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Force, membrane potential and cytoplasmic Ca^{2+} responses to cyclic nucleotides in rat anococcygeus muscle

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Abstract

Simultaneous recordings of membrane potential and force, and cytoplasmic calcium ($[Ca^{2+}]_i$) and force were made in rat anococcygeus to determine whether membrane hyperpolarisation plays a role in cyclic nucleotide-induced relaxation. In the presence of phenylephrine (0.2 μM), which evoked sustained contraction, an elevation in $[Ca^{2+}]_i$, and depolarisation, nitroprusside (5 μM) caused $96 \pm 3\%$ relaxation, $77 \pm 3\%$ decrease in suprabasal $[Ca^{2+}]_i$, and 16 ± 2 mV hyperpolarisation. Forskolin (1 μM) caused $98 \pm 1\%$ relaxation, $92 \pm 2\%$ decrease in suprabasal $[Ca^{2+}]_i$, and 18 ± 1 mV hyperpolarisation. These responses persisted in the presence of a variety of K^+ channel blockers or in ouabain. The decrease in $[Ca^{2+}]_i$ preceded the commencement of relaxation whereas the onset of hyperpolarisation lagged behind. Thus, cyclic nucleotide-mediated relaxation in rat anococcygeus is not dependent on hyperpolarisation mediated by the opening of K^+ channels. Rather, it is suggested that the decrease in $[Ca^{2+}]_i$ gives rise to hyperpolarisation, which reflects a decline in the Ca^{2+} dependent conductance(s) activated by phenylephrine.

Keywords: Anococcygeus; Ca^{2+}, intracellular; K^+ channel blocker; Membrane potential; Ouabain; Smooth muscle

1. Introduction

Relaxation in smooth muscle can be initiated by the activation of second messenger cascades involving the cyclic nucleotides, cyclic AMP and cyclic GMP. A lowering of cytoplasmic free Ca^{2+} ($[Ca^{2+}]_i$) associated with the relaxation mediated by these cyclic nucleotides may involve a number of cellular mechanisms including: (i) stimulation of Ca^{2+} sequestration by the sarcoplasmic reticulum (Luo et al., 1993; Ito et al., 1993), (ii) inhibition of the release of Ca^{2+} from the sarcoplasmic reticulum (Murthy et al., 1993), (iii) stimulation of Ca^{2+} extrusion across the cell membrane (Vrolix et al., 1988) and (iv) inhibition of Ca^{2+} influx into the cell (Ahn et al., 1992). It has also been proposed that mechanisms that are independent of a lowering of $[Ca^{2+}]_i$ may contribute to cyclic nucleotide-mediated relaxation in some smooth muscles (Karaki et al., 1988; Abe and Karaki, 1989; Lincoln et al., 1991).

The membrane potential plays a key role in controlling $[Ca^{2+}]_i$ and hence the contractile state in many smooth muscles (Nelson and Quayle, 1995). This is achieved predominantly by the voltage-dependent L-type Ca^{2+} channels, since depolarisation increases the open probability of these channels. Agonists may depolarise the membrane through activation of non-selective cation channels or Cl^- channels, or inhibition of K^+ channels (Karaki et al., 1997). Upon depolarisation, the L-type Ca^{2+} channels open to allow Ca^{2+} influx that subsequently elevates $[Ca^{2+}]_i$. Conversely, hyperpolarisation of the membrane reduces the open probability of L-type Ca^{2+} channels leading to an inhibition of Ca^{2+} influx, facilitating a lowering of $[Ca^{2+}]_i$ (Nelson and Quayle, 1995). The second messenger systems associated with cAMP and cGMP are associated with membrane hyperpolarisation in a variety of different smooth muscles (Smith et al., 1993; Parkington et al., 1995; Yuan et al., 1996). This raises the possibility that hyperpolarisation may play a role in cyclic nucleotide-mediated relaxation. In various smooth muscles, both Ca^{2+}-activated and ATP-sensitive K^+ channels have been implicated in the hyperpolarisation associated with elevation of cAMP (Parkington et al., 1995; Song and Simard, 1995). Similarly, nitric oxide donors that increase

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intracellular cGMP have also been reported to produce membrane hyperpolarisation as a result of the opening of K^+ channels in a variety of smooth muscles (Koh et al., 1995; Parkington et al., 1995; Watson et al., 1996).

The rat anococcygeus muscle contains an excitatory adrenergic and an inhibitory non-adrenergic, non-cholinergic innervation that is believed to utilise nitric oxide as a transmitter (Li and Rand, 1989). There is evidence that non-adrenergic, non-cholinergic relaxation in this tissue is accomplished via a decrease in $[Ca^{2+}]_i$ since both this and the relaxation are inhibited by nitric oxide synthase inhibitors (Raymond et al., 1995). The nitric oxide donor nitroprusside also lowers $[Ca^{2+}]_i$ and relaxes the rat anococcygeus muscle and this has been linked, at least in part, to stimulation of Ca^{2+} sequestration into the sarcoplasmic reticulum (Raymond and Wendt, 1996). Forskolin, which increases cAMP, relaxes intact rat anococcygeus, and it has also been reported that direct application of cAMP to permeabilised tissues causes relaxation (Chrichton et al., 1989). The forskolin-mediated relaxation is associated with a lowering of $[Ca^{2+}]_i$ generated in part by Ca^{2+} sequestration into the sarcoplasmic reticulum (Raymond and Wendt, 1996).

