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Short communication

Calyculin-A inhibits nitrergic relaxations of the mouse anococcygeus

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Abstract

The aim was to determine whether blockade of store-operated Ca^{2+} entry, or inhibition of Ca^{2+} sensitisation, is the predominant mechanism by which neuronally released nitric oxide mediates relaxation of the mouse anococcygeus. Nitrergic relaxations to field stimulation (10 Hz, 10 s trains) were unaffected by the sarcoplasmic reticulum Ca^{2+} ATPase blocking agent thapsigargin (100 nM), known to prevent nitric-oxide-induced inhibition of store-operated Ca^{2+} entry. Conversely, the myosin phosphatase inhibitor calyculin-A (1 μM) caused almost complete abolition of nitrergic relaxations. The results provide evidence that inhibition of Ca^{2+} sensitisation is the major cellular mechanism underlying nitrergic relaxation of the mouse anococcygeus.

Keywords: Anococcygeus, mouse; Calyculin-A; Nitrergic relaxation; Thapsigargin

1. Introduction

Over the past decade, experimental evidence has established that nitrergic nerves mediate smooth muscle relaxation throughout the respiratory, gastrointestinal and urogenital tracts (Martin, 2000). Attention has now focussed on identifying the post-junctional signalling mechanisms activated by the neurotransmitter nitric oxide released from these nerves. The cellular receptor for nitric oxide in the smooth muscle cell is soluble guanylyl cyclase, which produces the second messenger substance cyclic GMP. Numerous mechanisms have been proposed for the smooth muscle relaxant effects of cyclic GMP, including inhibition of inositol trisphosphate synthesis, decreased sensitivity of the inositol trisphosphate receptor, activation of K^+ channels, enhanced removal of Ca^{2+} from the cytoplasm and inhibition of Ca^{2+} sensitisation (Lincoln et al., 2001); Ca^{2+} sensitisation in smooth muscle is defined as an increase in contractile force that is independent of changes in cytoplasmic Ca^{2+} concentration (Sward et al., 2000). However, it is not clear which of the mechanisms mentioned above specifically contribute to the relaxations induced by nitrergic nerves. The experiments described here attempt to clarify this issue in the mouse anococcygeus, one of the first tissues in which a neurotransmitter role for nitric oxide was identified (Gibson et al., 1990). Previous work has established that nitrergic relaxations of the mouse anococcygeus involve activation of the soluble guanylyl cyclase/cyclic GMP pathway (Gibson and McFadzean, 2001). Experiments with nitric oxide donor drugs and 8-bromo-cyclic GMP have identified two possible mechanisms of relaxation. First, cyclic GMP inhibits store-operated Ca^{2+} entry, most probably by enhanced Ca^{2+} refilling of the sarcoplasmic reticulum; in support of this, the inhibitory effect of nitric oxide donors on store-operated Ca^{2+} entry can be blocked by the sarcoplasmic reticulum Ca^{2+} ATPase inhibitor thapsigargin, which would prevent store refilling (Ayman et al., 2001). Secondly, experiments with skinned anococcygeus preparations have demonstrated that the nitric oxide/cyclic GMP pathway may also cause relaxation by inhibition of Ca^{2+} sensitisation (Ayman et al., 2001). This latter action of cyclic GMP depends on the activation of myosin phosphatase (Lincoln et al., 2001), and so should be prevented by drugs known to inhibit myosin phosphatase, such as calyculin-A (Burdyga and Wray, 2002). Thus, the aim of the present study was to compare the effects of thapsigargin (which should prevent inhibition of store-operated Ca^{2+} entry) and calyculin-A (which should prevent inhibition of Ca^{2+} sensitisation) on nitrergic relaxations of

