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Force and intracellular Ca^{2+} during cyclic nucleotide-mediated relaxation of rat anococcygeus muscle and the effects of cyclopiazonic acid

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1 Simultaneous recordings of tension and $[Ca^{2+}]_i$ were made in rat anococcygeus muscle strips to investigate possible mechanisms involved during cyclic nucleotide-mediated relaxation. Relaxation of pre-contracted muscles was induced by sodium nitroprusside (SNP) or forskolin and the effects of cyclopiazonic acid (CPA) on these responses were examined.

2 In muscles pre-contracted with 0.2 μM phenylephrine addition of SNP (10 μM) caused a rapid and near complete relaxation of force. This was accompanied by a decrease in $[Ca^{2+}]_i$, however, this was not of a comparable magnitude to the decrease in force. The level of $[Ca^{2+}]_i$ in muscles relaxed with SNP was shown to be associated with substantially higher force levels in the absence of SNP. Forskolin (10 μM) caused a slower, essentially complete relaxation which was associated with a proportional decrease in $[Ca^{2+}]_i$.

3 In muscles pretreated with SNP or forskolin subsequent responses to phenylephrine were attenuated with both force and $[Ca^{2+}]_i$ rising slowly to attain eventually levels similar to those observed when the relaxant was applied to pre-contracted muscles.

4 Exposure of the muscles to the sarcoplasmic reticulum Ca^{2+}-ATPase inhibitor, CPA (10 μM), resulted in a sustained increase in $[Ca^{2+}]_i$ which, in most cases, was not associated with any force development. The relaxation and decrease in $[Ca^{2+}]_i$ in response to both SNP and forskolin were attenuated and substantially slowed in the presence of CPA. Overall the extent of this attenuation was greater for SNP. For both SNP and forskolin, CPA attenuated the decrease in $[Ca^{2+}]_i$ to a greater extent than the decrease in force. In some cases, SNP-mediated relaxation in the presence of CPA was observed with almost no detectable change in $[Ca^{2+}]_i$.

5 The results suggest that, in the rat anococcygeus muscle under normal circumstances, a lowering of $[Ca^{2+}]_i$ can fully account for the relaxation induced by forskolin but not for that induced by SNP, where mechanisms independent of changes in $[Ca^{2+}]_i$ appear to contribute. Whilst Ca^{2+} sequestration into the sarcoplasmic reticulum plays a role in the relaxation mediated by both SNP and forskolin other Ca^{2+} lowering mechanisms may also be involved, especially in the response to forskolin.

Keywords: Anococcygeus muscle; intracellular Ca^{2+}; cyclopiazonic acid; relaxation; sarcoplasmic reticulum; sodium nitroprusside; forskolin; fura-2

Introduction

Relaxation of smooth muscle following the elevation of intracellular levels of either adenosine 3',5'-cyclic monophosphate (cyclic AMP) or guanosine 3',5'-cyclic monophosphate (cyclic GMP) is a widely published phenomenon. Although it is generally agreed that these second messengers can mediate relaxation of smooth muscle, the precise mechanisms responsible for causing relaxation in different types of smooth muscle are still not clearly defined. Many of the suggested mechanisms centre on a decrease in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) leading to relaxation (Morgan & Morgan, 1984; McDaniel *et al.*, 1991;1992). Elevations in intracellular levels of both cyclic AMP and cyclic GMP have been linked to a lowering of calcium levels inside smooth muscle cells and the cellular mechanisms proposed to be involved can be broadly characterized into the following: (i) an enhancement of Ca^{2+} sequestration into the sarcoplasmic reticulum (SR), (ii) increased Ca^{2+} extrusion across the plasmalemma, (iii) a reduction in Ca^{2+} influx and, (iv) an inhibition of SR Ca^{2+} release.

There is evidence that SR Ca^{2+} uptake is stimulated following elevations of both cyclic AMP and cyclic GMP (Ito *et al.*, 1993; Luo *et al.*, 1993). This may be mediated by phosphorylation of phospholamban since this regulatory protein of the SR has been shown to be a substrate for both the cyclic AMP-dependent and the cyclic GMP-dependent protein kinases (Raeymaekers *et al.*, 1990; Cornwell *et al.*, 1991). Enhanced Ca^{2+} extrusion can be achieved by stimulation of either the Na^+/Ca^{2+} exchanger or the plasmalemmal Ca^{2+}-ATPase and both of these Ca^{2+} extrusion pathways may be stimulated by cyclic nucleotides (Vrolix *et al.*, 1988; Furakawa *et al.*, 1988). $[Ca^{2+}]_i$ may also be reduced by inhibition of the processes responsible for liberating Ca^{2+} into the cytoplasm. Again there is evidence that both cyclic AMP and cyclic GMP can reduce Ca^{2+} influx. This may result from an action on the Ca^{2+} channels themselves, or occur secondary to an activation of K^+ channels with subsequent hyperpolarization of the membrane (Ahn *et al.*, 1992; Blatter & Wier, 1994; Hiramatsu *et al.*, 1994). Inhibition of Ca^{2+} release from the SR by cyclic GMP has also been suggested, possibly resulting from inhibition of either inositol trisphosphate (IP_3) production or IP_3-dependent Ca^{2+} mobilization (Hirata *et al.*, 1990; Murthy *et al.*, 1993).

Mechanisms independent of changes in $[Ca^{2+}]_i$ have also been proposed to be involved in the relaxation of smooth muscle to both cyclic AMP and cyclic GMP. The involvement of cyclic AMP in Ca^{2+}-independent relaxation is based on the observation that myosin light chain kinase (MLCK) could be phosphorylated by cyclic AMP-dependent protein kinase,

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thereby decreasing the activity of MLCK at any given $[Ca^{2+}]_i$ (de Lanerolle *et al.*, 1984). A reduction in the level of myosin light chain phosphorylation, independent of a change in $[Ca^{2+}]_i$ during cyclic GMP-dependent relaxation has also been obtained (Lincoln *et al.*, 1991), giving support to the possible involvement of Ca^{2+}-independent mechanisms in cyclic GMP-mediated relaxation as well.

The rat anococcygeus muscle contains a motor adrenergic and an inhibitory non-adrenergic, non-cholinergic (NANC) innervation. Increases in cyclic GMP but not cyclic AMP levels have been found to be associated with NANC relaxation in this muscle (Mirzazadeh *et al.*, 1991), and the NO donor, sodium nitroprusside (SNP), appears to mimic NANC nerve stimulation (Li & Rand, 1989). There is evidence that NANC relaxation was accomplished via a decrease in $[Ca^{2+}]_i$ which was inhibited by the nitric oxide synthase inhibitor, L-NMMA (Saha *et al.*, 1993). Very little is known about the nature of cyclic AMP-mediated relaxation in the rat anococcygeus muscle. It is known that this muscle contains very few or no β receptors on the muscle surface. However, direct application of cyclic AMP to permeabilised rat anococcygeus has been shown to cause relaxation (Chrichton *et al.*, 1989).

