significantly longer to inhibit the carbachol-induced I_{oscil} than I_{DOC}. SKF 96365 took 24 ± 2 s to inhibit I_{DOC} compared with 51 ± 18 s to inhibit I_{oscil}, and Cd^{2+} took 20 ± 2 s to inhibit I_{DOC} compared with 50 ± 7 s to inhibit I_{oscil}. La^{3+} (400 μM; (Figure 6c) had no significant effects on either the carbachol-induced I_{oscil} or I_{DOC}.

Discussion

The muscarinic receptor agonist carbachol produced a complex pattern of inward currents in mouse anococcygeus smooth muscle cells; an initial transient current was followed by a smaller sustained current and, superimposed on the latter, were a series of inward current oscillations. Previous work (Wayman *et al.*, 1996a, b) has identified the initial transient current as a calcium-activated chloride conductance ($I_{Cl(Ca)}$) which results from the release of stored calcium ions from the SR and

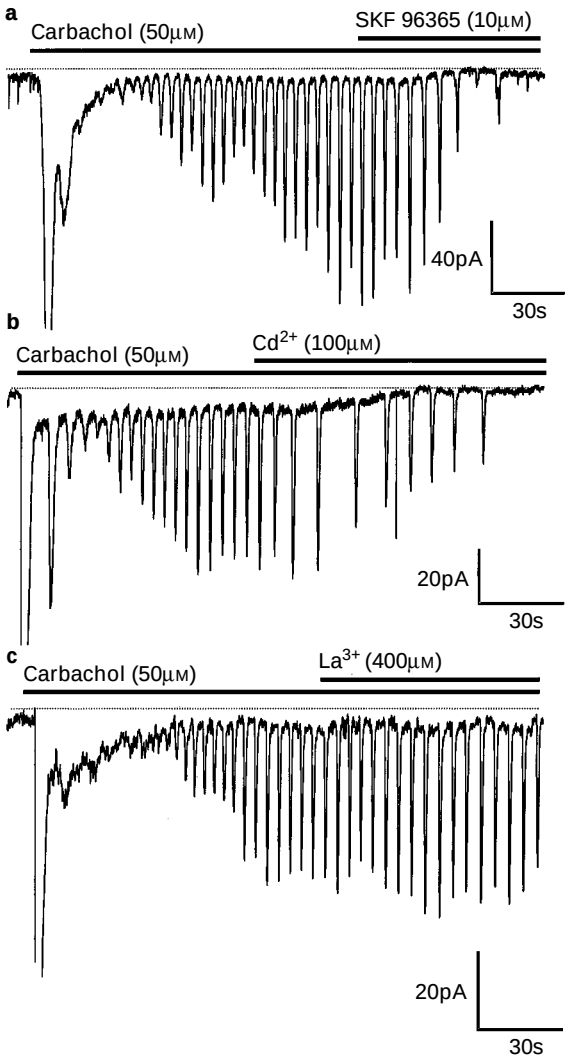

Figure 6 Capacitative calcium entry via the store depletion-operated current (I_{DOC}) is essential for the maintenance of the carbachol-induced oscillations. (a) and (b) The inhibitory effects of SKF 96365 (10 μM) and cadmium (Cd^{2+}; 100 μM), respectively, on both I_{DOC} and the carbachol (50 μM)-induced oscillations. (c) Lanthanum (La^{3+}; 400 μM) had no effect on either I_{DOC} or I_{oscil} activated by carbachol (50 μM). Cells were held at a membrane potential of -40 mV and zero current is indicated by the dotted lines. Drugs were applied for the duration of the solid bars.

the second sustained current as a non-selective cation conductance directly activated by the depletion of the calcium stores in the SR (I_{DOC}). The object of the present study was to investigate the cellular mechanisms underlying the oscillatory inward current (I_{oscil}).

