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A comparison of the effects of capsaicin with inhibitory nerve stimulation in the rat anococcygeus muscle in vitro

Richard E. Davies, Philippa M. Bashforth, Reginald J. Docherty *

Department of Pharmacology, St. Thomas's Campus, United Dental and Medical Schools of Guy's and St. Thomas's, Lambeth Palace Rd., London SE1 7EH, UK

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Abstract

Capsaicin was used to test whether centrifugal activation of sensory fibres in the rat anococcygeus muscle can contribute to non-adrenergic non-cholinergic (NANC) relaxation of the muscle. In a solution containing 0.5 mM Ca^{2+} and in the presence of carbachol (10 μM) capsaicin evoked a fast concentration-dependent relaxation of the muscle that was usually followed by a smaller, slower, relaxant response. The fast relaxant response was reduced when extracellular Ca^{2+} was raised to 2.5 mM, desensitized after a single application of capsaicin and was blocked by tetrodotoxin (1 μM) or ruthenium red (10 μM). The fast response was greatly reduced by haemoglobin, by cold storage of the muscles or by *N*-monomethyl-L-arginine (100 μM) in the absence but not in the presence of L-arginine (100 μM). It is concluded that centrifugal activation of sensory fibres evokes a nitric oxide-mediated relaxation of the anococcygeus muscles that probably contributes to electrically evoked NANC relaxation.

Keywords: Capsaicin; Sensory neuron; Nitric oxide (NO); Anococcygeus; NANC (non-adrenergic, non-cholinergic); Smooth muscle

1. Introduction

The non-adrenergic, non-cholinergic (NANC) inhibitory innervation of the anococcygeus muscle (Gillespie, 1972, 1980) is probably nitrergic (for recent reviews, see Gibson et al., 1995; Rand and Li, 1995). The inhibitory principal released from the nerves is either nitric oxide (NO) or a more stable intermediate compound such as nitrosocysteine from which NO is released (Gillespie et al., 1989; Li and Rand, 1989; Gibson et al., 1992; Li and Rand, 1993; Gibson et al., 1995; Rand and Li, 1996). Consistent with this, the anococcygeus has been shown by immunocytochemistry (Brave et al., 1993) to contain a dense innervation, distinct from the sympathetic innervation, that contains NO synthase.

A sub-population of peripheral sensory nerves of the dorsal root ganglia stain positively for NO synthase (Aimi et al., 1991; Verge et al., 1992). The NO synthase-positive dorsal root ganglion neurons are thought to be associated with sensory fibres innervating the viscera (Aimi et al., 1991). Since the anococcygeus (in common with vascular and other non-vascular smooth muscles) is likely to have a sensory innervation it is possible that some or all of the NO synthase positive fibres in the muscle are sensory rather than motor fibres. Hence the relaxant NANC responses observed when nerve fibres in the anococcygeus muscle are activated by an electric field could be due to centrifugal firing of NO synthase-containing sensory fibres. In the present study we have tested this hypothesis by using capsaicin as a specific excitant of sensory fibres (Holzer, 1991) in the muscle and comparing the responses of the muscle to capsaicin with electrically-evoked NANC responses. Capsaicin would be expected to excite some, though not all, of the NO synthase-positive sensory neurons (Ren and Ruda, 1995). Interestingly, the spinal origin of the NANC inhibitory innervation of the anococcygeus is between L5 and S2 (Gillespie and McGrath, 1973) which corresponds to the most sensitive region (L5 to S1) for capsaicin-induced induction of NO synthase in sensory neurons (Vizzard et al., 1995). A preliminary account of some of the results has already appeared (Bashforth et al., 1997).

* Corresponding author. Tel.: +44-171-922-8180; Fax: +44-171-922-8180.