In the present study, we have investigated whether a lowering of $[Ca^{2+}]_i$ can account for relaxation of the rat anococcygeus muscle in response to cyclic AMP and cyclic GMP stimulation. In particular, we have examined the importance of the SR Ca^{2+}-ATPase in lowering $[Ca^{2+}]_i$ in cyclic nucleotide mediated relaxation of this muscle by using cyclopiazonic acid (CPA) to inhibit the SR Ca^{2+} uptake. Our findings lead us to conclude that a lowering of $[Ca^{2+}]_i$ is associated with both cyclic AMP and cyclic GMP mediated relaxations and that uptake of Ca^{2+} into the SR plays an important role in these responses. It appears, however, that more than one cellular mechanism is employed to lower cytosolic Ca^{2+} levels during relaxation in this muscle, and that the decrease in $[Ca^{2+}]_i$ in response to cyclic GMP elevation is more reliant on stimulation of the SR Ca^{2+}-ATPase than is that in response to cyclic AMP elevation. It is also clear that mechanisms independent of those that lower intracellular Ca^{2+} contribute to relaxation, particularly that mediated by cyclic GMP.

Methods

Muscle preparation

Anococcygeus muscles were dissected from male Sprague-Dawley rats (150–250 g) that had been killed by chloroform overdose. The paired bands of the muscle were exposed, carefully freed of connective tissue and one band was tied at both ends with 5.0 non-capillary silk. The muscle strip was subsequently excised and mounted in a 2.5 ml organ bath continuously superfused with Krebs-Henseleit solution maintained at 27°C. The muscle strips were orientated horizontally and fixed via the silk threads between two stainless steel pins. One pin acted as a fixed attachment point and the other was connected to a force transducer (AE801 semi-conductor element, SensoNor, Horten, Norway). An assembly of four micromanipulators allowed adjustment of muscle length as well as movement of the whole preparation in all three planes. Once mounted on the force recording apparatus the muscle strips were stretched to a passive force of 5–10 mN and left to equilibrate for 15 min in Krebs-Henseleit solution containing 1.5 mM Ca^{2+}. The muscles used in this study ranged in length from 5.5 to 11.0 mm and weighed between 1.3 and 5.2 mg.

All contractions in this study were isometric. Following the equilibration period stable contractile responses to the α-adrenoceptor agonist phenylephrine were established. During the experiments, unless otherwise stated, 0.2 μM phenylephrine applied in the presence of 1.5 mM Ca^{2+} was used as the standard contractile stimulus. Both SNP and forskolin were applied at a concentration of 10 μM. This combination of stimulus and relaxant concentrations was chosen since it resulted in reproducible submaximal force levels that, under control conditions, could consistently be relaxed completely or near completely by both of the relaxants employed.

Tissue fluorescence measurements

Simultaneous recordings of tension and tissue fluorescence were obtained with a system previously used in this laboratory (Munro & Wendt, 1994; Raymond *et al.*, 1995). Tissue fluorescence was recorded with a spectrophotometer system (Cairn Research Limited, Kent, U.K.) coupled with an inverted fluorescence microscope (Olympus IMT-2). The organ bath, which had a coverslip as its base, was mounted on the stage of the microscope and the muscle strip was carefully positioned over the microscope objective. Tissue autofluorescence of each preparation was measured at the beginning of the experiment. The muscle strips were then loaded with the fluorescent Ca^{2+} indicator, fura-2, by incubation in a solution containing the acetoxymethyl ester form of fura-2 (fura-2/AM) at a concentration of 5 μM for 3 h at room temperature. Following incubation, the muscle strips were washed with Ca^{2+}-free Krebs-Henseleit solution for 20 min.

A high pressure Xenon lamp (75 W) supplied excitation light that was directed through a rotating filter wheel containing four 340 and two 380 nm filters and then, via a light guide, to the epifluorescence attachment of the microscope. For these experiments the filter wheel was spun at 20 revolutions per second. The alternating excitation light was focussed onto the muscle surface with a ×40 u.v. objective (Nikon Fluor) and the emitted fluorescence at 510 nm was collected back through the objective and monitored by a photomultiplier tube. Excitation light was prevented from reaching the muscle during non-recording periods by closure of a solenoid-driven shutter. Following current to voltage conversion the signals from the photomultiplier tube during 340 and 380 nm excitation, together with the concurrent force signal, were digitised and recorded by personal computer. The fluorescence signals were subsequently corrected for tissue autofluorescence and the ratio of the fluorescence at 340 nm excitation to that at 380 nm ($R_{340/380}$) was taken as an indicator of $[Ca^{2+}]_i$. The autofluorescence at 380 and 340 nm excitation was consistently in the order of 5–10% and 10–20%, respectively, of the fluorescence signals recorded following loading of the muscles with fura-2.

Tissue fluorescence calibration

Since the relationship betwen $R_{340/380}$ and $[Ca^{2+}]_i$ is not linear, precise quantitation of the fura-2 fluorescence signals in terms of $[Ca^{2+}]_i$ requires calibration of these signals. There are, however, several assumptions and concomitant uncertainties in such calibrations which have led some authors to express reservations about presenting absolute $[Ca^{2+}]_i$ values from similar whole tissue fura-2 measurements (Karaki *et al.*, 1988; Lincoln *et al.*, 1990; Chen & van Breemen, 1993). In the present study an internal calibration was performed at the completion of the experiment in the majority of the muscles used. The maximum fluorescence ratio (R_{max}) was obtained by exposing the muscle to 10 μM of the Ca^{2+} ionophore, ionomycin, in the presence of Krebs-Henseleit solution containing 1.5 mM Ca^{2+}. Immediately after determining R_{max} the solution in the muscle bath was replaced with Ca^{2+}-free solution containing 2 mM EGTA and the minimum fluorescence ratio (R_{min}) determined. For the muscles on which this calibration was performed the mean values for R_{min} and R_{max} were 0.37 ± 0.03 and 2.71 ± 0.34, respectively ($n = 25$). Throughout these experiments the maximum value of $R_{340/380}$ recorded during stimulation of the muscles with 0.2 μM phenylephrine was on average $39 \pm 4\%$ of the R_{max} subsequently determined at the end of the experiment. $R_{340/380}$ values were converted to Ca^{2+} concentrations according the formula described by Grynkiewicz *et al.* (1985) with the assumption that the dissociation constant of fura-2 and Ca^{2+} is 224 nM.

The fluorescence data are presented as values of both $R_{340/380}$ and $[Ca^{2+}]_i$, where the latter is available from calibration information. However, given that there are some uncertainties about the precise $[Ca^{2+}]_i$ values, and since not all muscles were calibrated, the following approach was adopted to facilitate statistical analysis of changes occurring during the relaxation responses. For each muscle $R_{340/380}$ was assigned a value of 0% under resting conditions and 100% during the steady-state in response to stimulation with 0.2 μM phenylephrine and, within each muscle, changes during relaxation are expressed relative to this.

