Variable potency of nitroergic-nitrovasodilator relaxations of the mouse anococcygeus against different forms of induced tone

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1 U46619 (thromboxane A₂ receptors; 0.002–1 μ M), carbachol (muscarinic M₃ receptors; 0.1–100 μ M), cyclopiazonic acid (CPA; Ca²⁺-ATPase inhibitor; 0.1–30 μ M) and K⁺ (5–100 mM) produced concentration-dependent contractions of the mouse isolated anococcygeus muscle. Equi-effective, sub-maximal concentrations of each agent were used in further experiments (40 nM U46619; 5 μ M carbachol; 5 μ M CPA; 70 mM K⁺).

2 Nifedipine (1 μ M) totally abolished contractile responses to K⁺; those to U46619, carbachol and CPA were reduced by only 20–30% in the presence of nifedipine, but were greatly reduced (>90%) by a combination of nifedipine and SKF 96365 (0.1–40 μ M).

3 In Ca²⁺-free medium, contractions to K⁺ and CPA were abolished. Small residual responses remained to both carbachol and U46619; those to carbachol were transient, could not be repeated in the continued absence of Ca²⁺ and were prevented by pre-incubation with CPA, but unaffected by SKF 96365; those to U46619 were sustained, could be repeated in the absence of Ca²⁺, and were resistant to CPA and SKF 96365.

4 Tone induced by all four agents could be relaxed by sodium nitroprusside (SNP), but with a clear order of potency. SNP (pIC₅₀) was most effective against U46619 (7.92), less so against carbachol (6.80) and CPA (6.68), and least potent against K⁺ (5.94). A similar order of potency was observed with 8Br-cyclic GMP (50 μ M) and nitroergic field stimulation (1–20 Hz).

5 The relaxant potency of SNP was similar in normal Krebs solution and in high K⁺ (70 mM) Krebs containing 1 μ M nifedipine.

6 Inclusion of SNP (0.01–1 μ M) or 8Br-cyclic GMP (50 μ M) in the Ca²⁺-free medium inhibited the transient residual response to carbachol. Inclusion of similar concentrations of SNP or 8Br-cyclic GMP, during Ca²⁺ re-loading, increased the subsequent residual contraction to carbachol in Ca²⁺-free medium.

7 At higher concentrations, SNP (0.1–10 μ M) produced a partial relaxation of the sustained contraction to U46619 in Ca²⁺-free medium.

8 Thus, the relaxant potency of the nitroergic stimuli was dependent on the agent and mechanism used to induce tone in the preparation. Examination of the contractile/relaxant interactions suggests that altered Ca²⁺ sequestration and inhibition of contractile protein function may underlie nitroergic relaxations of this tissue.

Keywords: Anococcygeus; 8Br-cyclic GMP; carbachol; cyclopiazonic acid; nifedipine; nitroergic stimulation; potassium; SKF 96365; sodium nitroprusside; U46619

Introduction

Anococcygeus muscles from rats and mice have proved useful models for the investigation of nitroergic neurotransmission in non-vascular smooth muscle (Rand, 1992). Field stimulation-induced relaxations are reduced by inhibitors of nitric oxide synthase (Gillespie *et al.*, 1989; Li & Rand, 1989; Ramagopal & Leighton, 1989; Gibson *et al.*, 1990) while relaxations to nitrovasodilator drugs, which mimic the effects of nitroergic stimulation via a postsynaptic action, are unaffected. Nitric oxide synthase has been detected in homogenates of the anococcygeus (Mitchell *et al.*, 1991) and has been localized by immunocytochemistry, to 6-hydroxy-dopamine-resistant nerves within the tissue (Brave *et al.*, 1993). Postsynaptically, nitroergic relaxations result from activation of the guanylyl cyclase/guanosine 3':5'-cyclic monophosphate (cyclic GMP) system since relaxations to field stimulation and nitrovasodilator drugs are reduced by inhibitors of guanylyl cyclase, enhanced by inhibitors of phosphodiesterase, and are associated with an increase in cyclic GMP within the tissue (Gibson & Mirzazadeh, 1989; Mirzazadeh *et al.*, 1991). However, the cellular mechanism(s) by which cyclic GMP effects relaxation is not known. In