The aim of the present study was to determine whether membrane hyperpolarisation plays a role in cyclic nucleotide-induced relaxation in the rat anococcygeus muscle and whether K^+ channels are involved in mediating these responses. Force and the associated changes in $[Ca^{2+}]_i$ and membrane potential were recorded during relaxations induced by nitroprusside and forskolin under control condi-

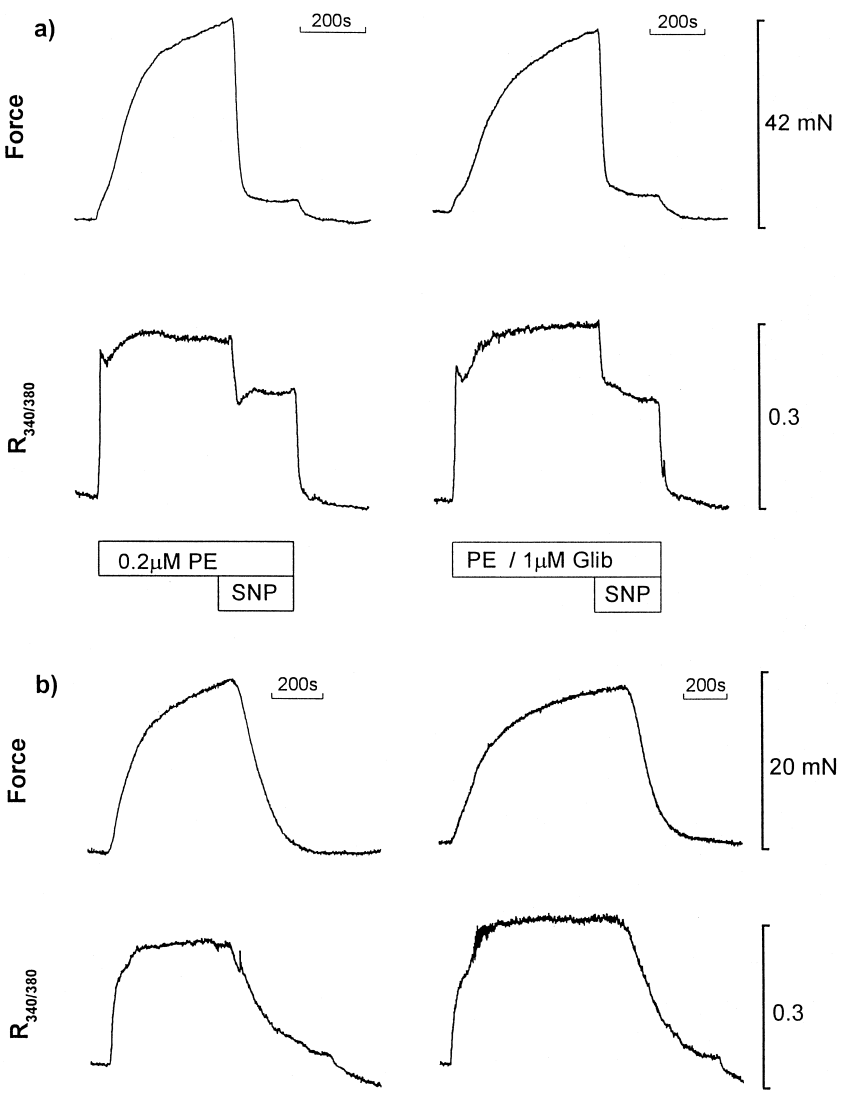

Fig. 1. Simultaneous recordings of force and $R_{340/380}$ from rat anococcygeus muscles pre-contracted with 0.2 μM phenylephrine and subsequently relaxed with (a) sodium nitroprusside (SNP, 5 μM) or (b) forskolin (FSK, 1 μM). Control responses are compared, in the same muscle in each case, with those in the presence of the K^+ channel blockers, glibenclamide (1 μM) for the SNP-induced relaxation and apamin (0.25 μM) for the forskolin-induced relaxation.

tions and in the presence of the K^+ channel blockers tetraethylammonium, glibenclamide, apamin and charybdotoxin.

2. Methods and materials

2.1. Tissue preparation

Male Sprague–Dawley rats (150–250 g) were killed by chloroform overdose and cervical dislocation, with the approval of the Physiology Animal Ethics Committee, Monash University (approval number 95107). The paired bands of the anococcygeus muscle were cleaned of connective tissue, excised, and transferred to the appropriate recording apparatus for simultaneous measurements of force and either tissue fluorescence or membrane potential.

2.2. Experimental protocol

Simultaneous recording of tension and fluorescence has been reported previously from this laboratory (Raymond et al., 1995). Intracellular $[Ca^{2+}]$ was monitored using the fluorescent Ca^{2+} indicator, fura-2. Tissue fluorescence was recorded using a spectrophotometer system (Cairn Research, UK) coupled with an inverted fluorescence microscope. The muscle strips were attached to a force transducer assembly and positioned in a 2.5 ml organ bath located on the stage of the microscope where they were continuously superfused at a rate of 30 ml/min with Physiological saline solution (PSS) maintained at 27°C. This lower temperature significantly slowed the leakage of fura-2 from the tissue and thus prolonged the period over which fluorescence recordings could be made from the preparations. Tissue autofluorescence was measured at the beginning of every experiment. The muscle strips were then incubated in a HEPES buffered solution containing 5 μM of the acetoxymethyl ester form of fura-2 (fura-2/AM) for 3 h at room temperature. Following this the muscles were washed for 15 min with PSS and stable isometric contractile responses to the α-adrenoceptor agonist phenylephrine were then established. Alternating excitation light of 340 and 380 nm was focused onto the muscle using a ×40 objective and the subsequent emitted fluorescence at 510 nm was monitored by a photomultiplier tube. The fluorescence signals were corrected by subtracting tissue autofluorescence and the ratio of the fluorescence at 340 nm excitation to that at 380 nm ($R_{340/380}$) was subsequently taken as an indication of $[Ca^{2+}]_i$. In selected experiments $[Ca^{2+}]_i$ was calibrated by exposing the muscle to the Ca^{2+} ionophore, ionomycin, and determining R_{min} and R_{max} as previously described (Munro and Wendt, 1994). For the muscles on which this calibration was performed the mean values for R_{min} and R_{max} were 0.42 ± 0.02 and 1.66 ± 0.17, respectively ($n = 10$). The maximum values of $R_{340/380}$ recorded during the experiments never exceeded 50% of the subsequently determined R_{max}.

