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Evidence against vasoactive intestinal polypeptide as the relaxant neurotransmitter in human cavernosal smooth muscle

*R.S. Pickard, *P.H. Powell & [1]M.A. Zar

The Departments of Pharmacological Sciences and *Urology, The Medical School, University of Newcastle upon Tyne NE2 4HH

1 The putative role of vasoactive intestinal polypeptide (VIP) as the relaxant neurotransmitter in human cavernosal smooth muscle has been studied in isolated tissue preparations.

2 Consistent neurogenic relaxations were evoked by electrical field stimulation (EFS; 2–64 pulses/train, 0.8 ms pulse duration, 10 Hz). VIP (0.1–3 μM) relaxed cavernosal smooth muscle in a dose-dependent fashion. Relaxant responses to both EFS and VIP were reduced in tissue from impotent men.

3 Neurogenic relaxant responses were not diminished in the presence of the VIP-inactivating peptidase, α-chymotrypsin (α-CT, 2 units ml^{-1}). In contrast VIP-induced relaxations were completely abolished.

4 Inhibition of nitric oxide synthase by N^G-nitro-L-arginine (30 μM), and of guanylate cyclase by methylene blue (50 μM) caused highly significant reductions of neurogenic relaxant responses whereas VIP-evoked relaxations were unaffected.

5 It is concluded that VIP-evoked relaxations are not mediated by the NO-guanosine 3′:5′-cyclic monophosphate (cyclic GMP) pathway and that VIP release is not essential for neurogenic relaxation of human cavernosal smooth muscle. VIP does not therefore act as the major relaxant neurotransmitter in this tissue.

Keywords: Vasoactive intestinal polypeptide; penile erection; nitric oxide

Introduction

Recent studies have provided good evidence that nerve-evoked relaxation of human cavernosal (penile) smooth muscle is mediated by the nitric oxide-guanosine 3′:5′-cyclic monophosphate (cyclic GMP) pathway (Kim *et al.*, 1991; Pickard *et al.*, 1991; Rajfer *et al.*, 1992; Holmquist *et al.*, 1992). However, the site of nitric oxide (NO) synthesis in this tissue remains unknown and hence the status of NO as a true neurotransmitter or as a secondary messenger is undecided.

Previous *in vitro* and *in vivo* findings lend some support for vasoactive intestinal polypeptide (VIP) as a relaxant neurotransmitter in this tissue. Nerves immunoreactive for VIP are found adjacent to the arterioles and vascular spaces of the corpora cavernosa (Polak *et al.*, 1981) and exogenous VIP relaxes isolated cavernosal tissue in a dose-dependent fashion (Willis *et al.*, 1983). In addition one study found increased levels of VIP in penile blood during erection (Ottesen *et al.*, 1984), although this finding was not confirmed by others (Kiely *et al.*, 1987). The putative role of VIP as a relaxant neurotransmitter has been strengthened by the finding that the VIP-inactivating peptidase, α-chymotrypsin attenuates neurogenic smooth muscle relaxation in other tissues (Angel *et al.*, 1983; Ellis & Farmer, 1989). In addition there is evidence that peptidergic (VIP) smooth muscle relaxation is mediated by the nitric oxide-cyclic GMP pathway in sheep cerebral artery and rat gastric fundus (Gaw *et al.*, 1991; Li & Rand, 1990).

In the present study we have investigated the possibility that VIP acts as the primary stimulator of NO release in human cavernosal tissue by examining the effect of α-chymotrypsin (α-CT), N^G-nitro-L-arginine (L-NOARG), an inhibitor of nitric oxide biosynthesis and methylene blue, an inhibitor of cyclic GMP formation, on relaxant responses evoked by VIP and nerve stimulation.

A preliminary account of this work was presented at a meeting of the Physiological Society (Pickard *et al.*, 1992).

[1] Author for correspondence.

Methods

The use of human tissue in this study was approved by the Newcastle Joint Ethics Committee.