Solutions

The Krebs-Henseleit solution had the following composition (mM): NaCl 118, KCl 4.75, $MgSo_4$ 1.18, KH_2PO_4 1.18, $NaHCO_3$ 24.8, glucose 10 and $CaCl_2$ 1.5. This solution was continuously aerated with 95% O_2 – 5% CO_2, was maintained at 27°C, and had a pH of 7.4. In some experiments the Ca^{2+} concentration was varied and, when required, Ca^{2+}-free solution was obtained by omitting the $CaCl_2$ from the solution. High K^+ solution was prepared by equimolar substitution of KCl for NaCl in the Krebs-Henseleit solution. The solution used during the fura-2 loading procedure was buffered with N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid] (HEPES), rather than bircarbonate, and had the following composition (mM): NaCl 135.5, KCl 5.9, $MgCl_2$ 1.2, HEPES 11.6 and Pluronic F127 0.01% to aid dispersal of the fura 2-AM. To this solution was added an appropriate aliquot of a 1 mM solution of fura-2/AM in dimethyl sulphoxide (DMSO) to give a final fura-2/AM concentration of 5 μM.

Drugs and reagents

L-Phenylephrine HCl, sodium nitroprusside, forskolin, caffeine and CPA were obtained from Sigma Chemical Co (St Louis, MO, U.S.A.). Fura 2-AM and pluronic F27 were supplied by Molecular Probes (Eugene, OR, U.S.A.).

Concentrated stock solutions of all drugs used were prepared and appropriate aliquots of these were added to the experimental solutions to achieve the desired final concentrations. Phenylephrine (1 mM) and sodium nitroprusside (10 mM) were dissolved in distilled water, forskolin (10 mM) in 95% ethanol, and CPA (10 mM) in DMSO.

Statistics

All data expressed as mean ± s.e.mean. Differences between means have been assessed by paired Student's t test where appropriate, and $P<0.05$ was taken to indicate differences that were statistically significant.

Results

Intracellular Ca^{2+} levels at rest and during stimulation

In unstimulated muscle strips superfused with nominally Ca^{2+}-free Krebs-Henseleit solution $[Ca^{2+}]_i$ was 20 ± 3 nM ($n=25$). The muscles generally displayed no increase in tone when superfused with 1.5 mM Ca^{2+} containing solution although $[Ca^{2+}]_i$ rose to 32 ± 6 nM ($n=25$). Although this increase was only slight, it was nonetheless significant ($P<0.05$, paired t test). Upon stimulation with phenylephrine in 1.5 mM Ca^{2+} containing solution, both force and $[Ca^{2+}]_i$ increased to reach relatively steady levels that were well sustained for stimulus periods of up to 10 min (see Figure 1). The steady-state level of $[Ca^{2+}]_i$ during stimulation with 0.2 μM phenylephrine was 194 ± 18 nM ($n=25$) while in muscles stimulated with 1 μM phenylephrine it rose to 331 ± 72 nM ($n=5$). The steady-state force levels were 46 ± 4% higher during stimulation with 1 μM as compared to 0.2 μM phenylephrine ($n=7$).

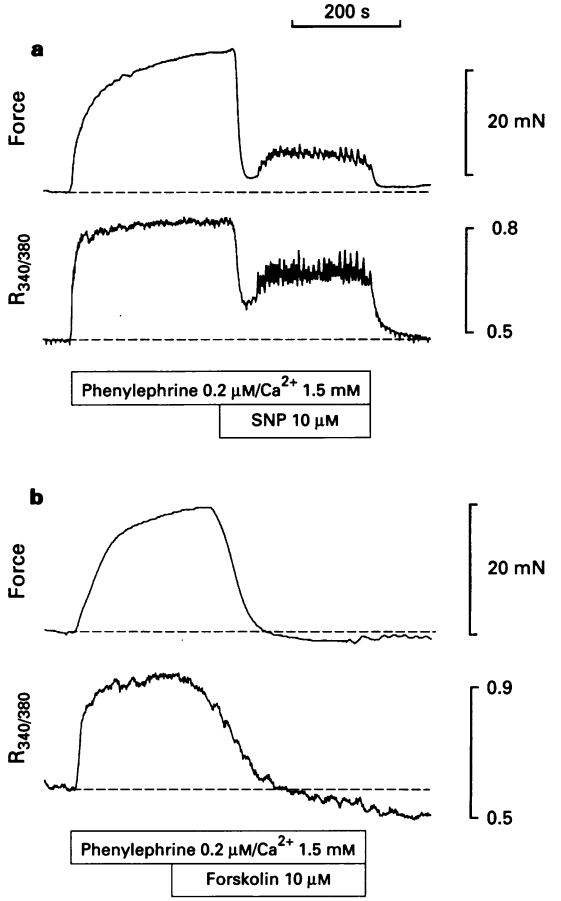

Figure 1 Simultaneous recordings of force and fura-2 fluorescence ratio ($R_{340/380}$) from a rat anococcygeus muscle contracted with 0.2 μM phenylephrine, and then relaxed with (a) sodium nitroprusside (SNP), or (b) forskolin. In most muscles, as seen here, SNP produced a biphasic response showing an initial rapid relaxation followed by a subsequent slight increase in force and $R_{340/380}$ which was associated with oscillations in both signals. Forskolin produced a slower relaxation with no evidence of any rebound or recovery during its application.

Relaxation induced by SNP

Addition of 10 μM SNP to muscles pre-contracted with phenylephrine caused a rapid decrease in both $[Ca^{2+}]_i$ and force (Figure 1a). In 18 out of 27 muscles, SNP produced a biphasic response consisting of an initial rapid relaxation followed by a rebound phase in which both the force and $[Ca^{2+}]_i$ recovered somewhat. The rebound phase of this response was often asssociated with the presence of relatively rapid oscillations in $[Ca^{2+}]_i$ and force as shown in Figure 1a. The extent of relaxation and decrease in $[Ca^{2+}]_i$ were assessed at the nadir of the response to SNP. This analysis revealed that, in muscles contracted with 0.2 μM phenylephrine, 10 μM SNP relaxed force by 93 ± 1% and decreased $R_{340/380}$ by 59 ± 4% ($n=27$). In those muscles in which the fura-2 signals were calibrated the $[Ca^{2+}]_i$ levels were 27 ± 7 nM at rest, 176 ± 25 nM during stimulation with phenylephrine, and 79 ± 11 nM at the nadir of the SNP relaxation response ($n=17$). The percentage decrease in suprabasal $[Ca^{2+}]_i$ produced by SNP was 64 ± 4%.

