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Characteristics of the NANC post-stimulus ('rebound') contraction of the urinary bladder neck muscle in sheep

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1 Strips of muscle from sheep bladder neck were set up for tension recording and subjected to electrical field stimulation (EFS) to stimulate their intramural nerves.

2 In the presence of atropine (1 μM) and guanethidine (1 μM), the response to 1 Hz EFS was biphasic, characterized by a relaxation during the stimulus period, followed by a post-stimulus contraction. A similar biphasic response was also seen following bolus application of nitric oxide (NO).

3 In the absence of atropine and guanethidine, the relaxations were masked by contractions during stimulation; however, the post-stimulus contractions were unaffected. L-NAME (100 μM) blocked the post-stimulus contractions and L-arginine (1 mM) restored them, suggesting that they were NO-mediated.

4 M&B 22948, a phosphodiesterase inhibitor, prolonged the relaxations and abolished the post-stimulus contractions. This suggests that rapid removal of cyclic GMP is required for post-stimulus contraction to occur.

5 When the number of pulses in the stimulus train was kept constant, the size of the post-stimulus contraction increased as the duration of the preceding period of stimulation increased. Maximal post-stimulus contractions were obtained following stimulation for >40 s.

6 The L-channel antagonist, nifedipine (1 μM) and verapamil (1 μM), had little effect on the amplitude of the post-stimulus contractions.

7 In contrast, ryanodine (8 μM) reduced the post-stimulus contractions by over 90%. Caffeine (20 mM) also abolished the post-stimulus contractions and cyclopiazonic acid (CPA, 10 μM) reduced them by 76%. However, in the presence of CPA a slower post-stimulus contraction developed. Nifedipine (1 μM) reduced this by 40%.

8 In conclusion, these results support a role for NO in the post-stimulus contraction of the sheep bladder neck muscle. The post-stimulus contraction depends more on release of intracellular Ca^{2+}, than Ca^{2+} influx through L-type channels.

Keywords: Nitric oxide; inhibitory innervation; bladder; rebound contraction; nifedipine; verapamil; ryanodine; cyclopiazonic acid, Ca^{2+} stores

Introduction

Stimulation of non-adrenergic, non-cholinergic (NANC) nerves in visceral smooth muscle has been reported in many instances to cause a contraction which occurs only after cessation of the stimulus (Bennett, 1966; Campbell, 1966; Wood & Marsh, 1973; Burnstock *et al.*, 1975; Den Hertog & Van Den Akker, 1979; Bywater *et al.*, 1981; Ward *et al.*, 1992). This has variously been referred to as a 'rebound' or 'post-stimulus' contraction. While the basis of the post-stimulus contraction remains uncertain, one common feature is that it occurs in tissues which characteristically show marked inhibitory responses during electrical field stimulation. It has thus been assumed to result from either release of an excitatory effect (normally masked by inhibition during stimulation) or to be a 'myogenic rebound' phenomenon, possibly due to depolarization following a preceding hyperpolarization, or some other consequence of a preceding period of relaxation/inhibition (Bennett, 1966; Campbell, 1966; Wood & Marsh, 1973; Bywater *et al.*, 1981).

Nitric oxide, however, appears to account for both the inhibitory and post-stimulus excitatory components of the NANC response in the canine colon (Ward *et al.*, 1992). The post-stimulus excitation in this tissue is characterized by a prolongation of the plateau phase of the electrical slow wave, which is sensitive to blockade with dihydropyridines (Ward & Sanders, 1992). This suggests that transmembrane Ca^{2+} influx is an important component of rebound contraction in this tissue. In the sheep bladder neck muscle, NANC stimulation results in a relaxation and post-stimulus contraction, both of which are blocked by N^G nitro-L-arginine methyl ester (L-NAME; Thornbury *et al.*, 1992). However, little is known about the processes which underlie the post-stimulus contraction in the bladder neck. For example, it is not known whether the post-stimulus contraction requires a preceding period of inhibition (i.e. is some kind of 'myogenic' rebound) or if it results from a more specific effect of NO. Also, the source of intracellular Ca^{2+} responsible for the contraction has not been investigated. The aims of the present study were to (i) confirm whether NO is likely to account for the post-stimulus contraction in the bladder neck and (ii) characterize further the response with a view to gaining more information regarding the underlying mechanisms.