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2. Methods

Male or female rats (Wistar strain; 200–250 g) were stunned by a blow to the head and killed by cervical dislocation. The anococcygeus muscles were dissected and suspended in 35 ml organ baths in a modified Krebs' solution of the following composition (in mM): NaCl, 118; KCl, 4.8; $MgSO_4$, 1.8; KH_2PO_4, 0.162; $CaCl_2$, 0.5; $NaHCO_3$, 25; glucose, 11) which was bubbled continuously with 95% O_2/5% CO_2 and maintained at 37°C. In some experiments the concentration of $CaCl_2$ was increased to 1.5 or 2.5 mM (see Section 3). One end of each muscle was tied to an anchor point in the organ bath and the other end was attached by a fine thread to a force transducer (Harvard Instruments). A resting tension of 1 g was applied to the muscles which were then allowed to equilibrate in the bath for at least 30 min before beginning the experimental protocol. Isometric tension was recorded on a Graphic 450 (Lloyd Instruments) chart recorder. Electrical field stimulation of the muscle was achieved by applying a voltage to two parallel platinum plate electrodes, one placed on either side of the muscle (Grass S88 stimulator; 5 ms pulse width; 70 V). In order to record NANC responses to electrical field stimulation or relaxant responses to drugs muscle tone was raised by applying 10 μM carbachol which produced a robust but sub-maximal contraction of the tissue (see, for e.g., Gibson et al., 1994). Either prazosin (5 μM) or phentolamine (20 μM) was added to block contractile responses due to electrical field stimulation-induced excitation of sympathetic nerves in the tissue (see Gibson et al., 1992). No differences in results were observed when either of these compounds were used to block sympathetic nerve responses.

For cold storage experiments muscles were dissected and threads were attached as normal. The muscles were placed in a beaker containing 30–40 ml of chilled (4°C) Krebs' solution and stored in a refrigerator for 3 days prior to use.

Relaxant responses to drugs were measured as the percentage reduction in carbachol-induced tone, e.g., 100% reduction would mean a return to the level of resting tone measured prior to carbachol application. Relaxant responses to drugs, including capsaicin, were measured as the maximum reduction of tone which occurred within 2 min of drug application irrespective of the duration of drug application. Drugs were usually added to the tissue for 2 min although in some experiments with capsaicin the drug was applied for longer, up to 10 min. When electrical field stimulation was applied during capsaicin responses the stimulus was applied as near as possible to the peak of the fast capsaicin response (see Section 3). When the effects of drugs or electrical field stimulation were measured after capsaicin application the stimulus was applied after capsaicin was washed out and as soon as possible (usually 10–15 min) after the tissue had reached a steady level of carbachol-induced tone. All drugs used were prepared as stock solutions and an appropriate amount added to the bath to achieve the desired bath concentration. Stocks of drugs were made up in distilled water except capsaicin and prazosin which were made up in dimethylsulphoxide (DMSO). In experiments where both prazosin and capsaicin were added to the bath the final concentration of DMSO was never more than 0.025% which concentration had no significant effect on electrical field stimulation evoked responses (either contraction or relaxation) or on carbachol-induced tone.

Haemolysate was prepared from blood collected from an anaesthetized rabbit using a method described by Bowman and Gillespie (1982). The haemolysate was stored at 4°C and used within 3 days of preparation (Bowman and Gillespie, 1982).

Data were expressed as the mean ± S.E.M. All statistical comparisons were made by two-tailed Student's *t*-test (paired or unpaired as indicated in text) using Microsoft Excel software.

The following drugs and chemicals were used: L-arginine, capsaicin, carbachol, phentolamine, prazosin, sodium nitroprusside (all from Sigma), ruthenium red (Aldrich), tetrodotoxin (Calbiochem) and *N*-monomethyl-L-arginine (a gift from Dr. J. Cunningham, Department of Pharmacology, UMDS).

3. Results

3.1. Effect of capsaicin

Capsaicin had no overt effect on anococcygeus muscles when the muscles were relaxed. When muscles were contracted by adding carbachol (10 μM) to the bathing solution then capsaicin (0.1 to 50 μM) caused a relaxation of the carbachol-induced tone (see Fig. 1). When capsaicin was first applied to the tissue the response was usually a fast, transient relaxation which peaked within 2 min and then decayed (Fig. 1a). This fast, transient response always desensitized after a single application. When capsaicin was re-applied to the tissue, the second or subsequent applications either did not produce a measurable response or more usually produced a smaller, slower and persistent relaxation which took up to 10 min to reach a maximum. This slow, persistent response was usually absent at concentrations of capsaicin less than 10 μM and was very variable between preparations. Fig. 1b and c show data from a preparation that displayed an especially prominent slow response. On average the slow response (at 10 μM capsaicin) was only 20 ± 3% ($n = 38$) as large as the fast response in a given preparation. Desensitization of the fast, transient response to capsaicin was not reversed by washing the tissue for up to two hours (Fig. 2). By contrast, the residual slow response persisted while capsaicin was present in the bath, usually reversed completely when the drug was washed out and did not desensitize further with