Force and membrane potential were recorded simultaneously as previously described (Coleman and Parkington, 1988). The anococcygeus was mounted horizontally in a 1 ml organ bath superfused with PSS at 3 ml/min at 35°C. One end of the tissue was pinned to the silicone rubber base of the bath while the other end was attached to a force displacement transducer (AE801; SensoNor, Horten, Norway). The strip was stretched until a maximal contraction to phenylephrine was achieved and was then allowed to equilibrate for 20 min. Membrane potentials were

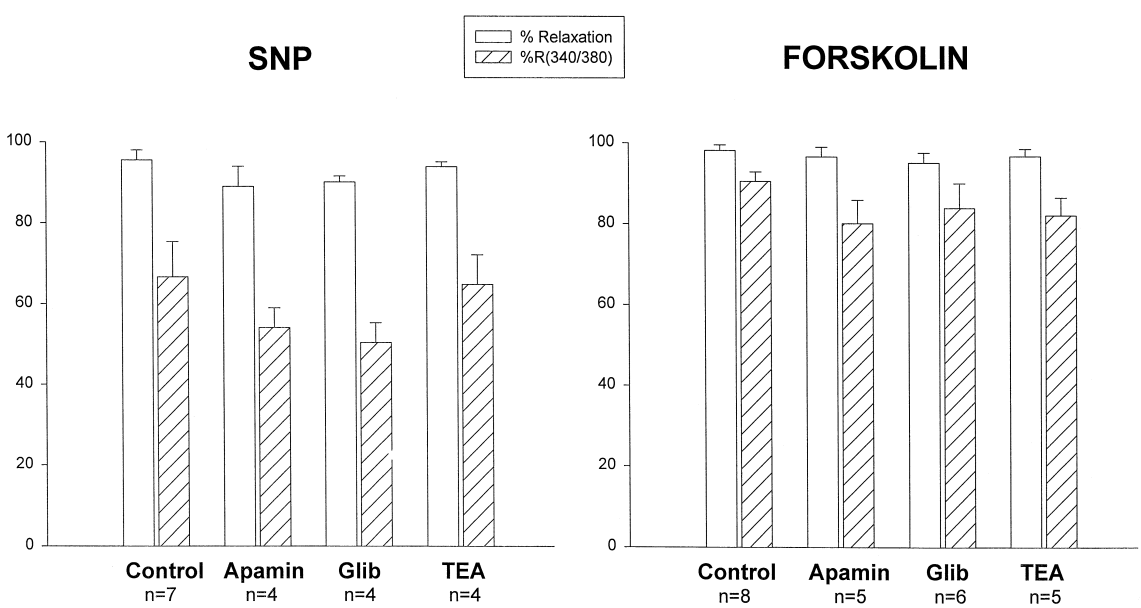

Fig. 2. Effects of K^+ channel blockers on force and $R_{340/380}$ responses during relaxation induced by sodium nitroprusside (SNP, 5 μM) and forskolin (1 μM). Shown are the means ± S.E.M. for relaxations and decreases in $R_{340/380}$, expressed as percent of phenylephrine-induced contraction and increase in $R_{340/380}$. There were no significant effects of apamin, glibenclamide (Glib) or tetraethylammonium (TEA) on the relaxations or decrease in $R_{340/380}$ produced by either SNP or forskolin ($P > 0.05$, paired t-test).

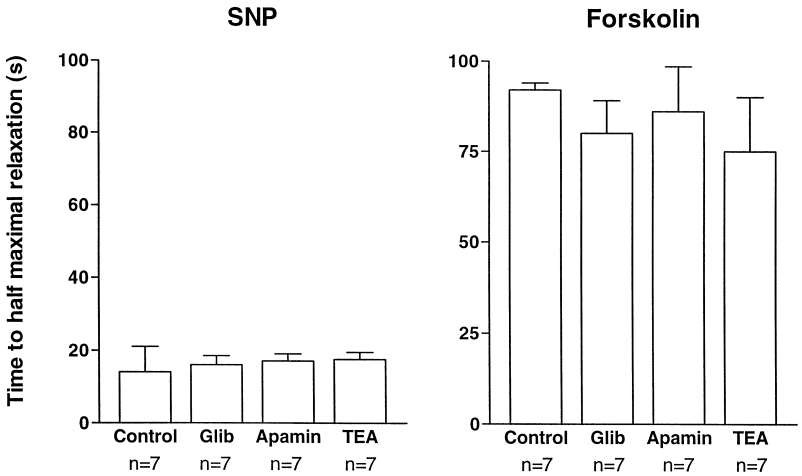

Fig. 3. The time to half maximal relaxation was shorter in response to sodium nitroprusside (SNP) than in response to forskolin. However, the times were unaltered in either case by the presence of glibenclamide (Glib), apamin or tetraethylammonium (TEA).

recorded from smooth muscle cells using conventional intracellular, glass microelectrodes filled with 1 M KCl and having resistances of 60–100 MΩ. Membrane potential and tension were also recorded at 27°C in three experiments. Oscillations in response to phenylephrine were less frequent but the hyperpolarisations and relaxations in response to nitroprusside and forskolin were unchanged (data not shown).

In all experiments, unless otherwise stated, 0.2 μM phenylephrine was used as the standard contractile stimulus. Other than in the concentration–response experiments, nitroprusside was applied at a concentration of either 5 or 10 μM and forskolin was applied at either 1 or 10 μM since these concentrations gave reproducible and essentially complete relaxation (see Raymond and Wendt, 1996).