vascular smooth muscle, nitrovasodilator drugs also act through cyclic GMP (Waldman & Murad, 1987) and several mechanisms have been proposed for the relaxations observed in these tissues, including membrane hyperpolarization, inhibition of phospholipase C, increased removal of Ca²⁺ from the cytosol and direct inhibition of contractile protein function (for reviews see Lincoln, 1989; Lincoln & Cornwell, 1993). In contrast, the mechanisms underlying NO/cyclic GMP-induced relaxations of non-vascular smooth muscle have received much less attention, mainly because the controlling influence of nitroergic nerves in such tissues has only recently been recognised. We have therefore begun a series of experiments to investigate the cellular mechanisms associated with nitroergic relaxations of the anococcygeus and, in the present study, we have investigated whether the ability of nitroergic activation (presynaptically via field stimulation, or postsynaptically via application of sodium nitroprusside [SNP] or 8Br-cyclic GMP) to cause relaxations of the mouse anococcygeus is dependent on the agent and mechanism, used to induce tone in the preparation. Four contractile agents, with varying cellular mechanisms, were used: (1) carbachol, which acts on muscarinic (M₃) receptors (Sideso *et al.*, 1994) believed to be linked to the phospholipase C/inositol 1,4,5 trisphosphate (IP₃)/diacylglycerol pathway; (2)

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U46619, a thromboxane A₂ receptor agonist, also believed to stimulate phospholipase C but which, in addition, directly sensitizes the contractile proteins to Ca²⁺, possibly via a G-protein (Himpens *et al.*, 1990; Crichton *et al.*, 1993; Kurata *et al.*, 1993); (3) cyclopiazonic acid (CPA), a reversible inhibitor of the Ca²⁺-ATPase of the sarcoplasmic reticulum (SR), which has been shown to increase cytosolic Ca²⁺ concentrations in several cell types via a Ca²⁺-release activated Ca²⁺-entry (CRAC) pathway (Low *et al.*, 1991; Demaurex *et al.*, 1992; Missaïen *et al.*, 1992) and (4) K⁺, which depolarizes the tissue leading to opening of voltage operated Ca²⁺ channels (VOCCs). A preliminary account of some of these results has been published (Gibson *et al.*, 1994b).

Methods

Male mice (LACA strain; 25–35 g) were killed by stunning and exsanguination. The anococcygeus muscles were dissected and set up in 1 or 2 ml glass organ baths containing Krebs-bicarbonate buffer (mM: NaCl 118.1, KCl 4.7, MgSO₄ 1.0, KH₂PO₄ 1.0, NaHCO₃ 25.0, glucose, 11.1 and CaCl₂ 2.5) which was maintained at 37°C and gassed continuously with 95% O₂:5% CO₂. A resting tension of 200–400 mg was placed on the tissue and changes in tension recorded with a Grass FT03 force-displacement transducer attached to a Graphtec pen recorder (WR3101). Muscles were allowed to equilibrate for 45 min before beginning the experiment. In all experiments, except those involving nitrenergic field stimulation, the Krebs solution also contained 1 µM phentolamine and 50 µM N^G-nitro-L-arginine (L-NOARG), and the tissues were preincubated for 10 min with 30 µM guanethidine (during the 45 min equilibration period); these drugs were used to prevent any effects due to release of transmitter from either the motor noradrenergic or inhibitory nitrenergic nerves within the tissue. In experiments involving nitrenergic field stimulation L-NOARG was omitted. Field stimulation (1 ms; 70 V) was applied via two parallel platinum electrodes running down either side of the tissue.

Concentration-response curves to contractile agents were constructed in a non-cumulative manner. The agents were left in contact with the tissue until the contraction had reached a plateau and the succeeding concentration was not added until tone had returned to baseline following washout.

To obtain relaxations, tone was first raised by the appropriate concentration of contractile agent. Once the plateau contraction had been established the relaxant stimulus was applied. Concentration-response curves to SNP were obtained in a cumulative fashion. Relaxations to 8Br-cyclic GMP were obtained with a single concentration of 50 µM, which has been shown to relax carbachol-induced tone of the mouse anococcygeus by about 50% (Gibson & Mirzazadeh, 1989). Results were expressed as % relaxation of induced tone. pIC₄₀ values (–log₁₀ of the molar concentration of relaxant required to produce a 40% reduction of induced tone) were calculated by regression analysis of individual concentration-response curves.