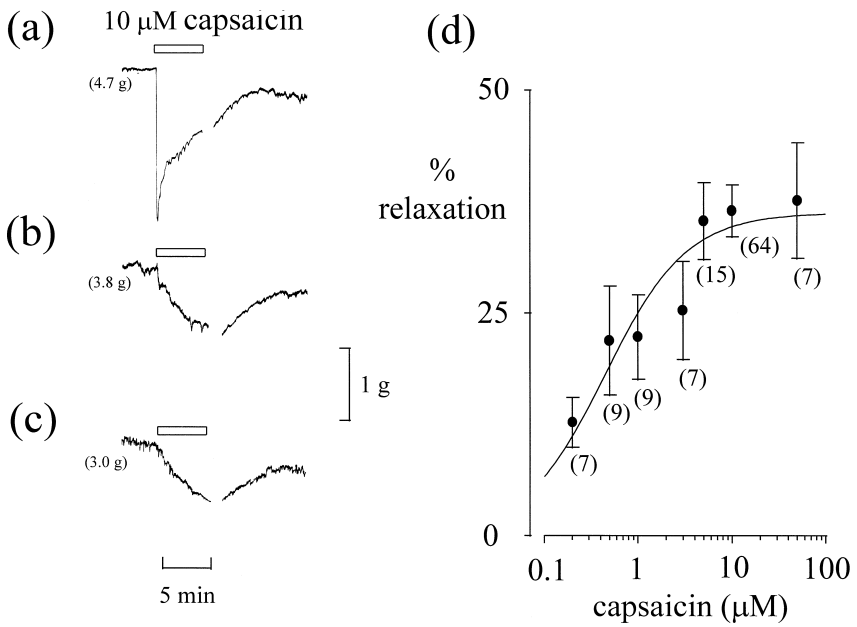

Fig. 1. Capsaicin-induced relaxation of the rat anococcygeus muscle. Part (a) shows the relaxant response evoked by a first application of capsaicin (10 μM) to the tissue and parts (b) and (c) show responses to subsequent applications applied about 30 and 60 min after the first response. Capsaicin was added to the bath during the period indicated by the bar and washed out during the gap in the trace. The resting tone just prior to capsaicin application (in grams) is indicated by each trace. Part (d) is a plot of the response to a first application of capsaicin against concentration. Each point shows the mean ± S.E.M. and the number of experiments is indicated in brackets beside each point. The solid line in (d) is the best fitting line drawn according to the equation $y = (a * x)/(k + x)$ where y = mean response, a = maximum response, x = capsaicin concentration and $k = EC_{50}$.

repeated applications of capsaicin. The fast response to capsaicin was probably due to an action on sensory neurons in the tissue while the residual slow response was due to a non-specific effect on the smooth muscle (see below). In this study, capsaicin was being used as a tool to study the effects of activation of sensory neurons so we focused on the fast response and the slow response was not considered further.

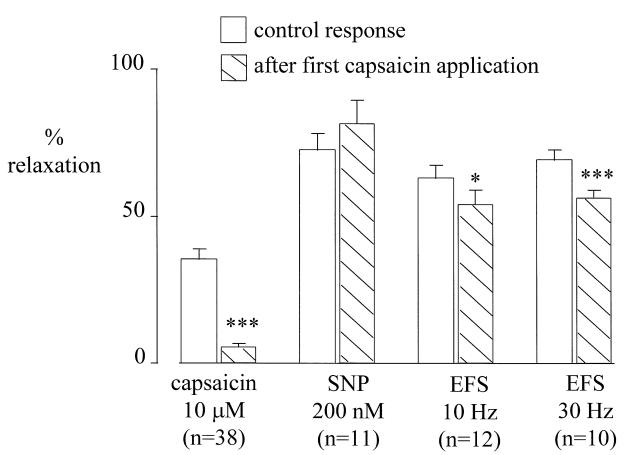

Fig. 2. The effect of capsaicin desensitization. Open columns represent the mean ± S.E.M. for the amplitude of responses to capsaicin (10 μM), sodium nitroprusside (SNP, 200 nM), or electrical field stimulation (EFS, 10 or 30 Hz) in tissues which had not previously been exposed to capsaicin and cross-hatched columns represent the response after prior exposure to capsaicin. Data were compared by paired t-test where * indicates $P < 0.05$ and *** indicates $P < 0.005$.