Methods

Urinary bladders of sheep of either sex were obtained from an abattoir approximately 15 min after slaughter and transported in oxygenated Krebs solution to the laboratory for dissection. Circularly-orientated rings were cut from the region of the bladder just below the trigone and these were opened and the mucosa removed by sharp dissection to give strips with approximate dimensions of 10 mm × 2 mm × 2 mm. The strips

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were mounted in organ baths (volume 5 ml), with initial tension adjusted to 6 mN, and perfused with Krebs solution of composition (mM): NaCl 120, $NaHCO_3$ 25, KCl 5.9, NaH_2PO_4 1.2, $CaCl_2$ 2.5, $MgCl_2$ 1.2 and glucose 11; gassed with O_2 (95%) and CO_2 (5%). Tension changes were measured with Statham UC3 and Dynamometer UF1 isometric transducers, the outputs of which were written on Gould TA240S or Lectromed MX 216 chart recorders. Electrical field stimulation (EFS) was applied via platinum ring electrodes mounted at either end of the tissue strip. Pulses of 0.3 ms duration were delivered in trains at constant frequencies of 0.2–16 Hz from a Grass S88 stimulator set at nominal output voltages of 70 V. Stimulation periods lasted from 5 s to 80 s and the recovery time between stimuli was usually 5 min. In most experiments the preparations were perfused continuously with Krebs solution and drugs were delivered in the perfusing solution. However, when testing the effect of ryanodine a different approach was used to reduce costs. In these experiments the tissue was mounted in 5 ml organ baths which were continuously bubbled with O_2 (95%) and CO_2 (5%). These baths were also perfused, but when ryanodine was applied the perfusion was switched off. Field stimulation experiments were then started after 30 min exposure. In experiments where the effect of ryanodine on responses to exogenous NO was tested a similar approach was used, with the exception that 10 ml baths were used.

Drugs

The following were used: guanethidine sulphate (Sigma), atropine sulphate (Sigma), noradrenaline acid tartrate (Sanofi Winthrop), N^G-nitro-L-arginine methyl ester (L-NAME; Sigma), L-arginine (Sigma); nifedipine (Sigma); verapamil (Sigma); caffeine (Sigma); ryanodine (Calbiochem); cyclopiazonic acid (CPA; Calbiochem); M&B 22948 (2-0-propoxyphenyl-8-azapurin-6-one; Zaprinast) was a gift from Rhone-Poulenc Rorer, Dagenham. Noradrenaline, atropine and guanethidine were made up to their final concentration in the Krebs solution from stock solutions also mixed in Krebs. CPA, caffeine, L-arginine and L-NAME were directly made up to their final concentrations in Krebs solution. Nifedipine and verapamil were diluted to final concentration from stock solutions (10^{-2} M) in methanol. M&B 22948 was diluted to final concentration from stock (10^{-2} M) in NaOH solution (0.1 M).

Statistics

Summarized results are expressed throughout as the mean response ± s.e.mean, and statistical comparisons were made with Student's paired t test (two-tailed), taking the $P<0.05$ level as significant.

Results

Effects of NO and field stimulation

Previous work has suggested that NO mediates the post-stimulus contraction in bladder neck muscle (Thornbury *et al.*, 1992). To substantiate this idea further, the response to exogenous NO was examined to see if it was similar to the effect of NANC nerve stimulation. These experiments were performed with guanethidine (1 μM) and atropine (1 μM) in the Krebs solution to block adrenergic and cholinergic responses. Neither of these drugs had any effect on the baseline tension in this tissue. Figure 1 shows a recording from an experiment where the effect of a 1 min period of field stimulation at 1 Hz was compared with that of bolus application of NO. EFS produced a relaxation throughout the stimulus period and this was followed by a sharp post-stimulus contraction similar to those reported previously. Responses such as these have been shown previously to be completely abolished by tetrodotoxin (1 μM; Thornbury *et al.*, 1992). When exogenous nitric oxide was applied to the tissue (0.1 ml of 1 mM solution to 5 ml bath) there was an initial relaxation followed by a contraction (Figure 1). The relaxation was similar in size to the one induced by EFS at 1 Hz, but the contraction was smaller in amplitude. Contraction following NO-induced relaxation was seen in only 7 of 20 preparations tested, even though all developed post-stimulus contractions following NANC nerve stimulation.

