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Short communication

Correlation between store-operated cation current and capacitative Ca^{2+} influx in smooth muscle cells from mouse anococcygeus

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Abstract

In mouse anococcygeus cells, simultaneous measurements of membrane currents and changes in intracellular Ca^{2+} were obtained using ''perforated-patch'' whole-cell recordings and Fura-2 microfluorimetry. Carbachol (50 μM) and cyclopiazonic acid (10 μM) produced a biphasic inward current; a transient Ca^{2+}-dependent chloride current (I_{ClCa}), followed by a smaller, sustained current (I_{DOC}). This response was mirrored by a biphasic increase in the intracellular Ca^{2+} concentration. SKF96365 (1-{β-[3-(4-methoxyphenyl) propoxyl]-4-methoxyphenethyl}-1*H*-imidazole; 10 μM) and Cd^{2+} (100 μM) inhibited both I_{DOC} and the sustained increase in intracellular Ca^{2+}; La^{3+} (400 μM) inhibited neither response. The results confirm that the non-selective cation current I_{DOC} underlies capacitative Ca^{2+} influx supporting sustained contractions in this tonic smooth muscle.

Keywords: Ca^{2+} entry, capacitative; Cation current; Smooth muscle

1. Introduction

The rise in free intracellular Ca^{2+} ($[Ca^{2+}]_i$) initiating smooth muscle contraction occurs as a result of both Ca^{2+} release from intracellular stores and Ca^{2+} entry from the extracellular space. Ca^{2+} entry pathways utilised by smooth muscle cells include dihydropyridine-sensitive (L-type) voltage-dependent Ca^{2+} channels and so-called ''receptor-operated'' Ca^{2+} channels, including the P_{2X} purinoceptor (Benham, 1989) and the non-selective cation conductance activated by G-protein-coupled receptors in gastrointestinal smooth muscle (Pacaud and Bolton, 1991). More recently, evidence has been accumulating to suggest that in some tonic smooth muscles, capacitative Ca^{2+} entry may also be important (Gibson et al., 1998). An example of such a tissue is the mouse anococcygeus muscle, which produces strong, well-maintained contractions to the muscarinic (M_3) cholinoceptor agonist carbachol. The contraction produced by carbachol is dependent upon Ca^{2+} entry, being effectively abolished in the absence of extracellular Ca^{2+}, but is reduced by only around 20% in the presence of nifedipine at a concentration sufficient to eliminate Ca^{2+} entry via L-type Ca^{2+} channels (Gibson et al., 1994). Evidence that capacitative Ca^{2+} entry might be important in this tissue came from the observation that cyclopiazonic acid, an inhibitor of the Ca^{2+} ATPase in the sarcoplasmic reticular membrane, also produces contractions which are of a magnitude comparable to those produced by carbachol (Gibson et al., 1994). Using the whole-cell patch–clamp technique, we have shown that both cyclopiazonic acid (Wayman et al., 1996) and carbachol (Wayman et al., 1998) activate a small, non-selective cation current (I_{DOC}) which we have postulated underlies capacitative Ca^{2+} entry in this muscle; the current is not dependent upon the rise in $[Ca^{2+}]_i$ produced by these drugs. Further, the pharmacology of the current (blocked by SKF96365 and cadmium; insensitive to lanthanum and nifedipine) parallels that of the contractions produced by cyclopiazonic acid (Wayman et al., 1996) suggesting that Ca^{2+} entry via I_{DOC} might be responsible for promoting sustained contractions in this tissue. While the results described above provide compelling evidence for our hypothesis that I_{DOC} underlies capacitative Ca^{2+} entry, they are not conclusive insofar as changes in $[Ca^{2+}]_i$ intimately associated with the activation of I_{DOC} have not yet been measured. In the

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present study, we have set out to rectify this situation by measuring I_{DOC} and changes in $[Ca^{2+}]_i$ simultaneously in single smooth muscle cells from the mouse anococcygeus. Our results show that activation of I_{DOC} by carbachol and cyclopiazonic acid results in a sustained rise in $[Ca^{2+}]_i$; furthermore, pharmacological inhibition of I_{DOC} produces a concomitant fall in $[Ca^{2+}]_i$.