Cavernosal tissue samples were obtained during penile operations from 5 potent and 10 impotent men and were transported to the laboratory in chilled Krebs-Henseleit solution (composition, mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 11). Tissue strips measuring approximately 2 × 3 × 5 mm were fashioned from each sample and suspended under 1 g of tension from a force transducer in a 1 ml organ bath. The bath contained Krebs solution at 37°C gassed with 95% O_2/5% CO_2 mixture. Each strip was left to equilibrate for 90 min. The bathing medium contained atropine 1 μM and guanethidine 10 μM in order to block muscarinic receptors and noradrenergic neuronal activity respectively throughout the experiments. To enable the recording of relaxant responses to electrical field stimulation (EFS) and VIP, the strips were precontracted by the addition of phenylephrine (PE) 10 μM.

Once a stable level of increased tone had been reached following the addition of PE, control relaxant responses to EFS were recorded. EFS comprised trains of pulses (0.8 ms pulse duration, 30 V) delivered by an electronic stimulator (Bell & Stein, 1971). EFS was applied both as trains of 2, 4, 8, 16, 32 and 64 pulses at a constant frequency of 10 Hz and as trains of 16 pulses at increasing frequencies of 2, 4, 8 and 16 Hz. The responses to the addition of cumulative doses of VIP from 0.1–3 μM were then recorded.

The strip was then washed repeatedly with fresh Krebs solution and allowed to re-equilibrate for 15 min. Next the strips were incubated with either α-CT (2 units ml^{-1}) for 10 min or L-NOARG (30 μM) for 10 min or methylene blue (50 μM) for 60 min. The strips were then re-contracted with PE and relaxant responses to both EFS and VIP recorded in the continued presence of the respective inhibitor. In experiments using methylene blue only one train length (16 pulses) at each frequency and one concentration of VIP (1 μM) was used.

Results are expressed as mean ± s.e.mean of data from *n* individuals. Statistical significance was tested by Student's *t*

test for paired and unpaired data and accepted if $P < 0.05$ (one-tailed).

Stock solutions of α-chymotrypsin (Sigma), atropine sulphate (Sigma), guanethidine sulphate (CIBA), methylene blue (Sigma), N^G-nitro-L-arginine (Sigma), phenylephrine (Sigma), porcine vasoactive intestinal polypeptide (Sigma) and tetrodotoxin (Sigma) were made up in distilled water and stored at − 20°C.

Results

Response to electrical field stimulation

Under conditions of sub-maximal contraction induced by PE, EFS produced relaxant responses that were rapid in onset with fast recovery to the original tension following cessation of the stimulus. The magnitude of the relaxation was dependent upon train length and, to a lesser extent, upon the pulse frequency (Figure 1a,b). The responses to EFS were fully sensitive to tetrodotoxin, 1 μM. Tissue strips from impotent men showed a reduction in the magnitude of relaxant responses to EFS at all train lengths (Figure 2).

Response to vasoactive intestinal polypeptide

The cumulative addition of VIP to the bath giving concentrations of 0.1, 0.3, 1 and 3 μM produced dose-dependent relaxation of tissue strips that was slow in onset with no recovery towards the original level of tone (Figure 1c). The rapidity of response varied between strips from different individuals. The response to VIP was reduced in strips from impotent men compared to those from potent men although the difference did not reach statistical significance (Figure 3).

Effect of α-chymotrypsin

Incubation with α-CT completely abolished the relaxant response to VIP at all concentrations tested. The presence of α-CT caused a small increase in the magnitude of relaxations evoked by EFS at most train lengths (Figure 4). This effect was observed at all frequencies tested. The increase was greatest and statistically significant when trains of 4 and 8 pulses were used at a frequency of 10 Hz, being 43 ± 14% and 15 ± 5% respectively ($n = 14$, $P < 0.05$).