Since the fast response to capsaicin desensitized each muscle preparation could be used to measure this response only once. As a consequence the concentration–response curve for the response was deduced from the average of single responses obtained from several muscles (see Fig. 1d). The threshold concentration of capsaicin required to evoke a measurable response consistently was about 0.2 μM. The EC_{50} of the response, which was obtained by fitting a curve to the data in Fig. 1d (MicroCal Origin v. 3.7 software), was 0.45 ± 0.14 μM and an estimated maximum relaxation of $36.3 \pm 2.3\%$ was achieved at concentrations ≥ 10 μM.

Responses to sodium nitroprusside (200 nM) and electrical field stimulation (10 or 30 Hz, 5–6 s) were obtained for comparison to capsaicin responses. In agreement with previous reports (see Rand and Li, 1995; Gibson et al., 1995) we found that sodium nitroprusside and electrical field stimulation caused a pronounced relaxation of the muscle that did not desensitize. In muscles that had not been exposed previously to capsaicin the response to 200 nM sodium nitroprusside or to electrical field stimulation at 10 or 30 Hz was significantly greater than the maximum response to capsaicin (Table 1).

Responses to sodium nitroprusside (200 nM) and electrical field stimulation (10 or 30 Hz) were compared before and after application of capsaicin to see whether desensitization of the capsaicin response had any effect on responses to these stimuli (Fig. 2). Responses to sodium nitroprusside were not significantly different after capsaicin desensitization (i.e., after prior exposure of the

Table 1
Comparison of the maximum response to capsaicin with responses to other stimuli

Stimulus	Percent inhibition of carbachol-induced tone[a]	n[b]	P[c]
Maximum response to capsaicin (10 μM)	36.5 ± 2.9	64	–
Electrical field stimulation (10 Hz)	55.9 ± 4.1	42	< 0.001
Electrical field stimulation (30 Hz)	72.4 ± 3.4	10	< 0.001
Sodium nitroprusside (200 nM)	63.8 ± 3.5	40	< 0.001

[a] Mean ± S.E.M.
[b] n = number of muscle preparations.
[c] Unpaired, two-tailed Student's t-test.

tissue to capsaicin and wash-out) but the response to 10 ($n = 12$) or 30 Hz ($n = 10$) stimulation was significantly reduced ($P = 0.030$ and 0.002, respectively, paired t-test).

Given that either capsaicin or electrical field stimulation would be expected to activate a proportion of the sensory nerves in the tissue experiments were performed to test whether the relaxant effects of capsaicin and electrical field stimulation were additive (Fig. 3). When electrical field stimulation (10 Hz) was applied during the capsaicin-induced relaxation there was still a small electrical field stimulation-induced relaxation (measured with respect to the muscle tone at the peak of the capsaicin response) but this was significantly reduced ($P = 0.003$, paired t-test, $n = 12$) compared to the response obtained immediately prior to capsaicin application. The combined relaxant effect of capsaicin and electrical field stimulation was significantly greater than electrical field stimulation alone ($P = 0.001$, paired t-test, $n = 12$) but was always much less than the sum of the capsaicin and electrical field stimulation responses. When capsaicin was re-applied to the tissue for a second time it had a relatively small effect on muscle tone (slow non-specific response seen after desensitization—see above) and no significant effect on responses to electrical field stimulation.

3.2. Specificity of capsaicin

Desensitization which is difficult to reverse is a characteristic feature of the response of sensory neurons to capsaicin (Holzer, 1991; Bevan and Docherty, 1993; Docherty et al., 1996). The fact that the fast response to capsaicin desensitized suggests that it is due to an action on vanilloid receptors. Consistent with this, the response to capsaicin was significantly inhibited ($P = 0.003$, unpaired t-test) by administration of tetrodotoxin (1.0 μM; Fig. 4). The electrical field stimulation-evoked NANC response was significantly reduced ($P < 0.001$, unpaired t-test) by tetrodotoxin but the response to sodium nitroprusside was not significantly changed (Fig. 4).