2.3. Solutions and drugs

PSS had the following composition (mM): 118 NaCl, 4.75 KCl, 1.18 $MgSO_4$, 1.18 KH_2PO_4, 24.8 $NaHCO_3$, 10 glucose and 1.5 $CaCl_2$, continuously aerated with 95% O_2–5% CO_2 and having a pH of 7.35. The HEPES buffered solution used when loading fura-2/AM contained (mM): 135.5 NaCl, 5.9 KCl, 1.2 $MgCl_2$, 11.6 HEPES, 10 glucose and Pluronic F127 (0.01%) to aid the dispersal of the fura-2/AM. The following drugs were used: apamin, forskolin, L-phenylephrine/HCl, N^{ω}-nitro-L-arginine methyl ester, ouabain, sodium nitroprusside and tetraethylammonium chloride (all from Sigma, St. Louis, MO, USA); charybdotoxin (Alomone Laboratory, Jerusalem, Israel); glibenclamide (a gift from Hoechst, Germany); fura 2-AM (Molecular Probes, Eugene, OR, USA). From concentrated stock solutions, appropriate aliquots of these drugs were added to the perfusing solution to achieve the desired final concentrations. Phenylephrine (1 mM), N^{ω}-nitro-L-arginine methyl ester (10 mM), charybdotoxin (0.1 mM), ouabain (10 mM), sodium nitroprusside (10 mM), apamin (500 μM) and tetraethylammonium (1 M) were dissolved in distilled water. Forskolin (10 mM) was dissolved in 95% ethanol and glibenclamide (10 mM) in dimethyl sulphoxide.

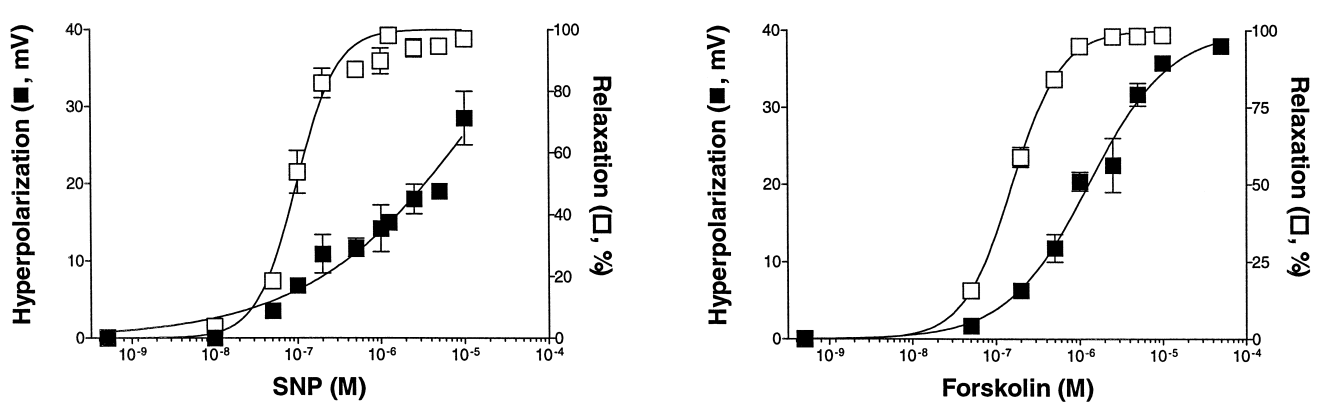

Fig. 4. Concentration–response curves of the hyperpolarisations (solid symbols) and relaxations (clear symbols) produced in rat anococcygeus muscles using a range of concentrations of sodium nitroprusside (SNP) and forskolin (muscles precontracted with phenylephrine). The data is expressed as a mean change in absolute membrane potential (ΔMP) and corresponding percent relaxation.

2.4. Analysis of the data

Relaxation was expressed as percent of the phenylephrine-induced contraction. To facilitate statistical analysis of changes in $R_{340/380}$ during the relaxation, $R_{340/380}$ was assigned a value of 0% under resting conditions in each muscle, and 100% during the steady-state response to 0.2 μM phenylephrine. Within each muscle, relaxation was then expressed relative to this.

Membrane potential data were expressed as absolute changes in membrane potential (ΔMP), that is, from the mean steady state level of depolarisation evoked by phenylephrine to the peak of the hyperpolarisation elicited by nitroprusside or forskolin. Only the maximum amplitudes of the hyperpolarisations and relaxations were measured. The nitric oxide inhibitor N^{ω}-nitro-L-arginine methyl ester (0.5 μM) (found to block nitric oxide production from vascular endothelium, unpublished observations) was present during most of the electrophysiological experiments to inhibit possible release of endogenous nitric oxide from intrinsic nerves, especially important when testing K^+ channel blockers.

All data are expressed as mean ± S.E.M. Student's *t*-test was used to test for significance between groups. A significance level of $P < 0.05$ was used throughout. In all situations, the number of tissues tested is signified by *n*.