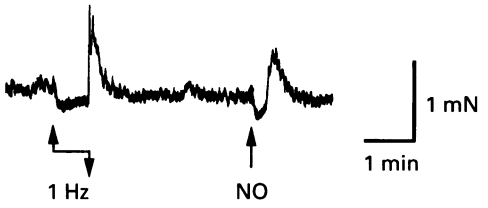

Figure 1 The effect of EFS at 1 Hz and bolus of NO (0.1 ml of 1 mM solution to 5 ml bath) in sheep bladder neck muscle. Atropine (1 μM) and guanethidine (1 μM) were present.

Post-stimulus contractions without a preceding period of inhibition

These experiments were performed without atropine and guanethidine present. Under these circumstances the NO-mediated relaxation is masked by a contraction during the period of stimulation (Figure 2a). Despite this, on cessation of the stimulus, there was a sharp post-stimulus contraction (top panel). Addition of L-NAME (100 μM) to the Krebs solution

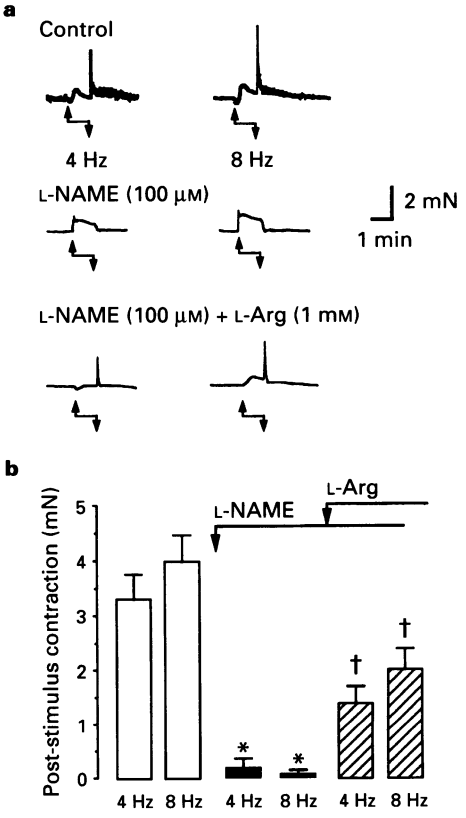

Figure 2 (a) Responses recorded without atropine and guanethidine present. Control (top trace) shows responses to 4 Hz and 8 Hz stimulation in absence of drugs (top trace), stimulations were then repeated in the presence of L-NAME (100 μM; middle trace) and then in the continued presence of L-NAME and L-arginine (1 mM; bottom trace). (b) Summary of 6 experiments where this protocol was followed; open columns; controls; solid columns; L-NAME (100 μM); hatched columns L-arginine (1 mM). *Significant depression in presence of L-NAME ($P<0.05$); †significant recovery in presence of L-NAME and L-arginine ($P<0.05$).

abolished the post-stimulus contraction, but potentiated the contraction during stimulation (Figure 2a, middle panel). In the continued presence of L-NAME, L-arginine (1 mM) restored the post-stimulus contraction. A summary of 6 such experiments is presented in Figure 2b, which shows that L-NAME significantly reduced the post-stimulus contraction ($P<0.05$), while L-arginine partially reversed this effect ($P<0.05$). These results suggest that a preceding period of relaxation is not a prerequisite for an NO-mediated post-stimulus contraction. Therefore, the post-stimulus contraction in this case may not represent a 'rebound' response to a period of inhibition *per se*, but a more specific effect of NO.

Effect of M&B 22948

In order to gain information as to the possible involvement of guanosine 3':5'-cyclic monophosphate (cyclic GMP) in the responses, the effect of the specific cyclic GMP phosphodiesterase inhibitor, M&B 22948, was tested. Initial experiments indicated that this drug almost completely inhibited spontaneous tone, making the responses to EFS difficult to interpret. To circumvent this problem, tone was induced with exogenous noradrenaline (10 μM) and 60 s periods of EFS at 1 Hz were delivered before and after the addition of M&B 22948. A single experiment is shown in Figure 3a, where M&B 22948 prolonged the relaxation and abolished the post-stimulus contraction. This result was typical of a total of 7 such experiments where M&B 22948 had little effect on the amplitude of relaxation (3.30 ± 0.73 mN before, and $3.91.5\pm1.03$ mN after exposure, $P>0.2$), but the duration of relaxation was prolonged from 1 min to an average of 4.2 ± 0.4 min ($P<0.001$). Before M&B 22948 the post-stimulus contraction was 2.26 ± 0.76 mN, and this was completely abolished in the presence of the drug ($P<0.03$). These results suggest that rapid removal of cyclic GMP was necessary in order to observe the post-stimulus contraction. In the light of this, it seemed possible that part of the difficulty in reproducing the post-stimulus contraction with exogenous NO (see above) might have been explained by more gradual 'wash out' of exogenous versus endogenous NO. To test this possibility, exogenous NO (0.5 ml of 1 mM solution to 5 ml bath) was added during period of EFS (Figure 3b). This reduced the EFS-induced post-stimulus contraction, compared to the control (0.5 ml of Krebs solution injected into the bath).