2. Materials and methods

2.1. Isolation of single smooth muscle cells

Experiments were performed on single smooth muscle cells obtained by enzymatic dissociation of the mouse anococcygeus as described previously (Wayman et al., 1996). Briefly, the anococcygeus muscles from male mice (LACA strain) were incubated for 10 min at 37°C in a physiological salt solution (PSS) containing zero added Ca^{2+} plus, in mM: NaCl 120, KCl 6, $MgCl_2$ 1.2, glucose 11, HEPES 10, pH 7.2. Following this, the muscles were incubated for approximately 12 min (depending on enzyme batch) in PSS to which had been added (all from Sigma) bovine serum albumin (fatty acid free, 3.0 mg ml^{-1}), papain (0.6 mg ml^{-1}), collagenase (Type 1A; 0.8 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.

2.2. Measurement of $[Ca^{2+}]_i$

Cells were loaded with Fura-2 during a 9-min incubation at 37°C in PSS to which had been added 0.75 mM $CaCl_2$ plus Fura-2 acetoxymethyl ester (Fura-2-AM; 5 μM). After loading, the cells were washed in fresh PSS, plated on poly-lysine-coated glass coverslips (alcohol-washed) and stored at 4°C for at least 1.5 h. $[Ca^{2+}]_i$ was measured using standard ratiometric methodology, utilising a Cairn spectrophotometer and filter control unit (Cairn Research, Faversham, UK). Experiments were performed at room temperature (19–23°C). $R_{340/380}$ is the ratio of Fura-2 fluorescence (measured at 510 nm) with excitation wavelengths of 340 nm and 380 nm.

2.3. Electrophysiological measurements

Whole-cell membrane currents were measured using the ''perforated-patch'' configuration of the patch–clamp technique using an Axopatch 200A amplifier (Axon Instruments, Foster City, USA). The chamber containing the cells was continually perfused with PSS containing (mM): NaCl 120, KCl 6, $MgCl_2$ 1.2, glucose 11, HEPES 10, $CaCl_2$ 2.5, pH 7.2 (with NaOH) plus nifedipine (1 μM). The patch pipette filling solution contained (mM): CsCl 130, $MgCl_2$ 1.2, tetraethylammonium chloride 20, HEPES 10, ATP 0.5, GTP 0.5, pH 7.2 (with CsOH) plus nystatin (0.2 mg ml^{-1}). When filled with this solution, patch pipettes had DC resistances of 4–7 MΩ. Following formation of the ''giga-seal'', adequate perforation of the patch was considered to have occurred when the series resistance had stabilised at a value less than 20 MΩ. All experiments were performed at room temperature.

2.4. Data handling

Membrane currents and Fura-2 fluorescence values (including values of $R_{340/380}$) were recorded directly onto the optical disk of a personal computer running Axotape® software (Axon Instruments). All results are expressed as mean ± S.E.M. Statistical analysis was carried out using Student's *t*-test; $P < 0.05$ was considered significant.

2.5. Materials

Fura-2-AM was obtained from Calbiochem and stored at −20°C in 10 μl aliquots of 0.5 mM stock solution in dimethylsulphoxide (DMSO). Other drugs used were (all from Sigma unless stated otherwise); carbachol, cyclopiazonic acid, nifedipine and 1-{β-[3-(4-methoxyphenyl) propoxyl]-4-methoxyphenethyl}-1*H*-imidazole HCl (SKF96365; Affiniti Research Products). All drugs were prepared as stocks in deionised water with the exception of cyclopiazonic acid (10 mM stock in DMSO) and nifedipine (10 mM stock in ethanol).

3. Results

Carbachol and cyclopiazonic acid each produced a biphasic inward current in mouse anococcygeus cells held at a membrane potential of −40 mV (Fig. 1). We have shown previously that the initial, transient inward current results from activation of a Ca^{2+}-dependent chloride conductance (I_{ClCa}), while the second, smaller, but sustained component reflects a non-selective cation current (I_{DOC}) activated as a result of Ca^{2+} store depletion, and which we have suggested underlies capacitative Ca^{2+} entry in these cells (Wayman et al., 1996, 1998). The peak amplitudes of I_{ClCa} activated by carbachol (50 μM) and cyclopiazonic acid (10 μM) were 200 ± 45 pA ($n = 14$) and 26.1 ± 6.5 pA ($n = 20$), respectively, while the corresponding figures for I_{DOC} in the same cells, measured when a stable sustained current had been established, were 6.6 ± 0.7 pA ($n = 15$) and 7.8 ± 1.3 pA ($n = 21$), respectively.