K⁺-rich solutions were prepared by substituting the appropriate amount of KCl for NaCl; Ca²⁺-free solutions were prepared by omission of CaCl₂ and addition of EGTA (1 mM). For experiments in Ca²⁺-free solution, control contractions were first established in normal medium. The muscle was then bathed in Ca²⁺-free medium for 5 min before challenging with contractile agent; 5 min was found to be the minimum time for complete loss of the response to 70 mM K⁺. If Ca²⁺ was to be re-admitted, the muscle was returned to normal medium for 15 min.

Results are expressed as mean ± s.e.mean. Statistical analysis was by Student's *t* test (unpaired) with *P* < 0.05 taken as indicating a significant difference.

The following drugs were used: 8-bromo-cyclic guanosine

monophosphate (Sigma); carbachol (Aldrich); cyclopiazonic acid (Sigma); guanethidine sulphate (Ciba); nifedipine (Sigma); N^G-nitro-L-arginine (Sigma); phentolamine mesylate (Ciba); SKF 96365 (1-[β-[3-(4-methoxyphenyl) Propoxy]-4-methoxyphenethyl]-1H-imidazole HCl; Smithkline Beecham); sodium nitroprusside (Sigma); U46619 (9,11-dideoxy-11α,9α-epoxymethano prostaglandin F_{2α}; Sigma).

All drugs were dissolved in distilled water except: cyclopiazonic acid (10 mM stock in dimethyl sulphoxide); nifedipine (10 mM stock in ethanol); U46619 (1 mM stock in ethanol).

Results

Contractions to U46619, carbachol, CPA and K⁺

U46619 (0.002–1 µM), carbachol (0.1–100 µM), CPA (0.1–30 µM) and K⁺ (5–100 mM) produced concentration-related contractions of the mouse anococcygeus (Figure 1). The contractions to all four agents were sustained, and were reversed on washout. The onset of contraction following addition of U46619, carbachol and K⁺ was much faster (2–3 s) than that observed with CPA (1–2 min). In all subsequent experiments, equi-effective sub-maximal concentrations of each agent were used: 40 nM U46619, 5 µM carbachol, 5 µM CPA and 70 mM K⁺ (Figure 1).

A number of experiments were performed to determine the source of Ca²⁺ utilized by each of the agents to produce contraction. Following 5 min incubation in Ca²⁺-free medium, contractions to both K⁺ and CPA were absent (Figure 2a); in the case of both U46619 and carbachol small residual contractions remained which amounted to about 5% of those obtained in normal medium (Figures 2a and 3). Although of similar magnitude, the characteristics of the two responses were quite different (Figure 3). Those to carbachol were transient and could not be repeated in the continued absence

Figure 1 Concentration-response curves for contractions of the mouse anococcygeus in response to U46619 (●), carbachol (○), cyclopiazonic acid (▲) and K⁺ (△). Each point represents the mean ± s.e.mean from at least five different muscle preparations.

Figure 2 Histograms of contractions of the mouse anococcygeus to sub-maximal concentrations of K⁺ (70 mM), U46619 (40 nM), carbachol (CCh; 5 μM) and cyclopiazonic acid (CPA; 5 μM) under control conditions (open columns), in Ca²⁺-free medium (cross-hatched columns in a), or in the presence of 1 μM nifedipine (cross-hatched columns in b). Each point represents the mean ± s.e. mean from at least five different muscle preparations. *Significantly different from adjoining column.

of Ca²⁺ (not shown). However, after re-admission of Ca²⁺ for 15 min, a normal response to carbachol was obtained and, following a 5 min exposure to Ca²⁺-free medium, a further transient contraction could be elicited (Figure 3a). Addition of 5 μM CPA, during the 5 min incubation in Ca²⁺-free medium, greatly reduced the residual response to carbachol and this inhibition was reversed on washout of the CPA; a higher concentration of CPA (20 μM) completely blocked the contraction to carbachol in Ca²⁺-free medium (Figure 3a). On the other hand, the residual responses to U46619 in Ca²⁺-free medium were sustained, could be repeated (at least four times) in the continued absence of Ca²⁺, and were unaffected by inclusion of 5 μM CPA (Figure 3b).