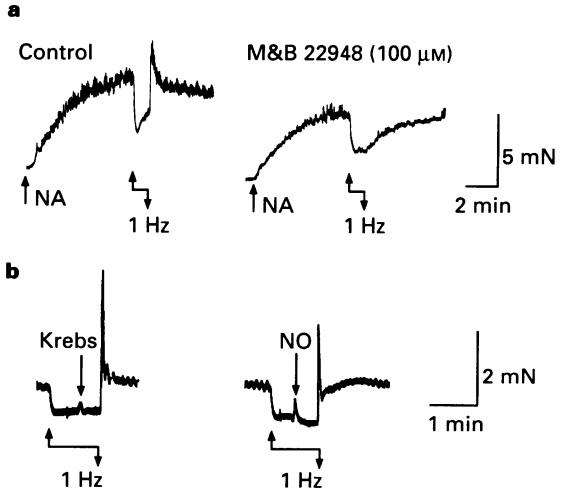

Figure 3 (a) Effect of M&B 22948: the preparation was precontracted with noradrenaline (10 μM; NA); atropine and guanethidine (both 1 μM) were present throughout. The preparation was stimulated at 1 Hz for 60 s, before (left trace), and after 30 min exposure to M&B 22948 (100 μM; right trace). (b) Effect of NO (0.5 ml bolus of 1 mM solution to 5 ml bath) on the EFS-induced post-stimulus contraction compared to injection of 0.5 ml Krebs solution. Atropine (1 μM) and guanethidine (1 μM) were present throughout.

Effect of varying duration of stimulus period

It was found that, when stimulating preparations with fixed frequencies of 1 Hz for periods of 10 to 60 s, the size of the post-stimulus contraction increased with increasing stimulus duration (e.g. Figure 5a). Since a fixed stimulus frequency of 1 Hz was used for these experiments, the contraction may have increased in response to an increase in the number of stimulus pulses rather than an increase in the duration of the stimulus period. In order to test the effect of stimulus duration more directly, experiments were carried out where the number of stimulus pulses was kept constant. When 40 pulses were delivered over periods of 5 s to 80 s, the post-stimulus contraction increased as the stimulus duration increased (Figure 4a). Summary data for 6 similar experiments indicated that the mean post-stimulus contractions increased with increasing stimulus durations above 5 s, and reached a plateau at 40 s (Figure 4b). The same protocol was repeated in the same tissues for 10, 20 and 80 pulses, the results are shown as separate line graphs in Figure 4c. It was not possible to test the effects of 10 pulses over time periods greater than 20 s as, under these conditions, each individual pulse elicited a separate relaxation and small rebound. Note that the values for 40 and 80 pulses overlapped, suggesting that they represented the maximum post-stimulus contractions possible at each of the stimulus durations.

Effects of Ca^{2+} channel antagonists

The dependence of the post-stimulus contraction on Ca^{2+} influx through L-type channels was tested with nifedipine and verapamil. Neither of these drugs had a consistent effect on baseline tension in the preparations, although in a few cases small reductions in tension were noted. A typical record of the effect of nifedipine (1 μM) is shown in Figure 5a. During the control period the preparation was stimulated at a fixed frequency of 1 Hz for periods of 10, 20, 40 and 60 s. Following each period of stimulation, there was a post-stimulus contraction which increased in size as the stimulus period was increased. After 30 min exposure to nifedipine (1 μM), the post-stimulus contractions were reduced, but not blocked. Figure 5b shows a summary of 7 experiments where nifedipine (1 μM) was applied. While nifedipine significantly reduced the post-stimulus contraction ($P<0.05$), there was a large component which was nifedipine-resistant. This was especially true