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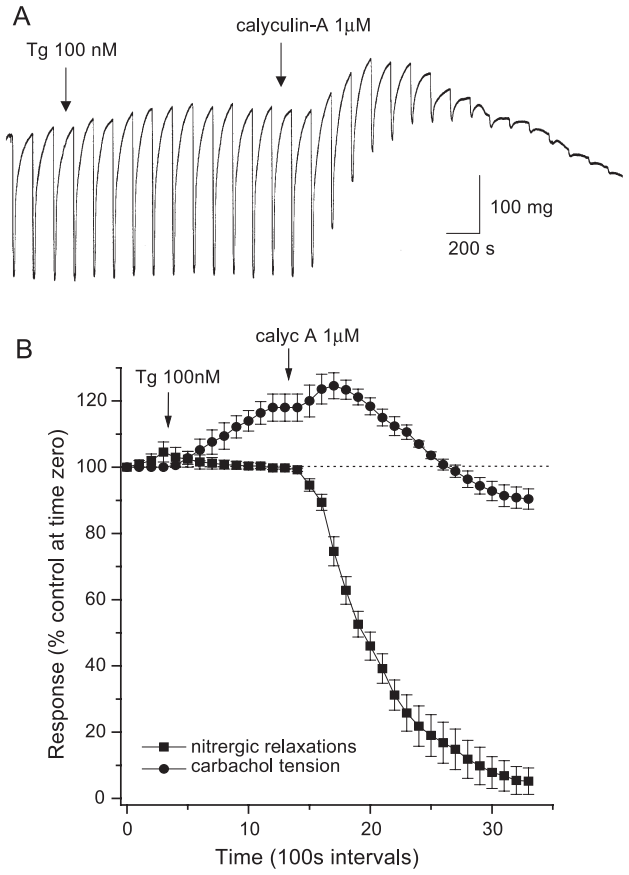

Fig. 1. (A) Trace showing nitrergic relaxations of the mouse anococcygeus in response to field stimulation (10 Hz, 10 s trains every 100 s) and the effect on these of sequential addition of thapsigargin and calyculin-A. Tone was raised with 50 μM carbachol. (B) Graphs showing the effects of sequential addition of thapsigargin (Tg) and calyculin-A (calyc A) on carbachol induced tension and nitrergic relaxation of the mouse anococcygeus (experiment as shown in the trace above). Each point is the mean ± S.E.M. from six individual muscle preparations.

the mouse anococcygeus in order to determine the predominant mechanism mediating the relaxation.

2. Materials and methods

The protocols used in the present study conform to the requirements of the King's College London Ethical Review Procedure. Male mice (LACA strain, 25–35 g) were killed by stunning and exsanguination. The two anococcygeus muscles were dissected and set up for the isometric recording of nitrergic relaxations to field stimulation as described fully elsewhere (Ayman et al., 2001). Muscle tone was raised with either 50 μM carbachol (484 ± 44 mg tension, $n = 6$) or 100 nM thapsigargin (404 ± 26 mg tension, $n = 6$). In all experiments, verapamil (10 μM) was included in the Krebs solution in order to remove the influence of voltage-operated Ca^{2+} channels. All drugs were obtained from Sigma with the exception of calyculin-A (Tocris). Thapsigargin and calyculin-A were dissolved in dimethylsulphoxide, and in the concentrations used, the vehicle had no significant effect on the tissue. Results are given as mean ± standard error of the mean (S.E.M.), and statistical analysis was by Student's t test.

3. Results

As shown in Fig. 1A and B, field stimulation (10 Hz, 10 s train every 100 s) of the mouse anococcygeus resulted in reproducible relaxations of carbachol-induced tone (47 ± 5% relaxation, $n = 6$). We have shown previously that these relaxations are nitrergic (Gibson et al., 1990). Addition of 100 nM thapsigargin caused a slight further increase of induced tone but, over a period of 17 min, had no effect on the nitrergic relaxations (Fig. 1A and B). Subsequent addition of 1 μM calyculin-A resulted in a further transient increase in tone, but the most obvious effect was an almost complete abolition of the nitrergic relaxations (Fig. 1A and