In the presence of 1 μM nifedipine, responses to K⁺ were again totally blocked (Figure 2b), but those to U46619, carbachol and CPA were reduced by only 20–30% in each case. However, the nifedipine-resistant component of the contractions to U46619, carbachol and CPA was reduced, in a concentration-dependent manner, by SKF 96365 (0.1–40 μM; Figure 4), which inhibits both voltage- and non-voltage-dependent Ca²⁺-entry processes (Merritt *et al.*, 1990); contractile responses to CPA were more sensitive to the inhibitory effect of SKF 96365 than those to either carbachol or U46619 (Figure 4). SKF 96365 had no effect on the residual responses to either carbachol or U46619 in Ca²⁺-free medium (against carbachol, response in presence of 30 μM SKF 96365 was 96 ± 18% of that in its absence; against U46619, response in presence of 20 μM SKF 96365 was 109 ± 14% of that in its absence; *n* = 5, *P* > 0.05 in both cases).

Relaxations to SNP, 8Br-cyclic GMP and nitroergic field stimulation

In these experiments, tone was again raised by the equi-effective, sub-maximal concentrations of the contractile agents mentioned previously.

Figure 3 Traces showing contractions of the mouse anococcygeus muscle in response to carbachol (5 μM in a; ●) or U46619 (40 nM in b; ●). The first trace in each panel shows a control response in normal medium. In (a), the muscle was then bathed in Ca²⁺-free medium (0Ca) for 5 min and then challenged with carbachol, which produced a small, transient contraction; following re-admission of Ca²⁺ for 15 min a normal response to carbachol was obtained. Inclusion of cyclopiazonic acid (CPA), during the 5 min incubation in Ca²⁺-free medium, reduced the residual response to carbachol. In (b), following the control response, the muscle was bathed in Ca²⁺-free medium for 5 min and then challenged with U46619, which produced a small, sustained contraction. This response to U46619 could be repeated in the continued absence of Ca²⁺, and was unaffected by CPA.

Figure 4 Concentration-response curves for inhibition by SKF 96365 of the nifedipine-resistant contraction to cyclopiazonic acid ($5 \mu\text{M}$; $\blacktriangle$), U46619 (40 nM ; $\bullet$) and carbachol ($5 \mu\text{M}$; $\circ$) in the mouse anococcygeus. Experiments were carried out in the presence of $1 \mu\text{M}$ nifedipine. Each point represents the mean $\pm$ s.e.mean from at least five different muscle preparations.

Figure 5 Concentration-response curves for relaxations of the mouse anococcygeus to sodium nitroprusside against tone induced by U46619 (40 nM ; $\bullet$), carbachol ($5 \mu\text{M}$; $\circ$), cyclopiazonic acid ($5 \mu\text{M}$; $\blacktriangle$) and K^+ (70 mM ; $\triangle$). Each point represents the mean $\pm$ s.e.mean from at least five different muscle preparations.

SNP produced concentration-related relaxations of tone induced by each agent (Figure 5), but with a clear order of potency; SNP was most potent against tone induced by U46619 ($\text{pIC}_{50} 7.92 \pm 0.13$; $n = 5$), equipotent against tone induced by carbachol ($\text{pIC}_{50} 6.80 \pm 0.07$; $n = 5$) and CPA ($\text{pIC}_{50} 6.68 \pm 0.10$; $n = 5$), and least potent against tone induced by K^+ ($\text{pIC}_{50} 5.94 \pm 0.02$; $n = 5$).

A similar order of potency was observed with $50 \mu\text{M}$ 8Br-cyclic GMP, which relaxed U46619-induced tone by $97 \pm 1\%$, carbachol-induced tone by $55 \pm 5\%$, CPA-induced tone by $52 \pm 7\%$ and K^+ -induced tone by $34 \pm 3\%$ ($n > 5$ in each case).

Nitrgic field stimulation (10 s train every 100 s) also caused frequency-dependent relaxations of tone induced by U46619, carbachol and CPA (Figure 6) and again, was most potent against U46619. The $\log_{10}$ of the frequency-reducing tone by 40% was 0.5 ± 0.09 against U46619, 0.78 ± 0.09 against carbachol, and 0.81 ± 0.09 against CPA ($n = 5$ in each case). For obvious reasons, nitrgic relaxations of K^+ -induced tone could not be studied.