Muscarinic receptor-induced inward current oscillations have been found in other non-vascular smooth muscle cells (Komori *et al*., 1993; 1996; Zholos *et al*., 1994; Liu & Farley, 1996a, b, c). In general, it is believed that the currents measured reflect oscillations in cytoplasmic calcium ion concentration which regulate various membrane ion channels; in intestinal smooth muscle cells the main current regulated is a G-protein-linked non-selective cation current (I_{cat}), while in tracheal myocytes a calcium-activated chloride channel is involved. Two pieces of evidence suggest that carbachol-induced I_{oscil} in mouse anococcygeus cells may be similar to the oscillations observed in the trachea and involve a calcium-activated chloride conductance. Firstly, I_{oscil} was inhibited by the chloride channel blocker A-9-C and, secondly, I_{oscil} was absent in cells in which the calcium buffer EGTA had been included in the intracellular pipette solution. It is possible that the current oscillations might reflect an oscillatory sensitivity of the chloride channel to cytoplasmic calcium. However, this seems unlikely since I_{oscil} was not observed with either CPA or caffeine, which do activate both $I_{Cl(Ca)}$ and I_{DOC} (Wayman *et al*., 1996b). In addition, these observations suggest that I_{oscil} does not result from calcium overload of the cell. Certainly, the oscillation frequency was greatest in experiments with an extracellular calcium concentration of 10 mM and the intracellular patch-pipette solution did not contain a calcium buffer. However, overloading would only occur if unusually large amounts of calcium were entering the cell, or the endogenous intracellular calcium buffering system was disrupted. Under resting conditions, in the absence of activator drugs, the level of calcium in the cell was not sufficient to activate $I_{Cl(Ca)}$. During activation of the cell, in the presence of 1 μM nifedipine, the main calcium entry pathway is I_{DOC}; the small whole cell current and its non-selective nature, would suggest that calcium overload is unlikely to occur via this mechanism. Further, I_{oscil} is not observed with either caffeine or CPA, even though the former can reproducibly activate $I_{Cl(Ca)}$ and I_{DOC} (Wayman *et al*., 1996b). Finally, at an extracellular calcium concentration of 2.5 mM, carbachol-induced oscillations were observed in cells from which recordings were made with the 'perforated-patch' technique, which would be expected to produce minimal disruption of intracellular calcium buffering mechanisms. Thus, it is most likely that the current oscillations directly reflect oscillations in cytoplasmic calcium concentration as a result of carbachol-induced activation of the IP_3 receptor system (see below), and are not due simply to calcium overload.

In calcium-free medium, carbachol produced $I_{Cl(Ca)}$, I_{DOC} and an initial burst of I_{oscil} which soon decayed (after 94 s). This observation suggests that the cytosolic calcium oscillations which regulate the chloride conductance underlying I_{oscil} are primarily derived from intracellular stores, but that the maintenance of I_{oscil} requires entry of calcium ions from the extracellular space. This would be in agreement with the calcium-dependence of inward current oscillations demonstrated in other smooth muscles (Komori *et al*., 1993; Liu & Farley, 1996a). We therefore carried out a series of experiments to identify the intracellular stores and the calcium ion entry pathways, responsible for I_{oscil} initiation and maintenance, respectively.

Agonist-induced oscillations in cytoplasmic calcium ion concentrations have been identified in many cell types and are commonly initiated by the activation of the IP_3 receptor on the SR (Berridge & Galione 1988; Berridge & Irvine, 1989; Rink & Jacob, 1990; Berridge, 1990; 1993). In accord with this, in mouse anococcygeus cells, the IP_3 receptor antagonist heparin blocked carbachol-induced I_{oscil}. The involvement of the SR in the generation of I_{oscil} was confirmed by the experiments with CPA, which depletes the SR of calcium by inhibiting the SERCA pump (Seidler *et al*., 1989); pretreatment of cells with CPA prevented the appearance of I_{oscil} on application of carbachol, while addition of CPA once carbachol-induced I_{oscil} had been established led to the slow disappearance of the oscillations. The above results suggested that calcium ions from the IP_3 sensitive store in the SR are necessary for the initiation of I_{oscil} and replenishment of the stores, via the SERCA pump, is required for its maintenance. Previous work has shown that caffeine (10 mM) elicits both $I_{Cl(Ca)}$ and I_{DOC} in mouse anococcygeus cells (Wayman *et al*., 1996a, b), suggesting the presence of a caffeine/ryanodine-releasable calcium pool within the SR of this tissue; the involvement of this pool in the production of carbachol-induced I_{oscil} was investigated by use of both caffeine and ryanodine. Application of caffeine, by itself, produced both $I_{Cl(Ca)}$ and I_{DOC}, but not I_{oscil}; further, in the presence of caffeine, carbachol failed to induce I_{oscil}. Similarly, application of a low concentration of ryanodine (3 μM), which depletes the ryanodine-sensitive store by locking the release channels in a sub-conductance state (Ehrlich *et al*., 1994), led to a slow inhibition of I_{oscil}, while application of a high concentration of ryanodine (30 μM), which acts as an antagonist