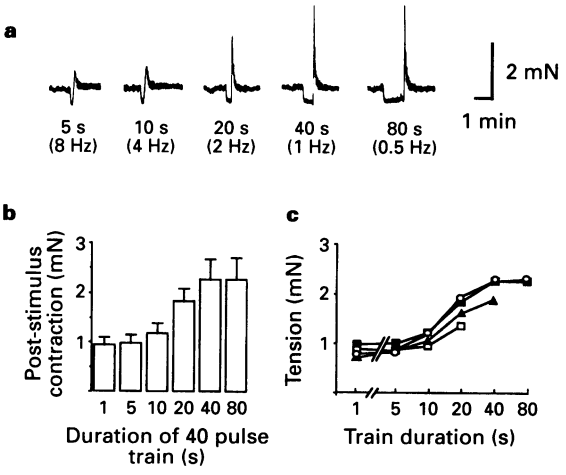

Figure 4 Effect of duration of stimulus train consisting of a fixed number of pulses: (a) 40 pulses delivered over periods of 5–80 s; (b) summary of the effect of 40 pulses delivered over periods of 1–80 s ($n=6$; means $\pm$ s.e.mean are shown); (c) summary of the effect of delivering 10 pulses (□), 20 pulses (▲), 40 pulses (○) and 80 pulses (■), over periods of 1–80 s in same 6 tissues. Error bars are omitted for clarity. Atropine (1 μM) and guanethidine (1 μM) were present throughout.

after the longer stimulus periods: e.g. the post-stimulus contraction following 60 s stimulation was reduced by only 22%, compared to a 50% reduction following 10 s stimulation. Exposure to nifedipine (10 μM) for periods of up to 90 min caused no further reduction in the post-stimulus contraction ($n=5$). The amplitudes of the post-stimulus contractions were also little affected by verapamil (1 μM; $n=7$). In some of the above experiments nifedipine or verapamil shortened the time course of the post-stimulus contraction by up to 50%, while having little effect on amplitude (4 out of 7 experiments with each drug).

Effect of ryanodine, caffeine and cyclopiazonic acid

The above experiments suggest that Ca^{2+} influx via L type channels plays a relatively small role in the generation of the post-stimulus contraction. It was decided, therefore, to examine the effects of several drugs which are known to interfere with intracellular Ca^{2+} stores. Figure 6a shows that effect of ryanodine (8 μM) in a typical experiment. The preparation was first stimulated at 1 Hz for 10, 20, 40 and 60 s and then exposed to ryanodine for 30 min and the stimuli repeated. The effect of the drug was to abolish the post-stimulus contractions, while producing little effect on relaxations. This action of ryanodine was confirmed in 7 experiments using this protocol, where the net effect was almost to abolish post-stimulus contractions while having only marginal effects on relaxations (Figure 6b). Ryanodine also had little effect on baseline tension in these preparations. On prolonged exposure to ryanodine (>2 h) there was some slowing of the rate of relaxation, and the rate of return to baseline was also slower ($n=3$). However, there was no evidence of a 'slow' post-stimulus contraction (see CPA below). Ryanodine (10 μM, 30 min exposure) also had a similar effect on the response to exogenous NO, reducing the rebound contraction, while having little effect on the relaxation (Figure 7).

In 4 control experiments, the effect of ryanodine was examined on K^+ contractures to test whether it was likely to be exerting its effect by blocking voltage-dependent Ca^{2+} influx. Before ryanodine, 60 mM K^+ Krebs induced contractures averaged 8.3±2.7 mN, while after ryanodine they averaged 7.1±1.3 mN ($P>0.05$). This suggests that ryanodine had little effect on voltage-dependent Ca^{2+} channels.