Experiments were also carried out to determine the effects of SNP and 8Br-cyclic GMP on the contractions to carbachol and U46619 obtained in Ca^{2+} -free medium; the results of these are given in Figure 7. Addition of SNP (0.01 – $1 \mu\text{M}$) during the incubation in Ca^{2+} -free medium, 1 min before challenge with carbachol, produced a concentration-related inhibition of the residual transient contraction to carbachol (Figure 7). However, if SNP (0.01 – $10 \mu\text{M}$) was included during the 15 min period of Ca^{2+} re-admission, but omitted during the subsequent 5 min in Ca^{2+} -free medium, then a concentration-related potentiation of the residual response to carbachol was observed (Figure 7). Similar effects were observed with 8Br-cyclic GMP; inclusion of $50 \mu\text{M}$ 8Br-cyclic GMP during the 5 min incubation in Ca^{2+} -free medium (added 4 min before carbachol) reduced the residual response to carbachol by $79 \pm 8\%$ ($n = 5$), but inclusion of $50 \mu\text{M}$ 8Br-cyclic GMP during the period of Ca^{2+} -reloading enhanced the residual response to carbachol by $98 \pm 18\%$ ($n = 5$).

Addition of SNP (0.01 – $10 \mu\text{M}$), during the sustained con-

Figure 6 Frequency-response curves for relaxations of the mouse anococcygeus to field stimulation (10 s train) against tone induced by U46619 (40 nM ; $\bullet$), carbachol ($5 \mu\text{M}$; $\circ$) and cyclopiazonic acid ($5 \mu\text{M}$; $\blacktriangle$). Each point is the mean $\pm$ s.e.mean from at least five different muscle preparations.

traction to U46619 in Ca^{2+} -free medium, produced a concentration-related relaxation of tone (Figure 7), but it was considerably less potent against the residual response to U46619 than against that to carbachol.

A number of experiments were carried out to determine whether the potency of SNP varied in the presence of nifedipine and in depolarizing medium. In these experiments, tone was raised with $5 \mu\text{M}$ carbachol. The potency of SNP

Figure 7 Concentration-response curves showing the effects of sodium nitroprusside (SNP) on contractions to carbachol ($5 \mu\text{M}$; ■ and ●) and U46619 (40 nM ; ▲) in Ca^{2+} -free medium (see Figure 3); (■) show the potentiating effect of SNP, present during the duration of the 15 min Ca^{2+} -reloading period but omitted during the subsequent 5 min in Ca^{2+} -free medium, on the residual response to carbachol; (●) show the inhibitory effect of SNP added during the Ca^{2+} -free incubation, 1 min before challenge with carbachol; (▲) show the relaxant effect of SNP on the sustained response to U46619 in Ca^{2+} -free medium. Each point is the mean $\pm$ s.e.mean from at least five different muscle preparations.

(0.05 – $5 \mu\text{M}$) against carbachol was similar ($P > 0.05$) whether the experiments were carried out in normal Krebs (pIC_{50} 6.90 ± 0.10 ; maximum relaxation $92 \pm 3\%$; $n = 6$), normal Krebs + $1 \mu\text{M}$ nifedipine (pIC_{50} 7.05 ± 0.05 ; maximum relaxation $95 \pm 1\%$; $n = 5$), or high K^+ (70 mM) Krebs + $1 \mu\text{M}$ nifedipine (pIC_{50} 7.06 ± 0.07 ; maximum relaxation $96 \pm 1\%$; $n = 6$).

Discussion

Actions of the contractile agents

K^+ -induced contractions were clearly due to entry of Ca^{2+} through VOCCs since responses were abolished in Ca^{2+} -free medium and in the presence of nifedipine. Although K^+ can cause complex effects on tone of the anococcygeus, due to non-selective depolarization of both smooth muscle cells and nerve terminals (Gibson & James, 1977), the effects of the latter were negated by the use of sympatholytic drugs (phen-tolamine and guanethidine) and a nitric oxide synthase inhibitor (L-NOARG).

Both U46619 (thromboxane A_2 receptors) and carbachol (muscarinic M_3 receptors) are believed to activate the phospholipase C/ IP_3 /diacylglycerol system and thereby release Ca^{2+} from the SR. However, in other smooth muscles, it has been found that U46619 produces a relatively weak activation of phospholipase C, as reflected in a lower release of Ca^{2+} from the SR, but that it also possesses a marked ability to sensitize the contractile proteins, possibly by way of a G protein (Himpens *et al.*, 1990; Crichton *et al.*, 1993; Kurata *et al.*, 1993). This difference was apparent in our experiments in Ca^{2+} -free medium. Carbachol produced a transient contractile response which could not be repeated in the continued absence of Ca^{2+} . This transient contraction is likely to be due to the release of Ca^{2+} from the SR since it was reduced by CPA, which depletes the SR of Ca^{2+} by inhibiting Ca^{2+} -ATPase (Demaurex *et al.*, 1992); refilling of