Effect of N^G-nitro-L-arginine

In the presence of L-NOARG, relaxations evoked by EFS were markedly diminished (Figure 5). This inhibition occurred at all frequencies tested and was highly significant when longer trains of stimulation were used, maximum inhibition being 90 ± 8% with trains of 64 pulses ($n = 11$, $P < 0.01$). In

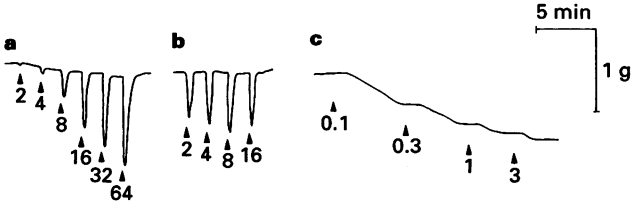

Figure 1 Relaxant responses of isolated preparations of human corpus cavernosum to electrical field stimulation (EFS, a,b) and vasoactive intestinal polypeptide (VIP, c). Atropine (1 μM), guanethidine (10 μM) and phenylephrine (10 μM) were present throughout. EFS given as a sequence of 2, 4, 8, 16, 32 and 64 pulse trains at a constant frequency of 10 Hz produced rapid relaxant responses of increasing magnitude (a). The magnitude of responses also increased when 16 pulse trains were delivered using increasing pulse frequencies of 2, 4, 8 and 16 Hz (b). The relaxant responses to cumulatively increasing VIP concentrations of 0.1, 0.3, 1 and 3 μM were slow in onset and concentration-dependent (c).

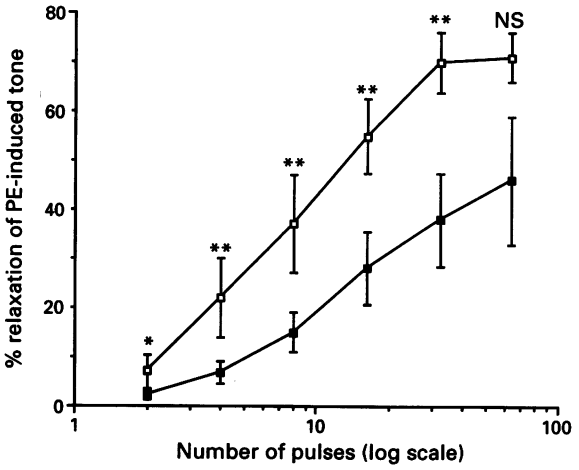

Figure 2 Relaxant responses to electrical field stimulation (2–64 pulse trains, 10 Hz) in isolated preparations of corpus cavernosum from 5 potent men (□) compared to those demonstrated in tissue from 10 impotent men (■). Each point is the mean relaxation of phenylphrine (PE, 10 μM)-induced tone with bars representing s.e.mean. NS = not significant; $*P < 0.05$; $**P < 0.01$.

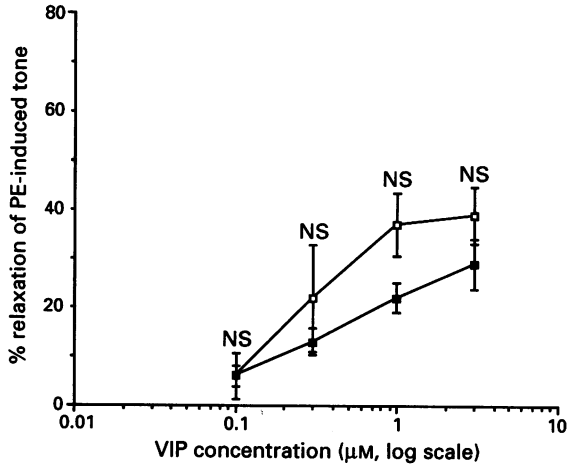

Figure 3 Comparison of relaxant responses to the cumulative addition of vasoactive intestinal polypeptide (VIP) giving concentrations of 0.1, 0.3, 1 and 3 μM in isolated preparations of corpus cavernosum from 5 potent (□) and 10 impotent men (■). Each point represents the mean relaxation of phenylephrine (PE, 10 μM)-induced tone with bars depicting s.e.mean. NS = not significant.