Proportionately, the decrease in $R_{340/380}$ produced by SNP was less than the extent of mechanical relaxation suggesting the possibility that the decrease in $[Ca^{2+}]_i$ may, by itself, not be able to account fully for the relaxation. To investigate this the $[Ca^{2+}]_i$ was graded in 4 muscles by stimulation with 0.2 μM phenylephrine in the presence of Ca^{2+} concentrations ranging from 0.1 – 1.5 mM. The force and $[Ca^{2+}]_i$ attained during a SNP relaxation response were then compared against the force

and $[Ca^{2+}]_i$ obtained during the graded stimulation. An example of such an experiment is shown in Figure 2a. It is clear that during the relaxation produced by SNP active force is completely abolished while $[Ca^{2+}]_i$, although reduced, is at a level that, during the graded stimulation, was associated with significant force development. In 4 muscles examined in this way, the force level during SNP-induced relaxation was $11 \pm 6\%$ of the control phenylephrine contraction while, at the equivalent $R_{340/380}$ value during the graded stimulation protocol, it was $56 \pm 14\%$ of the same control force. These values were found to be significantly different ($P < 0.05$, paired t test).

Relaxation induced by forskolin

Muscles contracted with phenylephrine could also be relaxed with forskolin as shown in Figure 1b. The relaxation to forskolin was considerably slower than that induced by SNP, the time to 50% relaxation being 58 ± 3 s ($n = 21$) as compared to 18 ± 1 s ($n = 27$) for the SNP (see Figure 7). The forskolin induced relaxation was also well sustained with there being no instances of the partial rebound often observed with SNP. In muscles contracted with 0.2 μM phenylephrine, 10 μM forskolin relaxed force by $99 \pm 1\%$ and decreased $R_{340/380}$ by $91 \pm 3\%$ ($n = 21$). The respective $[Ca^{2+}]_i$ levels, determined in those muscles in which the fura-2 signals were calibrated, were 34 ± 10 nM at rest, 230 ± 28 nM during stimulation with phenylephrine, and 44 ± 11 nM following relaxation with forskolin ($n = 9$). The percentage decrease in suprabasal $[Ca^{2+}]_i$ produced by forskolin was $93 \pm 3\%$. In contrast to SNP, forskolin appeared to lower $[Ca^{2+}]_i$ to levels below those required for active force development. This was confirmed in 3 muscles in which the force and $[Ca^{2+}]_i$ levels following relaxation with forskolin were compared directly with the levels attained during graded stimulation with 0.2 μM phenylephrine and varying solution Ca^{2+} concentrations (0.1 to 1.5 mM). In all 3 muscles forskolin produced complete relaxation of force and the associated $[Ca^{2+}]_i$ level was below that which was associated with detectable force development in the graded stimulation protocol. An example of such an experiment is shown in Figure 2b.

Effects of pretreatment with SNP or forskolin

Pretreatment of the muscle strips with SNP or forskolin was used to test further the effect of these relaxants in inhibiting contraction. Pre-incubation of the tissue with 10 μM SNP for 2 min before stimulation with 0.2 μM phenylephrine resulted in an attenuation of the force and $[Ca^{2+}]_i$ responses (Figure 3a). Both force and $[Ca^{2+}]_i$ rose slowly upon stimulation reaching final levels similar (within 10%) to those attained when SNP

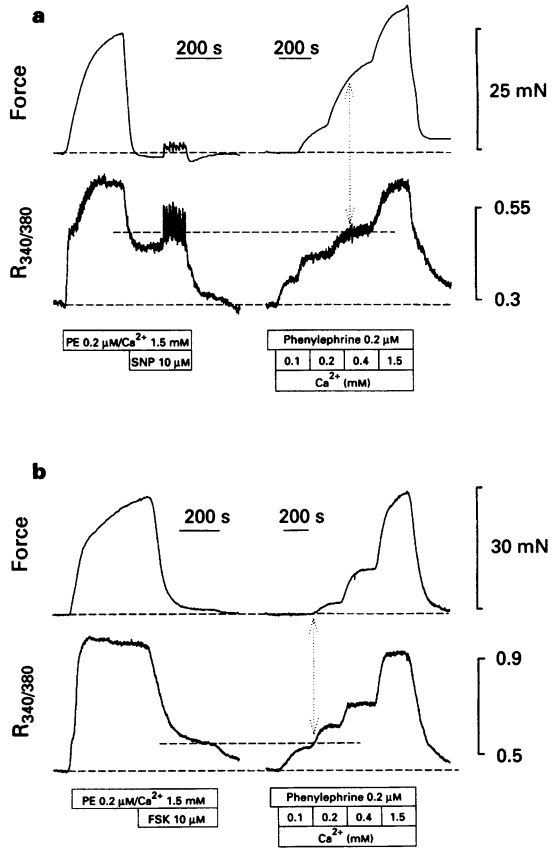

Figure 2 Records illustrating a comparison of the levels of force and $R_{340/380}$ attained during relaxation induced by (a) sodium nitroprusside (SNP), and (b) forskolin (FSK), with those attained during graded stimulation of the muscle. The left hand traces in each panel show the response of the muscle when initially contracted with 0.2 μM phenylephrine (PE) in the presence of 1.5 mM Ca^{2+}, and then relaxed with either SNP (a) or forskolin (b). The right hand traces are from the same muscle in each case and show the response when the muscle was stimulated with 0.2 μM phenylephrine in the presence of graded extracellular Ca^{2+} concentrations. (a) Shows that while SNP fully relaxed force, the $R_{340/380}$ level attained was, in the absence of SNP, associated with a substantial force response (right panel). In contrast, as shown in (b), forskolin reduced $R_{340/380}$ to a level below that associated with significant force development.

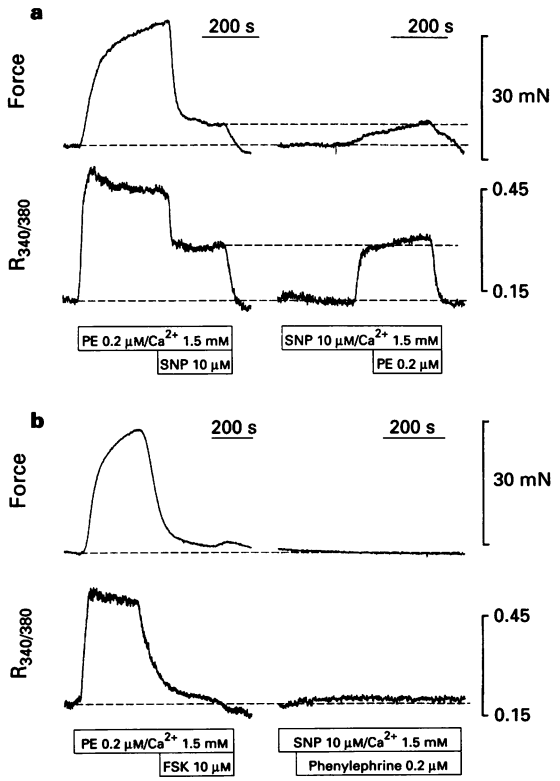

Figure 3 Records showing the effect of pretreatment with (a) sodium nitroprusside (SNP), and (b) forskolin (FSK) on phenylephrine contractions. In each panel the left hand records show the response when the relaxant was applied after the muscle had been contracted with 0.2 μM phenylephrine (PE) while the right hand records are from the same muscle and show the response when the muscle was stimulated with phenylephrine in the presence of the relaxant. Pretreatment with either SNP or forskolin markedly attenuated the subsequent response to phenylephrine, and in both cases force and $R_{340/380}$ rose slowly to levels similar to those attained when the relaxant was applied after contraction had been established.

was applied to the muscle after it had been precontracted with phenylephrine. Similar pretreatment of the muscle strips with 10 μM forskolin led to an essentially complete inhibition of the contractile and $[Ca^{2+}]_i$ responses upon the addition of 0.2 μM phenylephrine (Figure 3b). The addition of either SNP or forskolin to unstimulated muscles had no effect on the resting level of $[Ca^{2+}]_i$.