Ruthenium red is a non-competitive antagonist of capsaicin responses (Maggi et al., 1988; Wood et al., 1988) that acts by inhibiting the opening of a non-specific cation channel which is linked to the vanilloid receptor in sensory neurons (Dray et al., 1990). Ruthenium red (10 μM) caused a small (5.9 ± 0.2%, $n = 5$) transient (≤ 60 s) relaxation when applied to the muscle but had no sustained effect on carbachol-induced tone. The relaxant response to capsaicin was significantly reduced ($P = 0.006$, unpaired

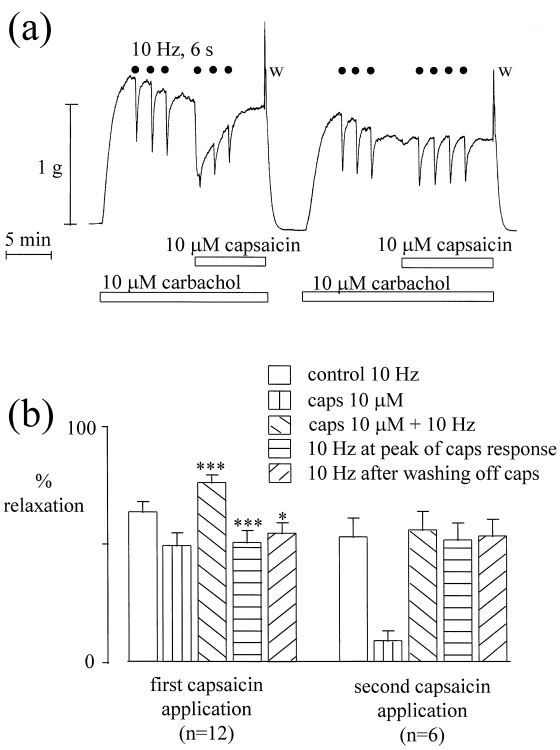

Fig. 3. Responses to capsaicin and electrical field stimulation are not additive. Part (a) shows a record of muscle tension with responses to electrical field stimulation (applied at 10 Hz for 6 s at 2 min intervals) applied at the solid black circles. Carbachol (10 μM) and capsaicin (10 μM) were applied during the periods indicated by the horizontal bars. The first response to capsaicin (applied for 8 min) was a rapid relaxation of muscle tone which reached a peak after about 20 s and desensitized in the continued presence of capsaicin. After washing the tissue and re-applying carbachol a second application of capsaicin (applied for 10 min), shown to the right of the figure, produced almost no response and had no effect on the response to electrical field stimulation. Part (b) of the figure is a histogram of the mean ± S.E.M. of the size of responses to electrical field stimulation alone (open columns), the size of the capsaicin response (vertical hatching), the total relaxation produced by electrical field stimulation and capsaicin together (downward sloping hatching), the response to electrical field stimulation measured from the peak of the capsaicin response (horizontal hatching) and the response to electrical field stimulation after washing everything off and re-applying carbachol (upward sloping hatching). Asterisks indicate the results of a statistical comparison (two-tailed, paired Students' t-test) with control data (***, $P < 0.005$; *, $P < 0.05$).

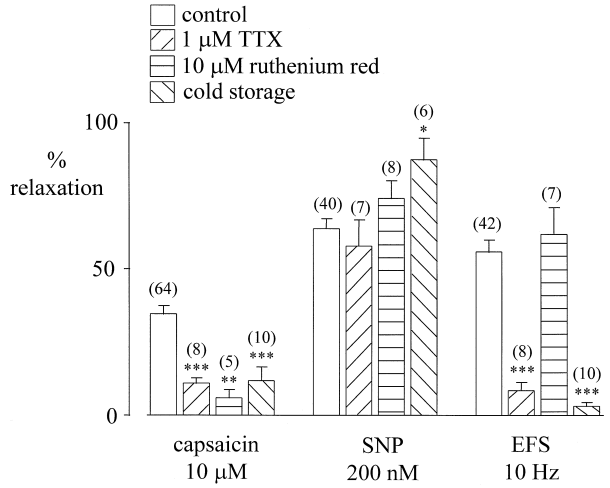

Fig. 4. The capsaicin response is due to activation of neuronal vanilloid receptors. The histogram shows the effect of tetrodotoxin (TTX, 1 μM), ruthenium red (10 μM) or cold storage of muscles (3 days at 4°C) on responses to capsaicin (10 μM), sodium nitroprusside (SNP, 200 nM) or electrical field stimulation (EFS, 10 Hz). Control responses for each stimulus are compared to test responses using an unpaired *t*-test and *** indicates $P < 0.005$, ** indicates $P < 0.01$ and * indicates $P < 0.05$.

t-test) by ruthenium red (10 μM) but it had no effect on either the electrical field stimulation-evoked NANC responses or on responses to sodium nitroprusside (Fig. 4).