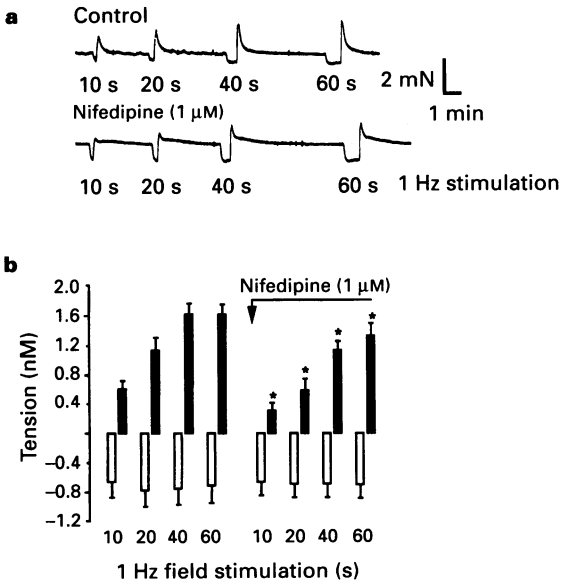

Figure 5 Effect of nifedipine (1 μM): (a) preparation was stimulated at a fixed frequency of 1 Hz for periods of 10–60 s under control conditions (atropine and guanethidine present), and again on addition of nifedipine (1 μM); (b) summary of the effect of nifedipine ($n=7$; $^*P<0.05$; means±s.e.mean are shown); post-stimulus contractions (solid columns); relaxations (open columns). Atropine (1 μM) and guanethidine (1 μM) were present throughout.

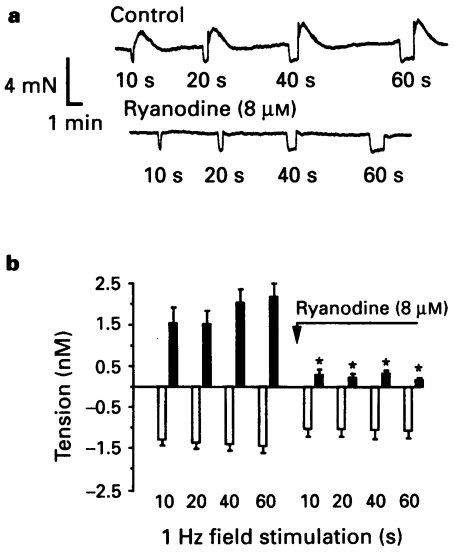

Figure 6 Effect of ryanodine (8 μM): (a) preparation was stimulated at fixed frequency of 1 Hz for periods of 10–60 s under control conditions (atropine and guanethidine present), and after 30 min exposure to ryanodine; (b) summary of the effect of ryanodine ($n=7$; $^*P<0.05$; means±s.e.mean are shown); post-stimulus contractions (solid columns); relaxations (open columns). Atropine (1 μM) and guanethidine (1 μM) were present throughout.

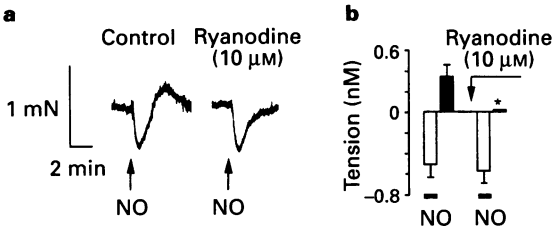

Figure 7 Effect of ryanodine (10 μM) on the response to exogenous NO; (a) NO bolus (1 ml NO gas to 10 ml bath) was applied before and after ryanodine; (b) summary of the effect of ryanodine on the response to NO (1–2 ml gas bolus; $n=5$; $^*P<0.05$; means±s.e.mean are shown); post-stimulus contractions (solid columns); relaxations (open columns).

Caffeine (20 mM) also abolished the post-stimulus contractions although, unlike ryanodine, it markedly affected the relaxations. In 7 experiments caffeine reduced the relaxations to 1 Hz stimulation for 60 s from 0.77±0.14 mN to 0.30±0.73 mN ($P<0.03$) and completely abolished the post-stimulus contractions from a control mean of 1.56±0.32 mN ($P<0.003$). Caffeine also caused a mean peak contracture of 3.02±0.59 mN which relaxed to 0.81±0.37 mN by the time EFS was repeated 30 min later.