this store would occur during the period of Ca^{2+} re-admission. On the other hand, in Ca^{2+} -free medium, U46619 produced a sustained contraction which could be repeated in the continued absence of Ca^{2+} and which was unaffected by CPA. This contraction, therefore, is likely to be due to the sensitizing action of U46619 on the contractile proteins; it is not clear whether the U46619 sensitizes the contractile proteins to the low levels of cytoplasmic Ca^{2+} which would exist in Ca^{2+} -free medium or whether the effect is Ca^{2+} independent (Matsuo *et al.*, 1989; Ozaki *et al.*, 1990). The lack of a rapid, transient component in the response to U46619 in Ca^{2+} -free medium would be consistent with a low release of Ca^{2+} from the SR, insufficient to be detected as a contraction.

The contractions to both carbachol and U46619 in Ca^{2+} -free medium were only some 5% of those obtained in normal medium, suggesting that the major source of Ca^{2+} used by these agents comes from the extracellular space. Entry of Ca^{2+} through VOCCs contributes only in small part to the overall response since contractions were reduced by only 20–30% in the presence of nifedipine, at a concentration which completely blocked contractions to K^+ . This suggests that U46619 and carbachol also activate a non-voltage-dependent Ca^{2+} entry process, a suggestion confirmed by the observation that the nifedipine-resistant contraction to both agents was greatly reduced by SKF 96365, which blocks both voltage- and non-voltage-dependent Ca^{2+} entry (Merritt *et al.*, 1990). The selectivity of SKF 96365 against Ca^{2+} entry processes was demonstrated by its lack of effect on contractions to U46619 and carbachol in Ca^{2+} -free medium. One possibility is that U46619 and carbachol, by reducing the Ca^{2+} content of the SR, stimulate the CRAC pathway which has been detected in several cell types (Penner *et al.*, 1993) including smooth muscle (Missaien *et al.*, 1992). Some evidence that this mechanism may exist in the anococcygeus was provided by the observation that CPA produced powerful, sustained contractions.

CPA inhibits the Ca^{2+} -ATPase of the SR, and thereby depletes the SR of Ca^{2+} and activates the CRAC pathway (Demaurex *et al.*, 1992); entry of Ca^{2+} through the CRAC pathway is prevented by SKF 96365. The contractions to CPA in the anococcygeus were dependent on extracellular Ca^{2+} , were only slightly reduced by nifedipine, but were completely blocked by a combination of nifedipine and SKF96365. The lack of contraction to CPA in Ca^{2+} -free medium, and the slower onset of contraction in normal medium, probably reflect the slower depletion of Ca^{2+} from the SR (Demaurex *et al.*, 1992). These results would therefore be consistent with CPA-induced opening of a CRAC pathway, but definitive evidence would come from patch-clamp studies of single smooth muscle cells, and such studies are in progress.

As mentioned, a small component of the contractions to U46619, carbachol and CPA was inhibited by nifedipine, suggesting a small contribution of Ca^{2+} entry through VOCCs. Opening of VOCCs probably results from depolarization caused by Ca^{2+} -activated Cl^- channels known to occur in several smooth muscle cell types (Hogg *et al.*, 1994), including the anococcygeus (Byrne & Large, 1987); any agent increasing cytosolic Ca^{2+} will tend to open such channels.

Despite the variety of mechanisms, and sources of Ca^{2+} , utilized by the four contractile agents, tone induced by each agent could be relaxed by nitrergic field stimulation, SNP and 8Br-cyclic GMP, although with a clear order of potency (U46619 > carbachol = CPA > K^+). These observations may be helpful in identifying the cellular mechanisms underlying nitrergic relaxations.

Actions of relaxant stimuli

Field stimulation, SNP and 8Br-cyclic GMP were potent relaxants of tone induced by U46619, carbachol and CPA, with SNP producing maximum relaxations of >90% in each

case; contractions to these agents were, however, much less sensitive to block by nifedipine. In contrast, K^+ -induced tone, which was totally blocked by nifedipine, was much less sensitive to relaxation by the nitroergic stimuli, with SNP producing a maximum relaxation of only 50%. In addition, both SNP and 8Br-cyclic GMP retained normal potency against carbachol in depolarizing medium. Although these results do not rule out a small contribution of membrane hyperpolarization to the overall relaxation to nitroergic activation such an action could be of only minor importance. In this respect, the mouse anococcygeus differs from the canine proximal colon in which membrane hyperpolarization is thought to be important for nitroergic relaxations (Thornbury *et al.*, 1991).