In all cells, the biphasic increase in membrane current was mirrored by a biphasic increase in $R_{340/380}$ (Fig. 1). The initial transient responses produced by carbachol and cyclopiazonic acid amounted to 133 ± 27.3% ($n = 15$) and 83 ± 27% ($n = 21$) increases over basal $R_{340/380}$ values, respectively, while the sustained increases amounted to 33 ± 3.1% ($n = 15$) and 37 ± 6.7% ($n = 21$), respectively.

The effects of carbachol on both current and fluorescence ratio were readily reversed on washout of the drug (data not shown). However, the effects of cyclopiazonic acid were not reversed, at least up to 5 min after washout; longer periods of washout were not recorded due to difficulties in maintaining an adequate seal with the patch pipette.

The main aim of this study was to determine whether Ca^{2+} entry via I_{DOC} underlies capacitative Ca^{2+} entry. To do this, we looked at the consequences of blocking I_{DOC} — using SKF96365 and Cd^{2+} — on the maintained rise in intracellular Ca^{2+}. As a control, we also looked at the effects of La^{3+}, which we have previously shown not to inhibit I_{DOC} (Wayman et al., 1996). The results are demonstrated in Fig. 1 and summarised in Fig. 2. Both SKF96365 (10 μM) and Cd^{2+} (100 μM) produced significant reductions in the sustained rises in Ca^{2+} produced by carbachol and cyclopiazonic acid while at the same time inhibiting I_{DOC}. Conversely, La^{3+} (400 μM) failed to either inhibit the current or produce a fall in the maintained increase in Ca^{2+}.

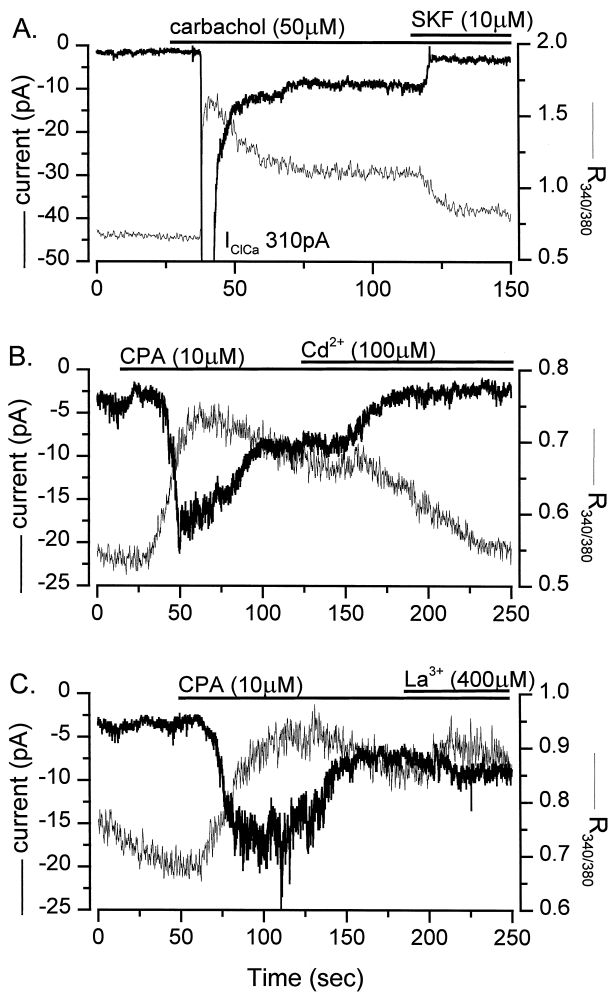

Fig. 1. Original recordings of membrane current (thicker trace) and Fura-2 fluorescence ratio (thinner trace) from single smooth muscle cells of the mouse anococcygeus held in the whole-cell configuration of the 'perforated' patch–clamp technique. Carbachol (top trace) and cyclopiazonic acid (lower traces) produce a biphasic inward current which is mirrored by a biphasic increase in fluorescence ratio. The sustained component of both responses is reduced by SKF96365 (SKF) and Cd^{2+}, but is unaffected by La^{3+}. The initial, transient Ca^{2+}-activated chloride current (I_{ClCa}) has been truncated in the top trace in order to highlight the sustained component.