An example of the effect of CPA (10 μM) is shown in Figure 8. The baseline steadily increased on exposure to this drug, reaching a plateau after about 30 min. CPA did not obviously interfere with the rate or amplitude of relaxation, as the muscle relaxed from the new increased baseline down to the same level as during the pre-CPA control period. The effect on the post-stimulus contraction was complex. Before CPA, the tissue demonstrated a sharp post-stimulus contraction similar to those described above. On exposure to CPA, this gradually declined, until it almost disappeared after 60 min (see arrows, Figure 8). However, a much slower post-stimulus contraction gradually developed over the same period. This was different from the normal 'fast' post-stimulus contraction in that it reached its peak some 30 s after the cessation of stimulation rather than 2 or 3 s. This result was typical of 8 preparations

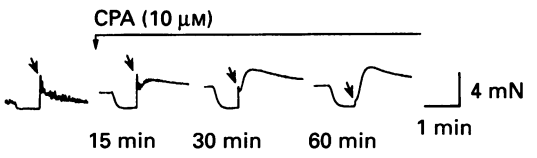

Figure 8 Effect of CPA (10 μM): preparation was stimulated at 1 Hz for periods of 60 s. Stimulation was repeated at times shown following perfusion of CPA. Arrows indicate the peak of the 'fast' post-stimulus contraction. Atropine (1 μM) and guanethidine (1 μM) were present throughout.

tested, where in each case there was a clear separation of 'fast' and 'slow' post-stimulus contractions after exposure to CPA. In every case the 'fast' component declined and the 'slow' component increased over the course of about 60 min. The amplitude of the 'fast' response was measured with reference to the tone during relaxation, rather than the baseline. This was necessary since the baseline tone increased (by 1.83 ± 0.31 mN, $n=8$). The mean 'fast' component before CPA was 2.53 ± 0.35 mN ($n=8$) and after CPA this was reduced to 0.60 ± 0.06 mN ($P<0.001$). The 'slow' phase which developed in the presence of CPA overshot the CPA contracture by a further 1.46 ± 0.28 mN, and this was reduced to 0.90 ± 0.02 mN by nifedipine (1 μM; not shown).

Discussion

Post-stimulation excitation is a well known feature of stimulation of NANC inhibitory nerves throughout the gastrointestinal tract (Bennett, 1966; Wood & Marsh, 1973; Burnstock *et al.*, 1975; Den Hertog & Van Den Akker, 1979; Bywater *et al.*, 1981; Ward *et al.*, 1992), and has also been observed previously in lower urinary tract muscle (Klarskov *et al.*, 1983; Thornbury *et al.*, 1992). Despite the volume of information available, no clear understanding of the mechanisms underlying post-stimulus contractions have emerged. Indeed, it is uncertain whether the post-stimulatory events in different tissues share common mechanisms, or if they represent a variety of phenomena. In the gastronintestinal tract, a 'myogenic' mechanism was proposed whereby the post-stimulus excitation followed automatically from a preceding period of inhibition or membrane hyperpolarization (Bennett, 1966; Campbell, 1966; Wood & Marsh, 1973). The contraction was thought to result from anode break depolarization and influx of Ca^{2+} through voltage-sensitive channels (Bennett, 1966; Wood & Marsh, 1973). However, doubts about this possibility emerged when it was found that the amplitude of the post-stimulus depolarization was not well correlated to the size of the preceding hyperpolarization (Bywater *et al.*, 1981). Moreover, post-stimulus depolarization still occurred even if the membrane potential was adjusted so that stimulation caused depolarization, rather than hyperpolarization.

The present study provides some evidence that NO is involved in producing the post-stimulus contraction in the sheep bladder neck. Firstly, L-NAME blocked the contraction, and this effect was partly reversed by L-arginine; secondly, a post-relaxation contraction was observed in one third of preparations following exposure to exogenous NO. The absence of the response in the other two thirds of preparations may have been related to differences in the delivery of exogenous versus endogenous NO. It is clear, for example, that both an adequately long stimulus period (Figure 4) and the rapid removal of cyclic GMP (Figure 3a) were necessary to achieve a post-stimulus contraction. It may be difficult, however, to achieve both of these conditions with exogenous NO, since it seems likely that the high concentrations of NO added to the bath would take longer to disappear than endogenously released NO. Indeed, as Figure 3b shows, addition of a bolus of exogenous NO during a period of field stimulation caused a reduction in the subsequent post-stimulus contraction.