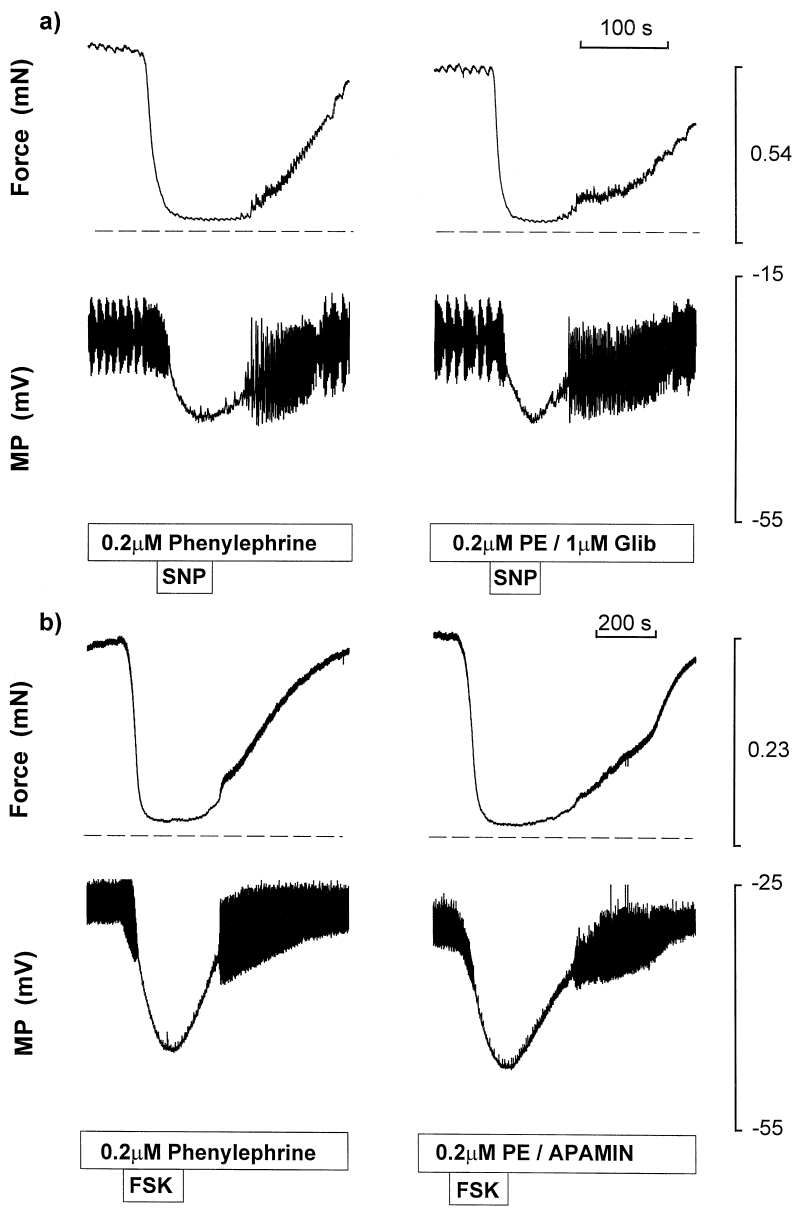

Fig. 5. The effect of K^+ channel blockers on simultaneous force and membrane potential responses initiated by either (a) sodium nitroprusside (SNP) or (b) forskolin (FSK) in muscles contracted with phenylephrine. The responses evoked by SNP in the presence of glibenclamide (Glib), and responses elicited by FSK in the presence of apamin are on the right. Each set of recordings in panels (a) and (b) were in the same muscle with the microelectrode in the same cell.

Concentration–response curves for nitroprusside and forskolin were fitted using the software package Prism (GraphPad).

3. Results

3.1. Force, $[Ca^{2+}]_i$ and membrane potential at rest and during α-adrenoceptor stimulation

At rest, the muscle strips showed no spontaneous contractile activity or active tone. Resting $[Ca^{2+}]_i$ was 28 ± 5 nM ($n = 9$), and the resting membrane potential was -65 ± 1 mV ($n = 30$). When the muscles were exposed to 0.2 μM phenylephrine, $[Ca^{2+}]_i$ increased to reach a stable level of 181 ± 31 nM ($n = 9$) which was sustained for up to 20 min. The rise in $[Ca^{2+}]_i$ always preceded the onset of force development. Phenylephrine induced depolarisation of the smooth muscle membrane to -29 ± 1 mV ($n = 29$) which also preceded the onset of force development. Spike oscillations generally occurred on top of the sustained depolarisation and these were often associated with small oscillations on top of the sustained tension. Examples are shown in Figs. 5 and 7.

3.2. Force and $[Ca^{2+}]_i$ responses during nitroprusside- and forskolin-induced relaxations and the effects of K^+ channel blockers

Application of 5 μM nitroprusside to muscles pre-contracted with 0.2 μM phenylephrine led to a substantial ($95.6 \pm 2.5\%$) and rapid relaxation (14 ± 7 s to half maximal relaxation) that was associated with a decrease in $R_{340/380}$ of $67 \pm 9\%$ (Fig. 1a, Fig. 2, $n = 7$). In four muscles in which a successful calibration was achieved, nitroprusside decreased suprabasal $[Ca^{2+}]_i$ by $76.7 \pm 3.4\%$.

Forskolin (1 μM) also produced near complete ($98.2 \pm 1.4\%$) relaxation of muscles pre-contracted with 0.2 μM phenylephrine and this was associated with a large ($90.6 \pm 2.3\%$, $n = 8$) decrease in $R_{340/380}$ (Fig. 1b, Fig. 2). In the muscles in which the fura-2 signal was calibrated the forskolin-induced decrease in suprabasal $[Ca^{2+}]_i$ was $91.6 \pm 2.1\%$ ($n = 6$). It is noteworthy that the relaxation (time to half maximal relaxation, 92 ± 2 s) and decrease in $[Ca^{2+}]_i$ evoked by forskolin were considerably slower than those in response to nitroprusside (Figs. 1 and 3).

The responses to both nitroprusside and forskolin were repeated in the presence of the K^+ channel blockers tetraethylammonium (1 mM), glibenclamide (1 μM) and apamin (0.25 μM). The results are summarised in Fig. 2. There was no significant difference between the control relaxation or $R_{340/380}$ responses and those repeated in the presence of any of these K^+ channel blockers ($P > 0.05$, paired *t*-test). An example of nitroprusside and forskolin responses in the presence of glibenclamide and apamin, respectively, are illustrated in Fig. 1. There was no difference in the times taken to achieve 50% relaxation during control nitroprusside and forskolin relaxations compared with those repeated in the presence of the K^+ channel blockers (Fig. 3).