Interestingly, the residual slow, persistent response seen after the initial response to capsaicin had desensitized (see above) was not blocked by either tetrodotoxin or ruthenium red (data not shown) which suggests that this response is probably a non-specific effect of capsaicin due to a direct action on the smooth muscle cells. A similar, non-specific smooth muscle relaxant effect of capsaicin is believed to give rise to the slow phase of capsaicin-induced relaxation of guinea-pig ileum (Bartho and Holzer, 1995).

3.3. Calcium dependence of the capsaicin response

In studies of the effects of capsaicin on isolated vagus nerve (Marsh et al., 1987) or on isolated dorsal root ganglion sensory neurons (Docherty et al., 1996) it has been found that the size of the response to capsaicin is increased when the concentration of Ca^{2+} in the bathing medium is reduced. For this reason the present experiments were performed with a lower concentration of Ca^{2+} (0.5 mM) in the bathing solution than is commonly used in studies of this type (e.g., see Gibson et al., 1992). When the concentration of Ca^{2+} was increased from 0.5 to 1.5 mM there was no significant change in the size of the capsaicin response ($36.5 \pm 2.9\%$, $n = 64$ and 52.4 ± 3.8, $n = 7$, respectively) but at 2.5 mM Ca^{2+} there was a significant decrease in the size of the capsaicin response ($11.3 \pm 3.5\%$, $n = 9$, $P = 0.002$, unpaired *t*-test).

3.4. Is the response to capsaicin NO-dependent?

Electrical field stimulation-evoked relaxant NANC responses in the rat anococcygeus are inhibited by drugs such as *N*-monomethyl-L-arginine (L-NMMA) which inhibit NO synthase or by haemoglobin which chelates free NO (Gillespie et al., 1989; Gillespie and Sheng, 1989; Li and Rand, 1989; Ramagopal and Leighton, 1989). We tested whether responses to capsaicin would be similarly affected.

In muscles exposed to L-NMMA (100 μM) the NANC response to electrical field stimulation (10 Hz) and the relaxant response to capsaicin (10 μM) were significantly reduced ($P = 0.006$, unpaired *t*-test) while the response to sodium nitroprusside (200 nM) was unchanged (Fig. 5). When L-arginine (100 μM) was added in addition to L-NMMA there was no significant difference in the size of responses to electrical stimulation, sodium nitroprusside or capsaicin, i.e., L-arginine abolished the inhibitory effect of L-NMMA. Addition of haemoglobin (Hb, 25 μM as haemolysate) to the bathing medium (Fig. 5) strongly inhibited responses to electrical stimulation ($P < 0.001$, unpaired *t*-test), capsaicin ($P < 0.001$, unpaired *t*-test), and sodium nitroprusside ($P < 0.001$, unpaired *t*-test).

3.5. Effect of cold storage

It has been shown that storage of isolated anococcygeus muscles at 4°C for 3 days results in the loss of NO synthase-positive nerve fibres in the muscle and a concomitant loss of NANC responses to electrical field stimu-

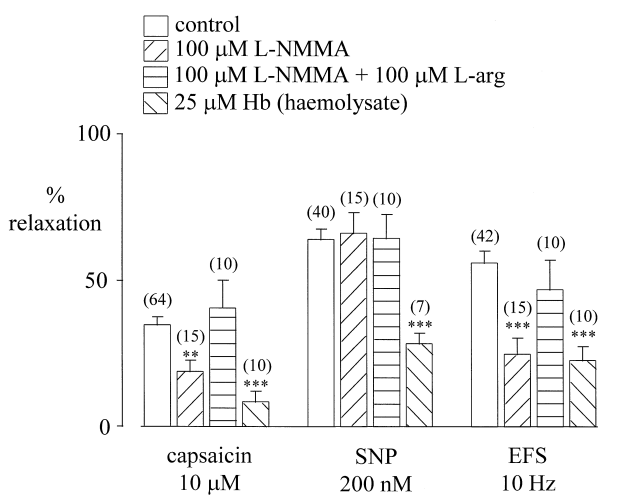

Fig. 5. The capsaicin response is NO-dependent. The histogram shows the effect of *N*-monomethyl-L-arginine alone (L-NMMA, 100 μM), L-NMMA in the presence of L-arginine (100 μM) and haemoglobin (Hb, 25 μM applied as haemolysate) on responses to capsaicin (10 μM), sodium nitroprusside (SNP, 200 nM) or electrical field stimulation (EFS, 10 Hz). Control responses for each stimulus are compared to test responses using an unpaired *t*-test and *** indicates $P < 0.005$, ** indicates $P < 0.01$ and * indicates $P < 0.05$.

lation although the muscles still display relaxant responses to nitrosothiol compounds (Gibson et al., 1992; Brave et al., 1993). In agreement with these studies we too found that the NANC response to electrical field stimulation (10 Hz) was greatly reduced in muscles stored at 4°C for 3 days ($P < 0.001$, unpaired *t*-test, $n = 10$) while the response to sodium nitroprusside (200 nM) was significantly larger than normal ($P = 0.017$, unpaired *t*-test, $n = 6$). The relaxant response to capsaicin (10 μM) was greatly reduced ($P = 0.002$, unpaired *t*-test, $n = 10$) in the muscles after cold storage (Fig. 4).