on the ryanodine receptors (Ehrlich *et al*., 1994) caused a much quicker inhibition. Taken together, these results suggest that the ryanodine-sensitive calcium store, which is associated with the process of calcium-induced calcium release (CICR), is necessary for the activation of I_{oscil}; similar inhibitory effects of caffeine and ryanodine on inward current oscillations have been observed in intestinal (Komori *et al*., 1993; Zholos *et al*., 1994) and tracheal smooth muscle cells (Liu & Farley, 1996a). Also, as observed in some other smooth muscles (Janssen & Sims, 1992; 1993; Martin & Shuttleworth, 1994; Liu & Farley, 1996a), caffeine did not by itself generate I_{oscil}. This may indicate that the release of calcium ions from the IP_3 sensitive store is the primary pacemaker for the oscillations; certainly there is good evidence that activation of the IP_3 receptor in many cell types demonstrates oscillatory behaviour, most probably related to the modulation of IP_3 receptor function by calcium ions (Berridge, 1993). Thus, a possible model for the involvement of SR calcium stores in the initiation of carbachol-induced I_{oscil} in the mouse anococcygeus is that IP_3 generated by activation of the muscarinic M_3 receptor (Sideso *et al*., 1994) stimulates release of calcium from the IP_3 sensitive pool. This calcium, while not sufficient by itself to activate the chloride current, then promotes further calcium release via the CICR pathway. It is this amplified calcium signal which both switches on the chloride conductance and also down-regulates the IP_3 system, thereby inhibiting IP_3-induced calcium release (Missaien *et al*., 1992a, b; Zholos *et al*., 1994). Re-filling of the IP_3-sensitive store via the SERCA pump and extrusion of calcium from the cell, reduces the cytoplasmic calcium concentration and re-activates the IP_3 receptor, thus setting up another oscillatory cycle.

While there is general agreement that the maintenance of muscarinic receptor-induced inward current oscillations in smooth muscle requires extracellular calcium, the pathway of calcium entry into the cell is uncertain. In some cases the, at least partial, involvement of L-type VOCCs has been demonstrated (Khoda *et al*., 1996; Komori *et al*., 1996; Liu & Farley, 1996a). In the mouse anococcygeus too, nifedipine caused a partial reduction in I_{oscil}, both at holding potentials of -20 mV and -60 mV. In addition, nifedipine produced a small inhibition (18%) of I_{DOC} at -60 mV. Interestingly, nifedipine also reduced the non-selective cation current (I_{cat}) on which inward current oscillations are superimposed in intestinal smooth muscle cells (Komori *et al*., 1996). Thus, in the anococcygeus, as in other smooth muscles (Komori *et al*., 1993; 1996; Khoda *et al*., 1996; Liu & Farley, 1996a), L-type VOCCs provide, at best, only a partial explanation for the entry of calcium ions to sustain muscarinic-receptor-induced current oscillations, and this entry can occur at holding potentials between -60 and -20 mV. The involvement of a non-VOCC pathway has been inferred, but its identification has remained elusive. The most important new finding of the present study is that I_{DOC} may be implicated, at least in the anococcygeus, as the non-VOCC pathway important for store re-filling. I_{DOC} is a non-selective cation current which is directly activated by depletion of the calcium stores in the SR, either via membrane receptor generation of IP_3 or by drugs acting directly on the SR, such as caffeine and CPA (Wayman *et al*., 1996a, b). The current has a distinctive pharmacology, being inhibited by the general calcium entry blocker SKF 96365 and Cd^{2+}, but resistant to La^{3+}. Sustained contractions of the mouse anococcygeus to carbachol or CPA show a similar pharmacology, suggesting that calcium entry through I_{DOC} is important for sustained contraction of this tissue. The present study shows that I_{oscil} is also blocked by SKF 96365 and Cd^{2+}, but not La^{3+} and therefore that calcium entering through I_{DOC} may also be important for replenishment of the stores and maintenance of I_{oscil}. This would explain our earlier observation that sodium nitroprusside (SNP) blocks carbachol-induced I_{oscil}, since SNP also inhibits I_{DOC} (Wayman *et al*., 1996b). The time-lag observed between the inhibition of I_{DOC} and the onset of decay of I_{oscil} indicates that the oscillations do not reflect an oscillatory property of the I_{DOC} mechanism itself and is consistent with the results obtained in calcium-free medium.

In conclusion, the results have shown that carbachol-induced inward current oscillations in mouse anococcygeus cells are due to a calcium-activated chloride current, and most likely reflect changes in cytosolic calcium ion concentrations. These calcium ions are derived primarily from the SR stores, but entry of calcium into the cell is necessary for replenishment of the stores and maintenance of the oscillations. The most important new finding is that capacitative calcium entry (via I_{DOC}) is important not only for sustained contraction of this smooth muscle but also for the maintenance of store-dependent calcium oscillations. Capacitative calcium entry in smooth muscle therefore represents an important target for drug action; the development of drugs which act specifically on this novel process should provide powerful tools with which to investigate smooth muscle function and with potentially important therapeutic applications.

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