Effects of CPA on SNP and forskolin-induced relaxation responses

CPA was employed in this study to inhibit the uptake of Ca^{2+} by the SR. To ascertain that it was effectively inhibiting Ca^{2+} accumulation by the SR the effect of CPA on caffeine-induced Ca^{2+} release responses initiated in Ca^{2+}-free solution was assessed. To load the SR with Ca^{2+}, muscles were first stimulated for 4 min with high K^+ solution (64 mM KCl) containing 1.5 mM Ca^{2+}. The extracellular Ca^{2+} was then washed out for 3 min with Ca^{2+}-free solution containing 0.5 mM EGTA and the muscle challenged with 25 mM caffeine in Ca^{2+}-free solution. The effect of CPA on such a caffeine-induced Ca^{2+} release response is illustrated in Figure 4. Following 20 min exposure of the muscle to 10 μM CPA the response to caffeine was almost completely abolished. In 6 muscles tested in this way CPA reduced the peak of the caffeine-induced Ca^{2+} release response by $86 \pm 3.9\%$. This was taken as evidence that CPA markedly inhibits Ca^{2+} uptake by the SR.

The addition of 10 μM CPA alone to resting muscles bathed in 1.5 mM Ca^{2+}-containing solution led to a significant and sustained increase in the resting $[Ca^{2+}]_i$, from 39 ± 10 nM to 93 ± 23 nM ($n = 9$). Interestingly, this rise was generally not associated with the development of any detectable force. In 2 of the 11 muscles tested the addition of CPA did lead to a significant contractile response. However, the force slowly returned to the pre-CPA resting level over a period of about 30 min even though $R_{340/380}$ remained elevated. Addition of either SNP or forskolin to muscles in which the resting $[Ca^{2+}]_i$ had been elevated by CPA resulted in a slight decrease in $R_{340/380}$, as shown in Figure 5. This indicates that both these agents are able to produce a slight lowering of $[Ca^{2+}]_i$ in these circumstances.

Upon stimulation with phenylephrine $[Ca^{2+}]_i$ rose to higher levels after treatment of the muscles with CPA (261 ± 46 nM after CPA versus 215 ± 30 nM before CPA, $n = 9$). However, this appeared to be attributable to the higher resting level of $[Ca^{2+}]_i$ in the presence of CPA since the magnitude of the suprabasal increase was not significantly different before and after CPA. Despite $[Ca^{2+}]_i$ rising to higher absolute values upon phenylephrine stimulation after CPA, the peak force levels were not significantly different from those before CPA.

As shown in Figure 6a and b, CPA attenuated the extent of both the mechanical relaxation and the decrease in $R_{340/380}$. These effects of CPA were evident with the relaxation responses to both SNP and forskolin, although the responses to SNP were attenuated to a greater extent. Mean data for the effects of CPA on the extent of relaxation and decrease in $R_{340/380}$ produced by SNP and forskolin are presented in Table 1. This also reveals that CPA appeared to have a more pronounced effect on the decrease in $R_{340/380}$ than on the relaxation of force. This was particularly so in the case of SNP where, in several muscles, substantial relaxation was still observed after CPA with only, however, a very slight decrease in $R_{340/380}$. As is evident in Figure 6a and b, a second clear effect of CPA was to slow the rate of relaxation. The time to achieve 50% relaxation of the force response was significantly prolonged by CPA for both SNP- and forskolin-induced relaxation responses as shown in Figure 7.

Discussion

The $[Ca^{2+}]_i$ levels observed at rest and during stimulation with phenylephrine are consistent with those obtained for other smooth muscles and in particular are in agreement with those observed in the mouse anococcygeus by Boland *et al.* (1992). Contractions were rapidly and markedly relaxed by the addition of SNP. SNP is known to release nitric oxide and has been shown to cause relaxation via stimulation of guanylate cyclase and a subsequent increase in intracellular levels of cyclic GMP in many smooth muscle types, including the rat anococcygeus (Mirzazadeh *et al.*, 1991). The SNP-induced relaxation was associated with a rapid lowering of $[Ca^{2+}]_i$. In the rat ano-

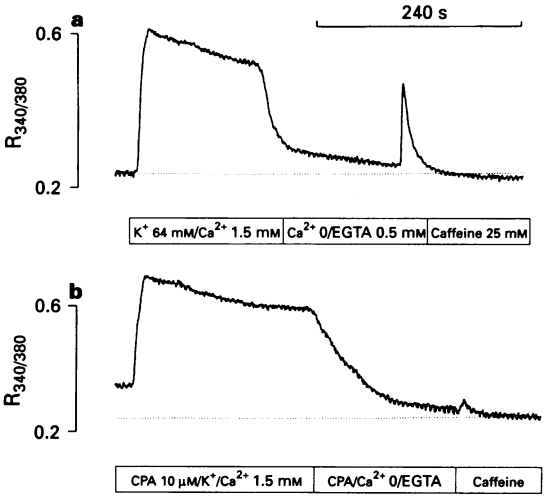

Figure 4 Recordings of $R_{340/380}$ showing the effect of cyclopiazonic acid (CPA) on Ca^{2+} loading of the sarcoplasmic reticulum (SR) in the rat anococcygeus muscle. The muscle was first stimulated with 64 mM K^+ to raise $[Ca^{2+}]_i$ and load the SR with Ca^{2+}. The muscle was then washed for 3 min with 0 Ca^{2+} solution containing 0.5 mM EGTA after which 25 mM caffeine was applied to release Ca^{2+} from the SR. (a) The control response while (b) is from the same muscle after it had been treated with CPA for 30 min. It is clear that CPA almost completely abolished the caffeine response. Two other features can be noted in the record following CPA treatment. Firstly, the elevated resting $[Ca^{2+}]_i$ when the muscle was in Ca^{2+} containing solution (i.e., the level before high K^+ stimulation in (b) and, secondly, the slower time course of the fall in $R_{340/380}$ during the 0 Ca^{2+} wash.