3.3. Force and membrane potential during nitroprusside- and forskolin-induced relaxations and the effects of K^+ channel blockers

In anococcygeus muscles pre-contracted with 0.2 μM phenylephrine, both nitroprusside (0.2–10 μM) and

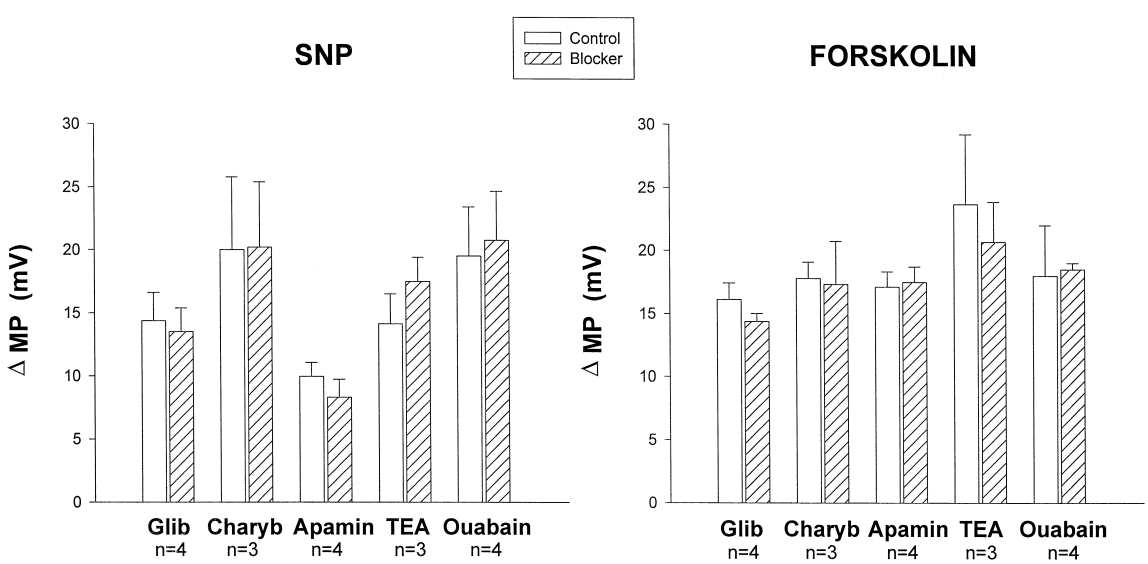

Fig. 6. The effect of K^+ channel blockers on the hyperpolarisations produced by sodium nitroprusside (SNP, 5 μM) and forskolin (1 μM) when applied to muscles precontracted with phenylephrine. Data is expressed as mean ± S.E.M. and the extent of hyperpolarisation is expressed as the change in membrane potential (ΔMP). K^+ channel blockers used were glibenclamide (Glib 1 μM), charybdotoxin (Charyb 20 nM), apamin (0.25 μM) and tetraethylammonium (TEA 1 mM). The effect of the Na^+/K^+ ATPase inhibitor, ouabain (2 μM), was also tested.

forskolin (0.2–50 μM) evoked concentration-dependent hyperpolarisation of the smooth muscle membrane and relaxation (Fig. 4). However, complete relaxation was achieved with concentrations of nitroprusside and forskolin that evoked only a fraction of the maximum possible hyperpolarisation (Fig. 4). Furthermore, the onset of relaxation preceded the hyperpolarisation and this is clearly seen in Fig. 5. This was consistent in all preparations tested. There was also a cessation of spike activity during the hyperpolarisation (Fig. 5).

The effects of a variety of K^+ channel blockers on the hyperpolarisations evoked by nitroprusside (5 μM) and forskolin (1 μM) are summarised in Fig. 6. Tetraethylammonium (1 mM), glibenclamide (1 μM), apamin (0.25 μM), charybdotoxin (20 nM) or the combination of apamin plus charybdotoxin had no measurable effect on either the nitroprusside- or forskolin-induced hyperpolarisations ($P > 0.05$, paired *t*-test). Examples of typical recordings are shown in Fig. 5.

The Na^+/K^+ pump is electrogenic, its activity can lead to hyperpolarisation of the plasma membrane, and its rate is modulated by cAMP in some smooth muscles (Scheid et al., 1979). Ouabain (2 μM), an inhibitor of the Na^+/K^+ ATPase, was used to test the possible involvement of this pump in the hyperpolarisations evoked by nitroprusside and forskolin. As shown in Fig. 6, ouabain had no effect on the hyperpolarisations evoked by either nitroprusside or forskolin.

3.4. Force and membrane potential following nitroprusside and forskolin pre-treatment

In addition to testing the effects of nitroprusside and forskolin on established phenylephrine responses, muscles were also pre-treated with either nitroprusside (10 μM) or