4. Discussion

The data presented above confirm that the rat anococcygeus muscle contains a NANC innervation that is activated by electrical field stimulation leading to relaxation of the muscle. Further, we have confirmed that the relaxant response to electrical field stimulation is strongly inhibited by L-NMMA which is a blocker of NO synthase, mimicked by the NO-donor sodium nitroprusside and inhibited by haemoglobin which chelates NO. These data are in complete agreement with several previous studies and strongly support previous suggestions that NO mediates electrical field stimulation-induced NANC relaxation in the anococcygeus (see Section 1). We have also shown that capsaicin, a stimulant of sensory neurons, mimics electrical field stimulation-evoked NANC responses.

4.1. The mechanism of the capsaicin response

The novel aspects of the present study are related to the effects of capsaicin. When capsaicin is applied to the anococcygeus muscle a relaxant response is evoked that displays similar pharmacological characteristics to the electrically evoked NANC response. The response to capsaicin is inhibited by L-NMMA in the absence but not in the presence of L-arginine. The response is blocked by haemolysate. These data strongly suggest that the response to capsaicin, like that to NANC nerve stimulation is mediated by the release of NO in the tissue. The question then arises as to the source of the NO released in the tissue. If the electrically-evoked NANC relaxation is due to release of NO from nerve fibres then this is also likely to be true of capsaicin responses. Nerve fibres that are distinct from the sympathetic innervation and which stain positively for NO synthase have been identified in the tissue (see Gibson et al., 1995). Since tetrodotoxin blocks the response to capsaicin it is reasonable to suppose that capsaicin-induced release of NO is due to stimulation of nitrergic nerve fibres in the tissue. This conclusion is supported by the data obtained from muscles after cold storage which showed a loss of both neurally-evoked and capsaicin-evoked responses.

The fast response to capsaicin showed a profound desensitization. The response was blocked by ruthenium red. These are characteristic features of the response of sensory neurons to capsaicin (see Holzer, 1991) and suggest that the capsaicin response was due to activation of vanilloid receptors on sensory neurons. Since unmyelinated sensory nerves express NO synthase and since sensory nerves are the only tissues in the periphery known to express vanilloid receptors (see Holzer, 1991; Szallasi, 1994; Caterina et al., 1997), it is probable that capsaicin-induced NANC responses in the anococcygeus are due to centrifugal firing of nociceptive sensory C-fibres. It follows that NANC responses induced by electrical field stimulation are also due, at least in part, to stimulation of sensory C-fibres. Interestingly, when electrical field stimulation was applied during the fast capsaicin evoked response the response observed was reduced as would be expected if some of the nerve fibres excited by electrical field stimulation were already activated by capsaicin. Also, capsaicin-induced NANC responses and electrical field stimulation-evoked NANC responses were less than additive as would be expected if the two stimuli activate overlapping populations of nerve fibres. Since the maximum response to capsaicin was smaller than that to electrical field stimulation it is unlikely that capsaicin activates all of the nitrergic nerves in the anococcygeus. It is difficult to tell whether the remaining nitrergic fibres are sensory fibres that are not sensitive to capsaicin or whether they are a distinct population of motor fibres. The possibility that the muscle contains other unmyelinated nitrergic nerves that have a motor rather than a sensory function cannot be ruled out (see below). However, only a proportion of NO synthase-containing sensory nerves are sensitive to capsaicin (Ren and Ruda, 1995). It is therefore possible to speculate that the NANC response to electrical field stimulation in the anococcygeus may be due entirely to centrifugal activation of sensory fibres since electrical field stimulation would be expected to stimulate capsaicin-insensitive, NO synthase-positive, sensory neurons in the tissue as well as capsaicin-sensitive neurons.