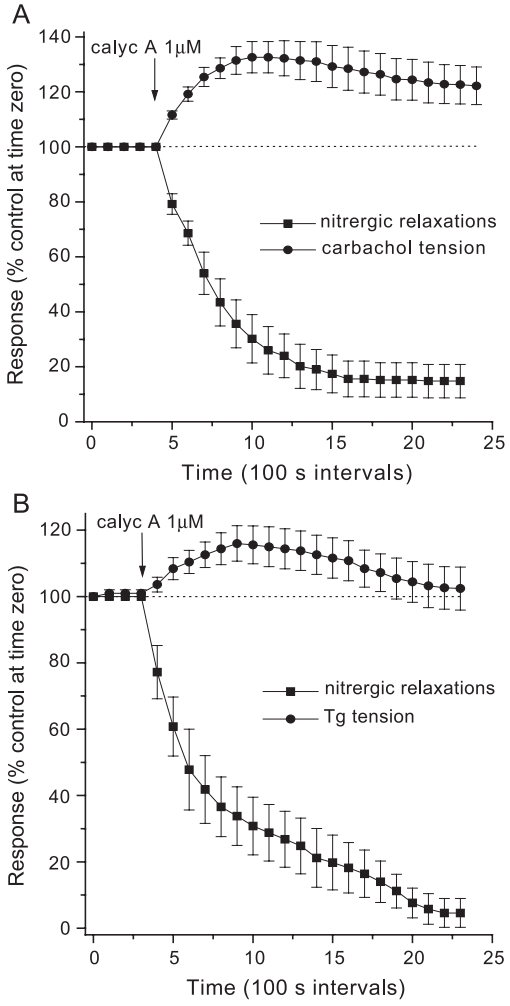

Fig. 2. Graphs showing the effects of calyculin-A (calyc A) on nitrergic relaxations and muscle tone when tension was raised initially with either 50 μM carbachol (A) or 100 nM thapsigargin (Tg; B). Each point is the mean ± S.E.M. from six individual muscle preparations.

B). The effects of calyculin-A were also determined when tone was raised by either carbachol or thapsigargin acting alone (Fig. 2A and B); when tone was raised with thapsigargin, the nitrergic relaxations (10 Hz; 10 s train every 100 s) were similar in size to those observed with carbachol (42 ± 7%, $n = 6$). Again, in both cases, calyculin-A produced a further small increase of induced tone, but the major effect was a marked inhibition of the nitrergic relaxations (Fig. 2A and B). To find out whether this inhibition of nitrergic function was exerted post-junctionally, we observed the effect of calyculin-A on relaxations induced by submaximal concentrations of sodium nitroprusside and papaverine. Relaxation of carbachol-induced tone by 1 μM sodium nitroprusside (86 ± 4%, $n = 6$) was greatly reduced in the presence of 1 μM calyculin-A (10 ± 4%, $n = 6$, $P < 0.05$), while the relaxation to 100 μM papaverine (84 ± 3%, $n = 6$) was unaltered (80 ± 6%, $n = 6$, $P > 0.05$).

4. Discussion

Previous work has shown that, in the presence of verapamil, both carbachol and thapsigargin activate store-operated Ca^{2+} entry and thereby raise cytoplasmic Ca^{2+} levels in mouse anococcygeus smooth muscle cells (Ayman et al., 2001). The nitric oxide/cyclic GMP signal can inhibit Ca^{2+} entry and reduce cytoplasmic Ca^{2+} when entry is activated by carbachol, but has no effect on either parameter when Ca^{2+} entry is activated by thapsigargin; this is because cyclic GMP inhibits store-operated Ca^{2+} entry by enhancing refilling of the sarcoplasmic reticulum, an action it cannot exert in the presence of thapsigargin which inhibits the Ca^{2+} ATPase (Ayman et al., 2001). Therefore, the observations of the present study that nitrergic relaxations were comparable whether muscle tone was raised with carbachol or thapsigargin supports a previous suggestion that inhibition of store-operated Ca^{2+} entry is not obligatory for nitrergic relaxation and indeed that relaxation can occur without a concomitant fall in cytoplasmic Ca^{2+} concentration (Ayman et al., 2001). On the other hand, the nitrergic relaxations were almost abolished by the myosin phosphatase inhibitor calyculin-A, using a concentration that has been employed in previous studies in smooth muscle (Burdyga and Wray, 2002). Addition of calyculin-A caused a further increase in the tone produced by either thapsigargin or carbachol. This would be expected given the sensitising effects of the myosin phosphatase inhibitor, but we have no explanation for the observation that this increase was more transient in the case of thapsigargin-induced tone. The inhibitory effect of calyculin-A on nitrergic relaxation appears to be exerted post-junctionally because the drug also reduced relaxations to sodium nitroprusside, although not to the general smooth muscle relaxant papaverine. The cellular mechanisms associated with the relaxant effects of papaverine are as yet unclear, but may involve nonselective inhibition of phosphodiesterase or interference with Ca^{2+} flux (Huddart et al., 1984; Beavo and Reifsnyder, 1990; Miller et al., 1994). The results of this study provide strong evidence that the predominant mechanism by which neuronally released nitric oxide causes relaxation is through inhibition of Ca^{2+} sensitisation via activation of myosin phosphatase. There is already evidence from other smooth muscles that the nitric oxide/cyclic GMP pathway can inhibit Ca^{2+} sensitisation, and activate myosin phosphatase, via phosphorylation of several proposed targets, including Rho A (Sauzeau et al., 2000), telokin (Wu et al., 1998) and myosin phosphatase itself (Surks et al., 1999). It will be important to determine which, if any, of these targets is phosphorylated during nitrergic relaxations of the mouse anococcygeus.

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