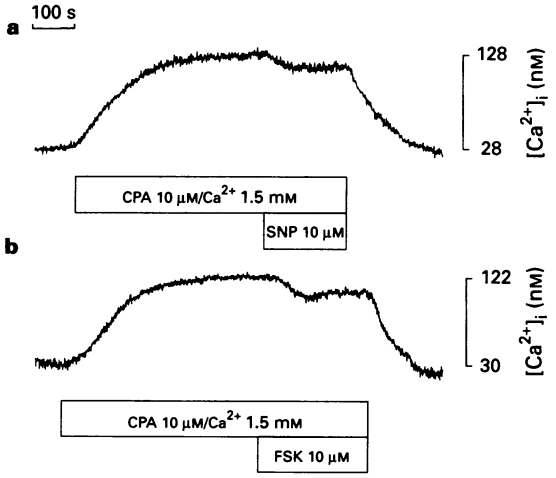

Figure 5 Recordings showing the effect of adding 10 μM cyclopiazonic acid (CPA) on the resting $[Ca^{2+}]_i$ of rat anococcygeus muscles bathed in Krebs-Henseleit solution containing 1.5 mM Ca^{2+}, and the effects of subsequent addition of sodium nitroprusside (SNP, a) or forskolin (FSK, b). Both SNP and forskolin were only able to produce a small lowering of $[Ca^{2+}]_i$ under these conditions.

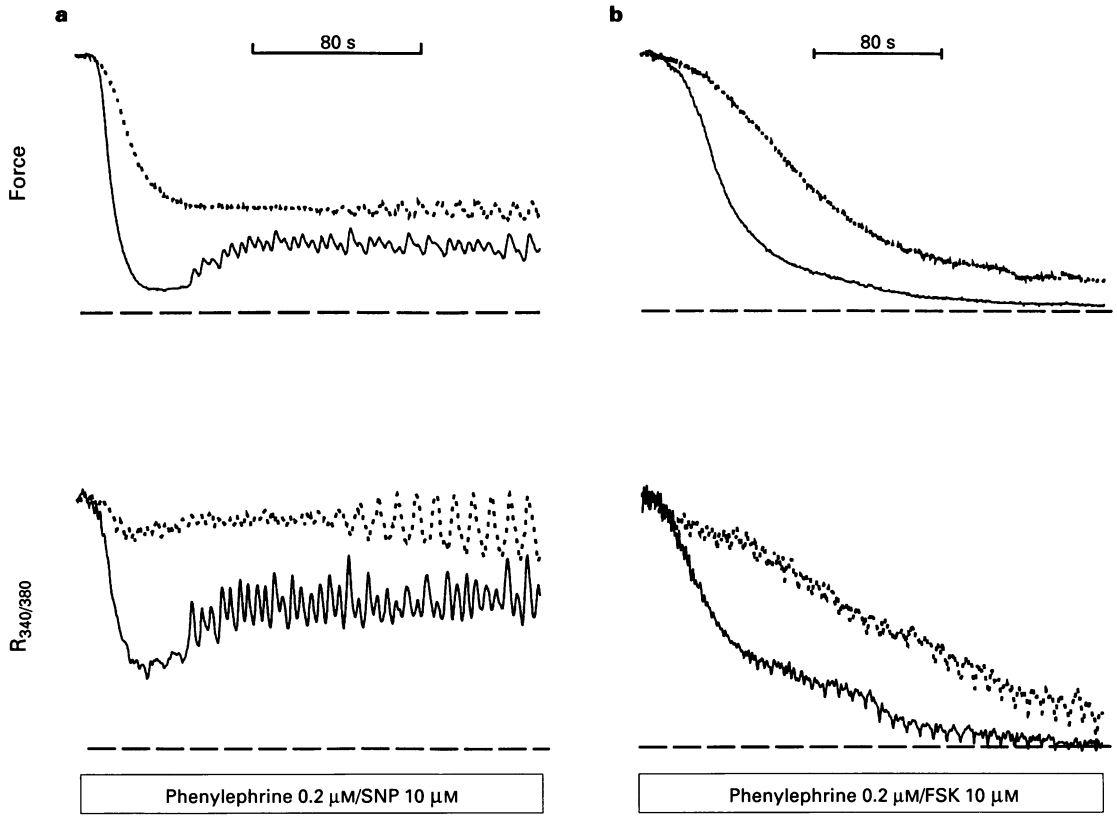

Figure 6 Records depicting the effect of cyclopiazonic acid (CPA) on the force (upper traces) and $R_{340/380}$ (lower traces) responses during (a) sodium nitroprusside (SNP), and (b) forskolin (FSK) mediated relaxation of a muscle contracted with phenylephrine. Only the relaxation profiles are shown and the dashed line in each panel represents the resting level of force or $R_{340/380}$ before stimulation with phenylephrine (defined as 0%). The responses before (solid lines) and after treatment of the muscle with CPA (broken lines) have been superimposed and normalised to the same amplitude (defined as 100%) just before the onset of the relaxation. For each relaxant the recordings before and after CPA are from the same muscle.

Table 1 Effects of cyclopiazonic acid (CPA) on the decreases in force and $R_{340/380}$ observed during sodium nitroprusside (SNP)- and forskolin-mediated relaxation of rat anococcygeus muscles contracted with phenylephrine

	SNP 10 μM ($n=10$)		*Forskolin 10 μM* ($n=11$)	
	Decrease in force (%)	*Decrease in $R_{340/380}$ (%)*	*Decrease in force (%)*	*Decrease in $R_{340/380}$ (%)*
Control	91.2±1.8	58.7±6.0	96.0±1.5	85.6±3.6
After CPA	72.9±2.2*	23.8±5.5*	90.4±2.6*	56.5±6.5*

Results are expressed as percentage decreases in the suprabasal force and fluorescence ratio levels generated by 0.2 μM phenylphrine. Data are shown as the mean±s.e.mean. Differences were assessed by Student's paired t test and *indicates the value after CPA is significantly different from control with $P<0.05$.

coccygeus, the relaxation in response to inhibitory NANC nerve stimulation is believed to be mediated by nitric oxide and has also been shown to be associated with a decrease in intracellular Ca^{2+} levels (Ramagopal & Leighton, 1989; Raymond *et al.*, 1995).

The decrease in $[Ca^{2+}]_i$ produced by SNP generally appeared to be proportionately less than the decrease in force. This suggested that the decrease in $[Ca^{2+}]_i$ alone may not be able fully to account for the relaxation and that some other mechanism independent of a change in $[Ca^{2+}]_i$ must also contribute to the response. Direct evidence for this was obtained in the experiment shown in Figure 2a where it is clear that the intracellular Ca^{2+} level attained during SNP-induced relaxation would normally give rise to a much greater force level when the muscle is not under the influence of this relaxant. This points towards a decrease in the apparent sensitivity of the contractile elements to Ca^{2+} being a contributing factor in cyclic GMP-mediated relaxation in the rat anococcygeus. In vascular smooth muscle it has also been shown that SNP-induced relaxation may not be associated with proportional decreases in $[Ca^{2+}]_i$ (Karaki *et al.*, 1988; McDaniel *et al.*, 1992). It has been proposed previously that relaxation independent of a lowering of $[Ca^{2+}]$ can occur as a result of the phosphorylation of myosin light chain kinase (Lincoln *et al.*, 1991). This reduces the activity of MLCK and effectively decreases the $[Ca^{2+}]_i$ sensitivity of myosin phosphorylation. It has been shown that cyclic GMP-dependent protein kinase is able to phosphorylate MLCK at one site. However, MLCK function did not appear to be altered by this phosphorylation (Nishikawa *et al.*, 1984). Alternatively SNP may lead to an uncoupling of stress from myosin phosphorylation. Recently, McDaniel *et al.* (1992) found that addition of nitrovasodilators to histamine-contracted arterial smooth muscle resulted in stress levels that were lower than expected for a given level of myosin phosphorylation. They suggested that thin filament, or cytoskeletal protein, regulation by either cyclic GMP or cyclic