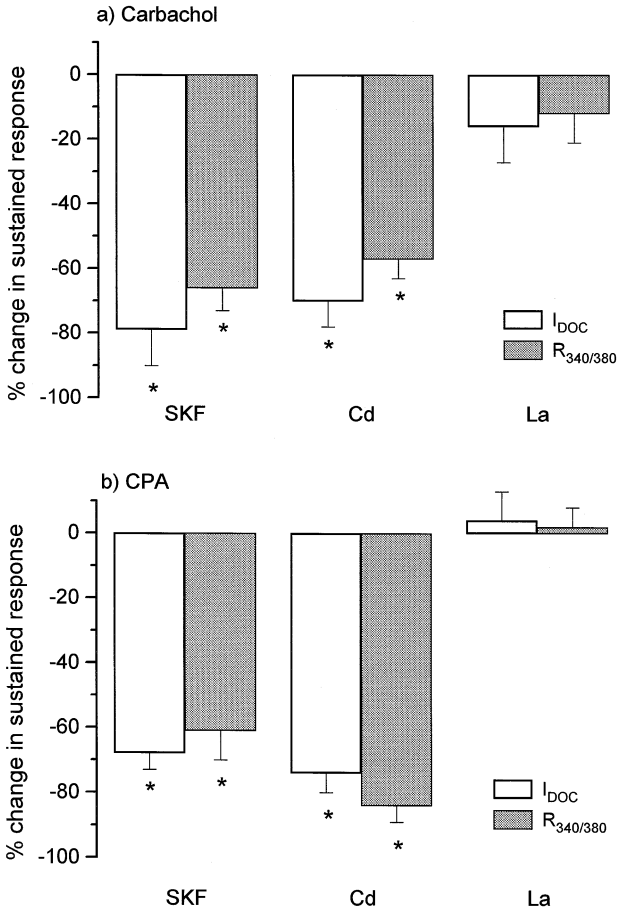

Fig. 2. Histogram showing the effect of SKF96365 (SKF; 10 μM), Cd^{2+} (Cd; 100 μM) and La^{3+} (La; 400 μM) on the sustained components of the inward current (I_{DOC}) and increased fluorescence ratio ($R_{340/380}$) activated by either carbachol (50 μM; a) or cyclopiazonic acid (10 μM; b) in single smooth muscle cells from the mouse anococcygeus. Results are expressed as mean ± S.E.M. (from at least five cells) and are measured as the percentage of change in the amplitude of each response following application of the test compound (see Fig. 1 for protocol). $^{*}P < 0.05$, significant change in the sustained component.

4. Discussion

The main finding of the present study is that pharmacological inhibition of the non-selective cation current I_{DOC} — activated as a result of Ca^{2+} store depletion in mouse anococcygeus cells — produces a parallel fall in the concentration of free intracellular Ca^{2+}, lending support to our hypothesis that I_{DOC} underlies capacitative Ca^{2+} entry in this tissue.

As expected, carbachol produced a biphasic inward current in mouse anococcygeus cells held at near-resting membrane potentials. The initial transient current is absent in cells internally dialysed with either ethyleneglycol-bis(β-aminoethyl ether)*N*,*N*,*N*′,*N*′,-tetraacetic acid (EGTA) or 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′,-tetraacetic acid (BAPTA) and is inhibited by the chloride channel blockers anthracene-9-carboxylic acid and 4,4′-diisothiocyanato-stilbene-2,2′-disulphonic acid (Wayman et al., 1996). These results suggested that the initial transient current was a Ca^{2+} activated chloride current (I_{ClCa}) activated as a result of Ca^{2+} release from intracellular stores (Gibson et al., 1998). The findings of the present study support this working model since carbachol produced an initial, relatively large increase in the free intracellular Ca^{2+} concentration, the time-course of which corresponded closely with that of I_{ClCa}. Cyclopiazonic acid also produced an initial transient rise in Ca^{2+} accompanied by activation of I_{ClCa}. Compared with carbachol, cyclopiazonic acid produced a smaller change in both intracellular Ca^{2+} and I_{ClCa}, presumably reflecting the indirect mechanism by which this inhibitor of the sarcoplasmic reticular Ca^{2+} ATPase releases Ca^{2+} from the store (Seidler et al., 1989).