The non-selective cation channel that is activated by capsaicin is permeable to Ca^{2+} (Wood et al., 1988; Holzer, 1991; Caterina et al., 1997). If NO is released from sensory nerves then it might be expected that Ca^{2+}-entry through capsaicin-activated ion channels in the sensory C-fibres would provide an adequate stimulus for NO synthesis and release. Curiously this does not appear to be the case. Since TTX blocks the capsaicin response then voltage-gated sodium channels must be activated, presumably secondary to capsaicin-induced depolarization, before NO is released. This suggests that the vanilloid-gated ion channels are located at sites that are distant from the sites at which NO is released or else the contribution to the total intracellular Ca^{2+} of Ca^{2+} entering through the vanilloid-gated ion channels is not by itself an adequate stimulus for NO synthesis and release. In keeping with this, raised

extracellular Ca^{2+} inhibited the capsaicin response. This procedure would be expected to reduce the depolarizing action of capsaicin (Marsh et al., 1987), since total membrane current would be reduced (Docherty et al., 1996), and hence Ca^{2+} entry through voltage-gated Ca^{2+} channels (secondary to depolarization) would also be reduced. However, Ca^{2+} entry through vanilloid-gated channels would not be expected to decrease in elevated extracellular Ca^{2+}. This is interesting since it suggests that either the vanilloid-gated ion channels are absent at NO release sites or else the synthesis and release apparatus for NO are very closely coupled to voltage-gated Ca^{2+} channels.

The fast, relaxant response to capsaicin in the anococcygeus is remarkably similar to the relaxant response of the rat duodenum save that the latter is less sensitive to tetrodotoxin (Maggi et al., 1986). Isolated ileum is relaxed by capsaicin in a tetrodotoxin-resistant manner and the response has been shown to be made up of a fast, desensitizing component which is probably mediated by CGRP release (Bartho et al., 1991) from extrinsic (presumably sensory) nerves and a slower component which is due to a direct effect on the muscle (Bartho et al., 1987). By comparison to most smooth muscle preparations the anococcygeus muscle represents a refreshingly simple system. There is no evidence to suggest that electrical field stimulation evoked NANC responses involve any intermediate cell type and there is good evidence that NO or an NO-donor molecule is neurally derived in this tissue. Whether the NANC inhibitory response is due entirely to NO or involves additional non-nitrergic mechanisms, especially when muscle tone is low (Selemidis and Cocks, 1997), is another question. Capsaicin or electrical field stimulation would be expected to release neuropeptides, especially CGRP and substance P, from sensory nerves in the tissue. So far as we are aware the effects of CGRP or CGRP antagonists on the anococcygeus are not known. Substance P causes contraction and potentiates contractile responses (Gibson et al., 1984) and is not therefore an attractive candidate as an inhibitory neurotransmitter. Vasoactive intestinal polypeptide (VIP) is a sensory neuropeptide which relaxes the anococcygeus muscle (Gibson and Tucker, 1982) but has been rejected as a likely candidate as an inhibitory transmitter (Carvajal et al., 1986). At the moment the simplest hypothesis to account for the data presented above is that electrical field stimulation or application of capsaicin to the anococcygeus muscle releases NO or an NO donor molecule which acts directly on smooth muscle cells to cause relaxation. Whether an additional substance contributes to the response is an interesting possibility which should be further investigated.

4.2. Is the inhibitory innervation of the anococcygeus motor or sensory?

It is not possible to rule out a contribution of an extrinsic or intrinsic nitrergic motor innervation to electrically evoked inhibitory responses in the muscle. However, there is no compelling reason to believe that a motor innervation exists. The best evidence that the inhibitory NANC innervation of the anococcygeus is motor in character rather than sensory comes from the in vivo study of Gillespie and McGrath (1973) who showed that the non-depolarizing ganglion blockers hexamethonium and mecamylamine transiently blocked relaxant responses of the anococcygeus to electrical stimulation of the spinal cord (S2 to L5). This suggested that the inhibitory pathway was efferent and interrupted by a ganglion. However, Gillespie and McGrath expressed their own reservations as to the presence of a ganglionic relay since, during their characteristically thorough experiments, they found that the effect of the ganglion blockers 'desensitized' which property is difficult to reconcile with the mechanism by which the ganglion blocking drugs are thought to act. The present experiments provide strong evidence for a sensory innervation in the anococcygeus which can cause muscle relaxation when stimulated. It is not necessary to postulate the existence of a separate motor inhibitory innervation to explain the phenomenon of the neurally evoked NANC relaxation.

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