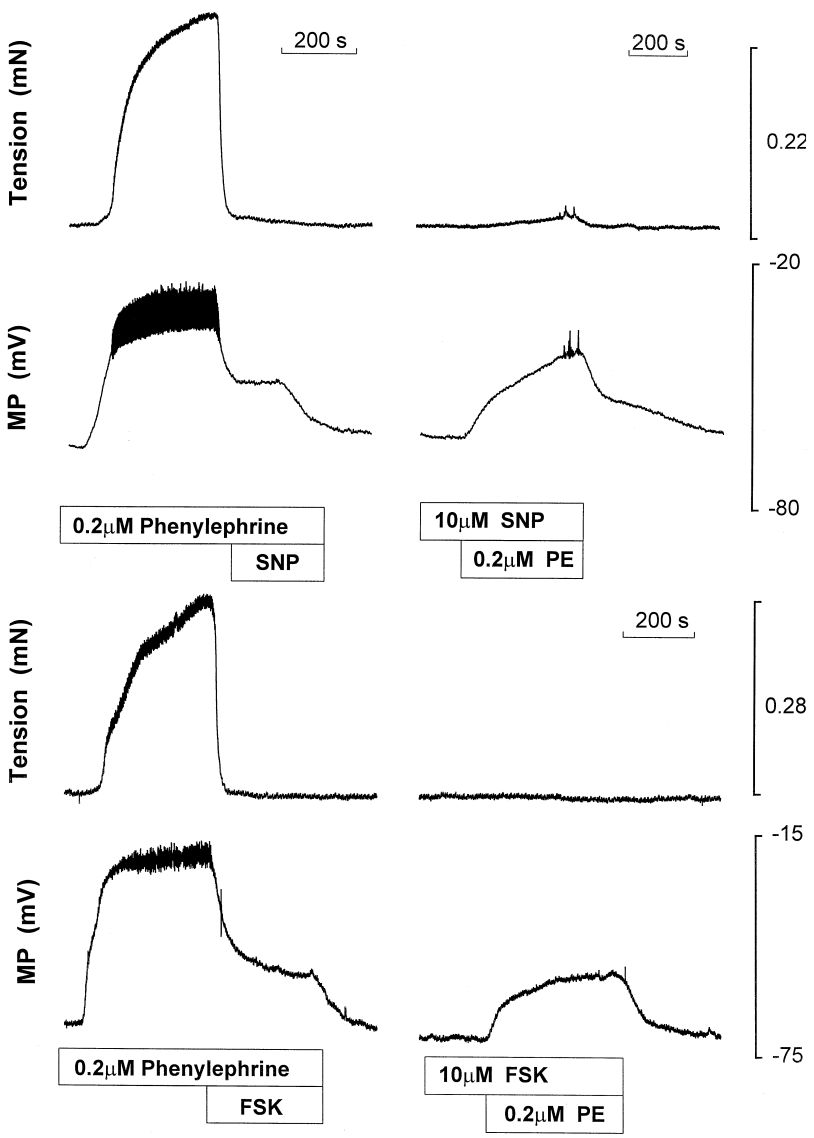

Fig. 7. The effect of pre-treatment of rat anococcygeus muscle with (a) sodium nitroprusside (SNP, 10 μM) or (b) forskolin (10 μM) on force and membrane potential upon subsequent stimulation with phenylephrine. Each set of recordings were in the same muscle with the microelectrode in the same cell.

forskolin (10 μM) prior to exposure to phenylephrine. There was no effect of pre-incubation with either relaxant on the level of the resting membrane potential ($n = 5$). Upon subsequent application of phenylephrine both force and membrane potential rose slowly to achieve final values (Fig. 7) that were similar to the final levels of relaxation and membrane potential attained when nitroprusside (10 μM) or forskolin (10 μM) were added to an established phenylephrine contraction and depolarisation (see above). Spike activity was generally not observed in tissues pre-treated with nitroprusside or forskolin.

4. Discussion

The rat anococcygeus muscle receives a motor innervation from the sympathetic nervous system and stimulation of these nerves or addition of noradrenaline produces contraction which is associated with membrane depolarisation. Both the membrane depolarisation and contraction are blocked by α-adrenoceptor antagonists (Creed et al., 1975; Byrne and Large, 1985). In the present study phenylephrine, a selective α-adrenoceptor agonist, also evoked a sustained depolarisation on which spike oscillations were superimposed. It is also known that the rat anococcygeus, when challenged with phenylephrine, responds with a sustained increase in $[Ca^{2+}]_i$ (Shimizu et al., 1995; Raymond and Wendt, 1996).

Several different conductances could contribute to the membrane potential response associated with α-adrenoceptor stimulation. Of particular importance in the anococcygeus muscle may be a Ca^{2+}-activated Cl^- conductance (Byrne and Large, 1987). Ca^{2+}-activated Cl^- channels have been found in many smooth muscles and are believed to mediate agonist-induced depolarisation leading to increased opening of voltage-dependent Ca^{2+} channels and resultant Ca^{2+} influx (Large and Wang, 1996). The existence of an inactivating outward current in the rat anococcygeus in response to depolarisation which shares properties of both A-type and delayed rectifier-type K^+ channels has also been demonstrated (McFadzean and England, 1992). It is believed that this current acts as a braking current to oppose depolarisation and therefore may be important in controlling Ca^{2+} entry into the cell via voltage-dependent Ca^{2+} channels. In the rat anococcygeus muscle phenylephrine can also inhibit L-type Ca^{2+} current, possibly involving an α-adrenoceptor-linked G protein (England and McFadzean, 1995). Stimulation of α-adrenoceptors in smooth muscle has also been proposed to increase the sensitivity of the contractile apparatus to Ca^{2+}. It is clear, therefore, that α-adrenoceptor activation in the rat anococcygeus may invoke multiple mechanisms to modulate membrane potential, $[Ca^{2+}]_i$ and force.

It is well known that agonists which generate increases in intracellular cAMP and/or cGMP can promote a lowering of $[Ca^{2+}]_i$ and relaxation in smooth muscle and this may also be accompanied by membrane hyperpolarisation. This has led to the suggestion that inhibition of Ca^{2+} influx, secondary to membrane hyperpolarisation, may contribute to lowering $[Ca^{2+}]_i$ and relaxation produced by these agents (Nelson and Quayle, 1995). Over recent years the hyperpolarisation induced by a variety of relaxants in smooth muscles has been shown to be linked to the activation of different types of K^+ channels. Large conductance Ca^{2+}-activated K^+ channels (Song and Simard, 1995) have been implicated most often, but activation of ATP-sensitive K^+ channels (Zhang et al., 1994) and small-conductance K_{Ca} channels (Martins et al., 1995) has also been reported.