Cyclopiazonic acid has been widely used in the study of capacitative Ca^{2+} entry since it causes receptor-independent depletion of intracellular Ca^{2+} stores (Berridge, 1995). In the mouse anococcygeus, cyclopiazonic acid causes strong, well-maintained contractions which are dependent upon extracellular Ca^{2+}. The contractions are inhibited by Cd^{2+} and SKF96365 but are largely insensitive to nifedipine and La^{3+}. In single cells, cyclopiazonic acid and carbachol activate a small (typically less than 10 pA) sustained inward current that is apparent after I_{ClCa} has decayed or when cells are dialysed with EGTA or BAPTA; in the presence of cyclopiazonic acid, concomitant application of carbachol produces no further increase in current amplitude (Wayman et al., 1998). The reversal potential for this current (around +30 mV in PSS containing 10 mM Ca^{2+}) moves to around 0 mV in zero extracellular Ca^{2+} suggesting that it has significant Ca^{2+} permeability. Cd^{2+} and SKF96365 block the current, but not nifedipine or La^{3+}. Its pharmacology, coupled to the fact that it is activated as a result of Ca^{2+} store depletion rather than receptor activation per se led us to suggest that this depletion-operated current (I_{DOC}) was responsible for capacitative Ca^{2+} entry in this smooth muscle (Wayman et al., 1996). The results of the present study add further weight to this hypothesis by demonstrating that inhibition of I_{DOC} leads to a concomitant lowering of the free intracellular concentration. As in many other cell types, the Ca^{2+} signal in response to carbachol was biphasic, the initial rise that results from the release of intracellular stores being followed by a smaller more sustained increase. This mirrored the electrophysiological response — I_{ClCa} followed by I_{DOC} — and cyclopiazonic acid produced qualitatively similar changes. More importantly, blockade of I_{DOC} using either SKF96365 or Cd^{2+} led to a parallel fall in the intracellular Ca^{2+} concentration; conversely, La^{3+}, which has been shown to inhibit capacitative Ca^{2+} entry in some cell types (Mendelowitz et al., 1992; Hoth and Penner, 1993), but has no effect on either I_{DOC} or the contraction of the mouse anococcygeus to cyclopiazonic acid (Wayman et al., 1996), failed to reduce the sustained increase in intracellular Ca^{2+}.

It now appears that capacitative Ca^{2+} entry occurs by way of a family of ion currents, the members of which differ in their relative selectivity for Ca^{2+} and in their sensitivity to blockade by the trivalent cations La^{3+} and Gd^{3+}. The first description of a current activated as a result of Ca^{2+} store depletion followed experiments in mast cells (Hoth and Penner, 1992). This ''Ca^{2+} release activated Ca^{2+} current'' (I_{CRAC}), was highly Ca^{2+}-selective and blocked by 1 μM La^{3+}. Since then, currents with properties similar to I_{CRAC} have been found in a range of cell types, but depletion operated non-selective cation currents have also been described, in smooth muscle cells (Wayman et al., 1996), endothelial cells (Mendelowitz et al., 1992; Zhang et al., 1994), pancreatic acinar cells (Krause et al., 1996), megakaryocytes (Somasundaran and Mahaut-Smith, 1994) and pancreatic β cells (Worley et al., 1994). The pharmacological profiles of these store-operated non-selective cation currents vary, particularly with respect to their sensitivity to block by cations; for example, the current in pancreatic acinar cells is unaffected by both La^{3+} and Cd^{2+} (Krause et al., 1996), whereas that in the mouse anococcygeus is blocked by Cd^{2+} but insensitive to La^{3+} (Wayman et al., 1996). This divergence is perhaps not surprising in light of the complex picture that exists with regard to the molecular pharmacology of store-operated Ca^{2+} channels. It is proposed that such channels are formed from proteins homologous to Drosophila Trp/TRP*l* (Berridge, 1995; Zhu et al., 1996; Montell, 1997) though a clear consensus on the molecular make-up of functional store-operated channels has yet to emerge. Another important question to be resolved concerns the nature of the signal which links store depletion to opening of store-operated channels; it is still not clear whether this involves a chemical messenger or a direct protein:protein interaction between the sarcoplasmic reticulum and the plasma membrane (Parekh and Penner, 1997)

In conclusion, these results lend strong support to our hypothesis that the non-selective cation current, I_{DOC}, underlies capacitative Ca^{2+} entry in the mouse anococcygeus. Identification of selective inhibitors of this pathway offers a potential route for the development of novel smooth muscle relaxant drugs.

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