Nitric oxide has also been implicated recently as the mediator of post-stimulus excitation in the canine proximal colon (Ward *et al.*, 1992) and a non-adrenergic, non-cholinergic contraction evoked *during* EFS of nerves in the rat small intestine (Bartho *et al.*, 1992). It seems unlikely, however, that the mode of action of NO in the sheep bladder neck is similar to its action in the dog colon as the latter response depends upon prolongation of the plateau phase of the electrical slow wave, which is a highly nifedipine-sensitive mechanism (Ward & Sanders, 1992). In contrast, the post-stimulus contraction in the bladder neck is not very sensitive to nifedipine or verapamil. It also seems unlikely that the mechanism is the same as the one underlying the contraction in the rat small intestine, since the response in this tissue occurs soon after the onset of stimulation. Interestingly, there is also an 'after contraction' in this preparation which is insensitive to inhibition of NO-synthase with N^G-nitro-L-arginine (Bartho *et al.*, 1992).

In contrast to the relative insensitivity of the bladder neck post-stimulus contraction to L-type Ca^{2+} channel blockers, the response was reduced or altered by drugs which interfere with intracellular Ca^{2+}. Ryanodine exposure for 30 min was sufficient to all but abolish the post-stimulus contraction. However, this drug had relatively little effect on relaxation, suggesting that its action was unlikely to have been due to reduced release of NO from nerves. Other possible prejunctional actions of ryanodine cannot be excluded in the present study, although in the rat vas deferens, nifedipine-sensitive contractions in response to EFS persisted in the presence of ryanodine, suggesting that transmitter output was not significantly reduced (Bourreau *et al.*, 1991b). Ryanodine also failed to reduce K^+ contractures significantly, suggesting that it did not block Ca^{2+} influx via voltage-dependent channels. Caffeine also abolished the post-stimulus contraction, but this result is difficult to interpret as the relaxation was reduced and could not therefore be used as an effective control against possible prejunctional actions on NO-producing nerves. Caffeine is also well known as a phosphodiesterase inhibitor, therefore it is possible that its effect on the post-stimulus contraction could have been mediated by a prolonged action of cyclic GMP (see Figure 3). Finally, the effect of CPA was examined. This drug acts as sarcoplasmic CaATPase inhibitor in smooth muscle (Bourreau *et al.*, 1991a; Uyama *et al.*, 1992), and has been shown to deplete the caffeine- and ryanodine-sensitive store (Suzuki *et al.*, 1992). In the present experiments CPA changed the post-stimulus contraction without blocking the nerve-induced relaxation. While the normal 'fast' component of the post-stimulus contraction gradually disappeared following exposure to CPA, a slower contraction emerged. The appearance of the 'slow' post-stimulus contraction was an unexpected finding and its basis remains unclear. Nifedipine, however, reduced it by 40%, suggesting that it partly depended on Ca^{2+} influx via L channels.

Other interesting features of the post-stimulus contraction were the fact that it could follow a period of contraction (Figure 2), and the dependence of its size on stimulus duration (Figure 4). The former observation suggests that it was unlikely to depend on a 'preconditioning' period of reduced intracellular Ca^{2+}, or hyperpolarization. The dependence on the duration of stimulation suggests that a cumulative process was involved. It is interesting to speculate that this might have represented loading of an internal Ca^{2+} store, since cyclic GMP is known to stimulate a sarcoplasmic reticulum Ca^{2+} pump (Twort & van Breemen, 1988; Raeymakers *et al.*, 1988). Thus a speculative mechanism relating the above pieces of experimental evidence would be: release of NO causes production of cyclic GMP which then causes Ca^{2+} sequestration in the sarcoplasmic reticulum; on cessation of stimulation, the cyclic GMP is removed by phosphodiesterases and the sequestered Ca^{2+} is released into the cytoplasm; this causes the rapid phase of the post-stimulus contraction which may later be supplemented to a lesser extent by Ca^{2+} influx. It has recently been proposed that a newly identified second messenger, cyclic ADP ribose, is produced in response to cyclic GMP and

causes Ca^{2+} release from a ryanodine-sensitive store in sea urchin eggs (Galione *et al.*, 1993; Berridge, 1993). If a similar system is identified in the bladder neck, it would be of interest to investigate whether it was responsible for releasing the Ca^{2+} necessary for the post-stimulus contraction.

In conclusion, the results of the present study suggest that the post-stimulus contraction following stimulation of NANC inhibitory nerves depends on the release of NO. The contraction was relatively insensitive to L-channel blockade but could be modified by drugs which are known to interfere with intracellular Ca^{2+} stores. It is proposed that NO may either release intracellular Ca^{2+} via intracellular mechanisms or may cause release of an extracellular messenger (possibly a NANC neurotransmitter) which then acts by releasing intracellular Ca^{2+}.

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