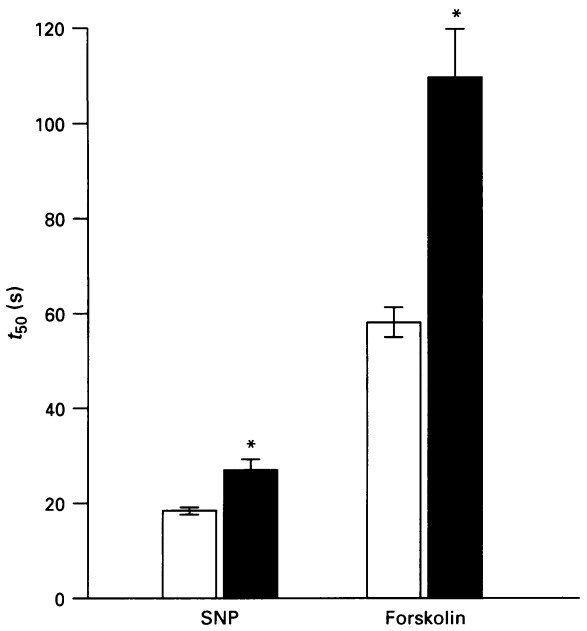

Figure 7 Effects of cyclopiazonic acid (CPA; solid columns) on time to 50% relaxation of force (t_{50}) during sodium nitroprusside (SNP) and forskolin mediated relaxation of phenylephrine contracted rat anococcygeus muscles (open columns). Data are expressed as mean ± s.e.mean with $n=10$ for SNP and $n=11$ for forskolin. *Indicates value after CPA is significantly different from control ($P<0.05$, Student's paired t test).

GMP-dependent protein kinase may be involved in this phenomenon. It seems likely, therefore, that mechanisms independent of a lowering of $[Ca^{2+}]_i$ contribute to cyclic GMP-induced relaxations in both vascular and non-vascular smooth muscle.

The rebound phenomenon observed during SNP relaxations has been observed previously in other gastrointestinal smooth muscles (Ward *et al.*, 1992) where it has been referred to as rebound excitation and has been shown to be associated with depolarization of the plasmalemma. In opossum oesophageal muscle Saha *et al.* (1993) believe that the rebound from SNP-induced relaxation may result from activation of a cyclo-oxygenase pathway, with stimulation of prostaglandin release that subsequently promotes a contractile response. Whether this occurs in the rat anococcygeus is not known. It is interesting, however, that this rebound from the relaxation was only seen with SNP and never occurred in the relaxations induced by forskolin.

Little is known about the cyclic AMP relaxation pathway in the rat anococcygeus. Forskolin, an activator of adenylate cyclase, elevates cyclic AMP levels in this muscle (Mirzazadeh *et al.*, 1991) and causes relaxation. From this it seems clear that the anococcygeus muscle contains the cellular machinery to facilitate cyclic AMP-mediated relaxation although it lacks β-adrenoceptors which are commonly associated with cyclic AMP-mediated relaxation in other smooth muscles. The relaxation to forskolin was consistently much slower than that to SNP. The reasons for this are not clear although two possibilities exist. Firstly, it may be that the mechanisms responsible for the forskolin relaxations are inherently slower in reducing the contractile force, and secondly, the slower response may simply reflect the fact that the activation of adenylate cyclase by forskolin and stimulation of cyclic AMP production is slower than is the activation of the cyclic GMP pathway by SNP.

Under control conditions the relaxation induced by forskolin was associated with a comparable decrease in $[Ca^{2+}]_i$. In muscles where forskolin completely relaxed the force response $[Ca^{2+}]_i$ was reduced to levels below those associated with detectable force development in the absence of the relaxant (Figure 2b). Based on this observation it would seem that with forskolin the decrease in $[Ca^{2+}]_i$ is, by itself, sufficient to account for the relaxation of force. This is consistent with observations in arterial smooth muscle where it was found that elevations of cyclic AMP caused relaxation primarily by decreasing $[Ca^{2+}]_i$, without changes in the Ca^{2+} senstivity of phosphorylation or the dependence of force on myosin phosphorylation (McDaniel *et al.*, 1991). Several other studies have demonstrated decreases in $[Ca^{2+}]_i$ during cyclic AMP-mediated relaxation (Abe & Karaki, 1989; Gunst & Bandyopadhyay, 1989). However, the fall in $[Ca^{2+}]_i$ alone does not in all cases appear to account fully for the relaxation (Morgan & Morgan, 1984; Takuwa *et al.*, 1988). It is important to bear in mind that differences in the strength of the contractile stimulus, the concentration of relaxant, and the type of smooth muscle used in these studies may influence the results obtained.

A possible involvement of a decrease in the Ca^{2+} sensitivity of the contractile elements in cyclic AMP-mediated relaxation must also be considered since it is known that MLCK can be phosphorylated by cyclic AMP-dependent protein kinase (de Lanerolle *et al.*, 1984; Nishikawa *et al.*, 1984). Whether this mechanism plays a physiological role in cyclic AMP-mediated relaxation has, however, been questioned (Stull *et al.*, 1990).

Pretreatment of muscles with either SNP or forskolin revealed that when subsequently challenged with phenylephrine the force and $R_{340/380}$ responses were markedly attenuated. In general, force and $[Ca^{2+}]_i$ rose slowly to levels that were similar to those attained when the relaxant was applied to precontracted muscles. With forskolin pretreatment subsequent contractile responses were essentially completely inhibited in the majority of muscles. This result indicates that the ability of the muscle to respond to excitatory stimulation is markedly inhibited by the relaxants which in turn suggests that some of the mechanisms involved in the activation of contraction may be shut down by pretreatment with these relaxants. There is a body of evidence suggesting that inhibition of Ca^{2+} influx as a result of blockage of voltage-gated Ca^{2+} channels plays an important role in the relaxation induced by both cyclic GMP (Clapp & Gurney, 1991; Blatter & Wier, 1994) and cyclic AMP (Ahn *et al.*, 1992; Hiramatsu *et al.*, 1994). The pretreatment results seem consistent in part with such a mechanism in the rat anococcygeus, since subsequent stimulation would result in attenuated Ca^{2+} influx with a blunted $[Ca^{2+}]_i$ and force response. If enhanced Ca^{2+} uptake and/or extrusion is primarily responsible for the relaxation, it might be expected that stimulation following pretreatment with the relaxants would still result in an initial mobilization of $[Ca^{2+}]_i$ and the onset of contractile activation which would then be overridden by the relaxing mechanisms. Alternatively it may be that the Ca^{2+} uptake and/or extrusion mechanisms are stimulated to such an extent by pretreatment with the relaxants that they are able to prevent $[Ca^{2+}]_i$ from rising upon subsequent stimulation.