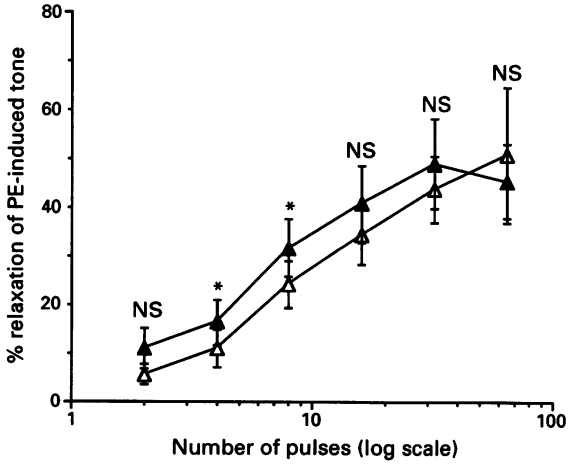

Figure 4 The effect of incubation of human isolated cavernosal strips in α-chymotrypsin (α-CT, 2 units ml^{-1}) on relaxations evoked by electrical field stimulation (2–64 pulse trains, 10 Hz). Each point shows the mean relaxation of phenylephrine (PE, 10 μM)-induced tone before (△) and following (▲) the addition of α-CT with bars representing s.e.mean ($n = 14$). NS = not significant; $*P < 0.05$.

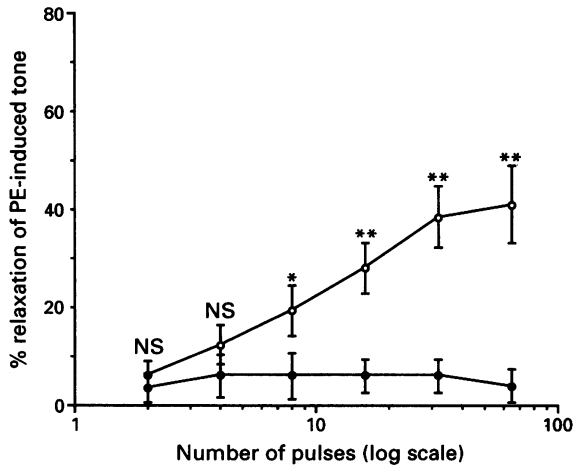

Figure 5 The effect of incubation with N^G-nitro-L-arginine (L-NOARG, 30 μM) on relaxations of human cavernosal strips evoked by electrical field stimulation (2–64 pulses/train, 10 Hz). Each point indicates mean relaxation of phenylephrine (PE, 10 μM)-induced tone before (○) and following (●) the addition of L-NOARG with vertical bars showing s.e.mean ($n = 11$). NS = not significant; $*P < 0.05$; $**P < 0.01$.

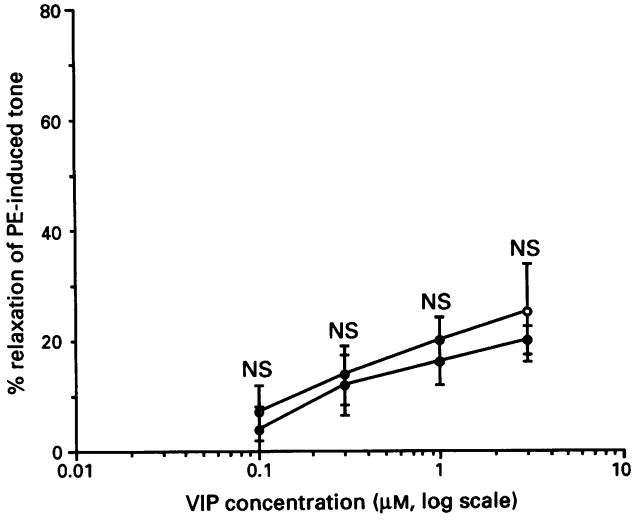

Figure 6 The effect of N^G-nitro-L-arginine (L-NOARG, 30 μM) on relaxations of human isolated cavernosal strips evoked by the cumulative addition of vasoactive intestinal polypeptide (VIP, 0.1, 0.3, 1 and 3 μM). Points show the mean relaxation of phenylephrine (PE, 10 μM)-induced tone before (○) and following (●) the addition of L-NOARG with bars representing s.e.mean ($n = 11$). NS = not significant.

contrast, only small, statistically insignificant reductions in the relaxant responses to VIP were observed at all concentrations tested (Figure 6), the maximum inhibition being 20 ± 20% at a VIP concentration of 1 μM ($n = 11$, NS).