The rat anococcygeus contains an inhibitory non-adrenergic, non-cholinergic innervation with nitric oxide as the likely neurotransmitter, since the relaxations evoked by nerve stimulation are blocked by nitric oxide synthase inhibitors, and mimicked by nitric oxide donors such as nitroprusside (Li and Rand, 1989). Relaxation by nitroprusside has been previously linked to a lowering of $[Ca^{2+}]_i$ (Raymond et al., 1995; Raymond and Wendt, 1996) which was found to be, at least in part, reliant on Ca^{2+} sequestration into the sarcoplasmic reticulum (Raymond and Wendt, 1996). A portion of the response is also due to desensitization of the contractile apparatus to Ca^{2+}, as previously reported (Raymond and Wendt, 1996) and as evident in Fig. 1a, where the near complete decrease in force is accompanied by only a partial decrease in $[Ca^{2+}]_i$. The present results reveal that there is also a distinct hyperpolarisation associated with the nitroprusside-induced relaxation. This is in accordance with an early study which found that stimulation of inhibitory nerves in the rat and rabbit anococcygeus led to hyperpolarisation of the cell membrane (Creed and Gillespie, 1977). The simultaneous recording of force and membrane potential employed in the present study revealed, however, that the hyperpolarisation always occurred secondary to the decrease in force (Fig. 5) and complete relaxation could be achieved with only modest changes in membrane potential (Fig. 4). This strongly suggests that the hyperpolarisation does not initiate the decrease in $[Ca^{2+}]_i$ or the relaxation, although a role in its maintenance cannot be ruled out.

Attenuation of cGMP-induced relaxation by blockers of Ca^{2+}-activated K^+ channels has been reported in tracheal smooth muscle (Jones et al., 1993), while in guinea-pig coronary artery, glibenclamide completely abolished the nitric oxide-induced hyperpolarisation while reducing the duration, but not the amplitude, of the relaxation (Parkington et al., 1995). In contrast, in the present study neither glibenclamide, tetraethylammonium, apamin, nor charybdotoxin altered the extent of the nitroprusside-induced hyperpolarisation or relaxation. This makes the involvement of K^+ channels in the response of the rat anococcygeus muscle to nitroprusside unlikely.

The role of cAMP-mediated relaxation in the rat anococcygeus muscle remains to be fully elucidated. It is

known that the adenylate cyclase activator forskolin lowers $[Ca^{2+}]_i$ and force in muscles pre-contracted with phenylephrine, and that this response is, in part, attributable to Ca^{2+} sequestration into the sarcoplasmic reticulum (Raymond and Wendt, 1996). The present study demonstrates that forskolin causes a substantial hyperpolarisation in phenylephrine-contracted muscles. Forskolin has been shown to cause relaxation and hyperpolarisation in several smooth muscle preparations (Honda et al., 1986; Smith et al., 1993) and both Ca^{2+}-activated K^+ channels (Song and Simard, 1995) and ATP-sensitive K^+ channels (Zhang et al., 1994) have been implicated. In the rat anococcygeus muscle, however, glibenclamide, tetraethylammonium, apamin, and charybdotoxin were all without effect on either the hyperpolarisation or the relaxation induced by forskolin. In addition, as was the case with the response to nitroprusside, the relaxation always preceded the hyperpolarisation. From these observations it would seem clear firstly, that hyperpolarisation is not a primary mechanism responsible for initiating cyclic nucleotide-mediated relaxation and secondly, that the hyperpolarisation produced by nitroprusside or forskolin does not result from activation of K^+ channels.

It has been suggested previously that stimulation of the Na^+/K^+-ATPase contributes to cAMP- (Scheid et al., 1979) and cGMP-mediated (Gupta et al., 1995) hyperpolarisation. In the present experiments ouabain, an inhibitor of this pump, had no effect on the relaxation or the hyperpolarisation induced by either forskolin or nitroprusside. Thus, it is unlikely that the Na^+/K^+-ATPase contributes to the hyperpolarisations seen during cyclic nucleotide-mediated relaxation in the rat anococcygeus muscle.

It may be that the hyperpolarisations observed during cyclic nucleotide-mediated relaxation of the rat anococcygeus reflect modulation of Cl^- channels. In guinea pig ileum, non-adrenergic, non-cholinergic nerve stimulation produces an inhibitory junction potential which is due to a decrease in Cl^- conductance (Crist et al., 1991). Cl^- channels play an important role in mediating α_1-adrenoceptor responses in the rat anococcygeus muscle (Byrne and Large, 1987), with activation of these channels, subsequent to an increase in $[Ca^{2+}]_i$, leading to depolarisation. It may well be that the hyperpolarisation evoked by nitroprusside or forskolin results from a decrease in Cl^- conductance secondary to a decrease in $[Ca^{2+}]_i$ brought about by mechanisms such as stimulation of Ca^{2+} uptake and/or extrusion. The resultant hyperpolarisation would then serve to limit any further Ca^{2+} entry and so help to sustain the relaxation. Whether the hyperpolarisations observed in response to nitroprusside and forskolin in the present study are in fact the result of Cl^- channel modulation remains to be determined.

Pre-treatment of rat anococcygeus muscles with nitroprusside or forskolin did not, on its own, result in any detectable changes in membrane potential or force. This is consistent with an action on Cl^- conductance since these channels are unlikely to be operational in the resting tissue. Pre-treatment with the relaxants however, led to significant attenuation of subsequent phenylephrine-induced force and depolarisation (Fig. 7). In the pre-treated tissues, force and depolarisation rose slowly to attain similar levels to those attained when the relaxants were applied to pre-contracted muscles. This result suggests that the ability of the muscle to respond to excitatory stimulation had been significantly inhibited, possibly through inhibition by nitroprusside and forskolin of mechanisms involved in the activation of contraction. Again, this is consistent with an inhibitory action of these relaxants on the activation of Cl^- conductance.

In conclusion, it is apparent that K^+ channels are unlikely to play a significant role in mediating nitroprusside- and forskolin-induced relaxation in the rat anococcygeus muscle. Although both nitroprusside and forskolin produced a hyperpolarisation this was not reduced by any of the K^+ channel blockers employed in the present study. In addition, the nitroprusside- and forskolin-induced hyperpolarisations appeared secondary to relaxation, suggesting that mechanisms other than a change in membrane potential are primarily involved in initiating cyclic nucleotide-mediated relaxation in this tissue.

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