One of the primary mechanisms that has been proposed to be involved in the lowering of $[Ca^{2+}]_i$ and relaxation induced by both cyclic AMP and cyclic GMP is an enhancement of Ca^{2+} sequestration by the SR due to stimulation of the SR Ca^{2+}-ATPase, possibly resulting from phosphorylation of phospholamban (Raeymaekers *et al.*, 1990; Cornwell *et al.*, 1991). CPA is believed to be a selective inhibitor of the SRCa^{2+}-ATPase (Uyama *et al.*, 1992) and was employed in this study to inhibit the uptake of Ca^{2+} by the SR. That it was effective in doing so was confirmed by the marked attenuation of caffeine-induced Ca^{2+} release responses following treatment of muscles with CPA. The relaxation and the decrease in $[Ca^{2+}]_i$ in response to both SNP and forskolin were reduced by CPA. In addition, relaxation was significantly slowed. This indicates that uptake of Ca^{2+} by the SR contributes to the lowering of $[Ca^{2+}]_i$ and relaxation mediated by both cyclic AMP and cyclic GMP. The responses to SNP were inhibited to a greater extent than those to forskolin suggesting that increased SR Ca^{2+} uptake may play a greater role in cyclic GMP-mediated relaxation. This is consistent with the recent

findings of McGrogan *et al.* (1995) which led them to conclude that, in canine tracheal smooth muscle, Ca^{2+} pumping into the SR plays a major role in cyclic GMP-induced but not cyclic AMP-induced relaxation.

The inhibitory effect of CPA was more noticeable on the decrease in $[Ca^{2+}]_i$ than the decrease in force. This was particularly noticeable for the SNP response where CPA reduced the decrease in $R_{340/380}$ by some 60% while the extent of mechanical relaxation was only reduced by 20%. Indeed in some muscles SNP produced almost no detectable decrease in $[Ca^{2+}]_i$ after CPA, although a substantial relaxation still occurred. This highlights the involvement of a mechanism(s) independent of a lowering of $[Ca^{2+}]_i$ by the SR in the relaxation mediated by cyclic GMP. A similar persistence of relaxation with little change in $[Ca^{2+}]_i$ following CPA treatment has also been seen with NANC nerve stimulation in this muscle (Raymond *et al.*, 1995).

Inhibition of Ca^{2+} uptake by the SR with CPA had less effect on the responses to forskolin, although again the decrease in $[Ca^{2+}]_i$ was affected to a greater extent, with the relaxation of force being reduced by only 6% while the decrease in $R_{340/380}$ was reduced by 34%. As a result, following CPA, the forskolin-induced decrease in force was no longer accompanied by a comparable decrease in $[Ca^{2+}]_i$ and so appeared to unmask a contribution of mechanisms independent of a decrease in $[Ca^{2+}]_i$ to the cyclic AMP-mediated response as well. The relaxation, although not inhibited to a great extent, was nevertheless considerably slowed by CPA, which suggests that SR Ca^{2+} uptake does contribute to the response under normal circumstances. It is possible that multiple mechanisms exist to produce relaxation and that if one becomes unavailable others can compensate to a large degree.

A substantial decrease in $[Ca^{2+}]_i$ in response to forskolin was still observed after CPA, and some lowering of $[Ca^{2+}]_i$ by SNP also persisted. This indicates the involvement of other $[Ca^{2+}]_i$ lowering mechanisms beside SR Ca^{2+} uptake. Candidates include inhibition of Ca^{2+} influx and/or release and enhanced Ca^{2+} extrusion. There is evidence for some contribution of each of these mechanisms to cyclic nucleotide-mediated relaxation in certain smooth muscles (Vrolix *et al.*, 1988; Hirata *et al.*, 1990; Ahn *et al.*, 1992; Blatter & Wier, 1994). Their precise contribution in the rat anococcygeus remains unknown although it has been shown that SNP has no effect on IP_3 release (Gibson *et al.*, 1994). Incomplete inhibition of SR Ca^{2+} uptake cannot also be ruled out.

In recent years evidence has accumulated supporting a role for the filling state of the SR in determining Ca^{2+} influx. In particular, depletion of SR Ca^{2+} appears to activate Ca^{2+} entry from the extracellular space (Putney 1990; Chen & van Breemen, 1993) which may aid in the refilling of Ca^{2+}-depleted stores or contribute to raising the cytoplasmic Ca^{2+} concentration. In rat urinary bladder smooth muscle it has been shown that depletion of SR Ca^{2+} caused by application of CPA results in Ca^{2+} influx and a sustained elevation of $[Ca^{2+}]_i$ (Munro & Wendt, 1994). In our study, the application of CPA also initiated a sustained rise in $[Ca^{2+}]_i$ and, interestingly, both SNP and forskolin were only able to lower slightly $[Ca^{2+}]_i$ under these conditions (Figure 5). This may simply be due to the fact that the SR Ca^{2+}-ATPase is of course inhibited by CPA, or it may also indicate that these relaxants have a limited ability in attenuating Ca^{2+} entry through this Ca^{2+} release-activated Ca^{2+} entry pathway which appears not to involve voltage-gated Ca^{2+} channels (Munro & Wendt, 1994).

Recent studies have suggested that both cyclic AMP and cyclic GMP may relax smooth muscle by activating cyclic GMP-dependent protein kinase. Cyclic GMP-dependent protein kinase can be activated by both cyclic GMP and cyclic AMP although much higher concentrations of the latter are required (Lincoln *et al.*, 1990). In the present study there were differences between the responses to SNP and forskolin suggesting that the underlying mechanisms could not be entirely common. However, that a part of the relaxation in response to forskolin is in fact mediated by cyclic GMP-dependent protein kinase certainly cannot be ruled out. It may be that the differences can be accounted for by the fact that forskolin activates both cyclic AMP-dependent protein kinase and cyclic GMP-dependent protein kinase while SNP predominantly activates cyclic GMP-dependent protein kinase.

In conclusion, the results show that relaxation responses observed in the rat anococcygeus muscle to both SNP and forskolin involve a lowering of intracellular Ca^{2+}. Under normal circumstances, the lowering of $[Ca^{2+}]_i$ can fully account for the relaxation induced by forskolin but not for that induced by SNP, where mechanisms independent of changes in $[Ca^{2+}]_i$ appear to contribute. Sequestration of Ca^{2+} by the sarcoplasmic reticulum plays a substantial role in the relaxation mediated by both cyclic AMP and cyclic GMP; however, other $[Ca^{2+}]_i$ lowering mechanisms may also be involved, especially in the response to forskolin.

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