As discussed in the preceding section, CPA induces Ca^{2+} influx via inhibition of the Ca^{2+} -ATPase of the SR, an action which by-passes phospholipase C and which is not accompanied by an increased metabolism of inositol phosphates (Demaurex *et al.*, 1992; Fasolato *et al.*, 1994). The observation that nitroergic field stimulation, SNP and 8Br-cyclic GMP were potent relaxants of tone induced by CPA suggests that inhibition of phospholipase C is unlikely to underlie nitroergic relaxations of the mouse anococcygeus. This suggestion is supported by the recent finding that carbachol-induced inositol phosphate generation in the rat anococcygeus is unaffected by nitroergic field stimulation, SNP or 8Br-cyclic GMP (Gibson *et al.*, 1994a) and is consistent with observations in several other non-vascular smooth muscles in which it has been shown that inhibition of inositol phosphate metabolism does not underlie relaxations to nitrovasodilators or cyclic GMP (Akhtar & Abdel-Latif, 1991; Chilvers *et al.*, 1991; Garcia-Pascual & Triguero, 1994).

In Ca^{2+} -free medium, the transient, residual contraction to carbachol is likely to reflect release of Ca^{2+} from the SR (see previous discussion). The observation that inclusion of SNP or 8Br-cyclic GMP during the period of Ca^{2+} -reloading increased the subsequent residual response to carbachol suggests that these relaxant drugs increased the carbachol-releasable store of Ca^{2+} in the SR. The concentrations of SNP required to increase the residual response to carbachol were similar to those which relaxed carbachol-induced tone in normal medium, and which inhibited the residual response to carbachol when included in the Ca^{2+} -free medium. Thus, modification of the cellular mobilization of Ca^{2+} may be an important mechanism of nitroergic relaxations of the mouse anococcygeus, which would be consistent with the observed reduction in cytosolic Ca^{2+} associated with nitroergic relaxa-

tions of the rat anococcygeus (Ramagopal & Leighton, 1989). There is considerable evidence that nitrovasodilators and cyclic GMP can increase the pumping of Ca^{2+} into the SR, possibly through phosphorylation of the regulatory protein phospholamban and consequent activation of Ca^{2+} -ATPase (Lincoln, 1989; Cornwell *et al.*, 1991; Lincoln & Cornwell, 1993). Pumping of Ca^{2+} into the SR would reduce cytosolic concentrations of the ion leading to relaxation; it would also indirectly affect any CRAC pathway, since filling of the SR would reduce the stimulus for CRAC opening. One difficulty in accepting that increased pumping of Ca^{2+} into the SR is important in nitroergic relaxations of the anococcygeus is the observation that field stimulation, SNP and 8Br-cyclic GMP were potent relaxants of tone induced by CPA, which is an inhibitor of the Ca^{2+} -ATPase of the SR. It may be that the nitroergic stimuli increase Ca^{2+} loading of the SR through a CPA-insensitive pathway (Bourreau *et al.*, 1993), or that other mechanisms are involved, such as reduced release of Ca^{2+} from the SR (Lincoln & Cornwell, 1993).

Also in Ca^{2+} -free medium, SNP could relax the residual, sustained contraction to U46619, which probably results from a direct action of the thromboxane-mimetic on the contractile proteins. SNP may, therefore, exert a direct inhibitory effect on the contractile proteins, as has been suggested in several vascular smooth muscle preparations (Pfitzer *et al.*, 1984; Nishimura & van Breemen, 1989). This effect, however, was observed with concentrations of SNP considerably higher than those which relaxed tone induced by U46619, carbachol or CPA in normal medium, although similar to those which relaxed K^+ -induced tone. Thus, inhibition of contractile protein function may be more important against K^+ -induced tone than against the other agents used. Experiments using chemically skinned preparations are in progress to examine this possibility.

In conclusion, the results of this study have shown that the relaxant potency of nitroergic stimuli in the mouse anococcygeus muscle is dependent on the cellular mechanisms activated to induce tone in the preparation. Analysis of the contractile/relaxant interactions suggests that neither membrane hyperpolarization nor inhibition of phospholipase C can play major roles in nitroergic relaxations of this tissue, but that further study of the effects of nitroergic activation on Ca^{2+} fluxes and on contractile protein function is warranted.

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