Effect of methylene blue

Treatment with methylene blue significantly attenuated the relaxant response to EFS by 38 ± 12% at a frequency of 10 Hz ($n = 5$, $P < 0.01$, Figure 7). This inhibitory effect was similar at all frequencies used, reaching a maximum of 54 ± 19% at 8 Hz ($n = 4$, $P < 0.01$). In contrast, the magnitude of VIP-evoked relaxations was slightly increased by 4 ± 19% ($n = 5$, NS, Figure 7). Following treatment with methylene blue the contractile response to PE was reduced by 11 ± 25% ($n = 5$, NS).

The effects of α-CT, L-NOARG and methylene blue on relaxant responses to EFS and VIP were similar in tissues from potent and impotent men.

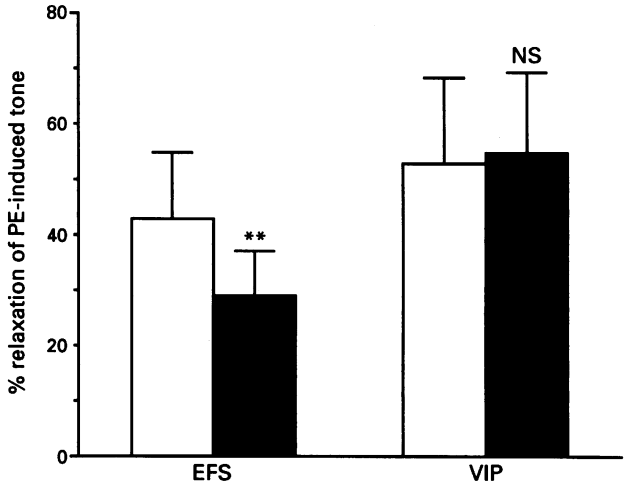

Figure 7 The effect of incubation with methylene blue (50 μM) on relaxant responses of isolated strips of human corpus cavernosum evoked by electrical field stimulation (EFS, 16 pulse trains, 10 Hz) and vasoactive intestinal polypeptide (VIP, 1 μM). Each pair of columns represents the mean relaxant responses before (open columns) and after (solid columns) the addition of methylene blue expressed as a percentage of phenylphrine (PE 10 μM)-induced tone. Vertical bars show s.e.mean ($n = 5$). NS = not significant; $**P < 0.01$.

Discussion

The neurogenic relaxation of cavernosal smooth muscle is essential for the initiation and maintenance of physiological penile erection in man (Krane *et al.*, 1989). The nerves responsible for cavernosal relaxation are overwhelmingly non-adrenergic, non-cholinergic (NANC) in nature (Saenz de Tejada *et al.*, 1988) and although the identity of the NANC neurotransmitter has yet to be unequivocally determined, VIP has been proposed as a strong candidate for this role (Willis *et al.*, 1983; Ottesen *et al.*, 1984; Adaikan *et al.*, 1986). The present investigation has tested the putative role of VIP in the neurogenic relaxation of human cavernosal smooth muscle using two different approaches and the results are incompatible with VIP being a major relaxant neurotransmitter.

In the first series of experiments incubation of corpus cavernosum strips with the peptidase, α-chymotrypsin (α-CT), which has been shown to abolish the smooth muscle relaxant effects of both endogenous and exogenous VIP in canine stomach and guinea-pig trachea (Angel *et al.*, 1983; Ellis & Farmer, 1989), caused no inhibition of neurogenic relaxant responses; on the contrary they were significantly increased by this treatment when short trains of stimuli were used. The failure of α-CT to reduce or abolish neurogenic relaxant responses was not attributable to weak peptidase activity since in these experiments under identical conditions, treatment with α-CT led to complete abolition of relaxant responses to exogenous VIP. Immunohistochemical studies have shown that the density of peptidergic (VIP) nerves is reduced in cavernosal tissue from impotent men, particularly those with diabetes (Gu *et al.*, 1984; Lincoln *et al.*, 1987). In our study although the magnitude of both neurogenic and VIP-evoked relaxations was somewhat reduced in tissue from impotent men the effects of α-CT were similar in strips from potent and impotent men. It is therefore unlikely that the lack of effect of α-CT was due to the pathological absence of peptidergic (VIP) nerves. These findings therefore indicate that neurogenic relaxation of human cavernosal smooth muscle is not dependent upon the release of VIP.

There is incontrovertible evidence that NANC nerve-evoked relaxation of human cavernosal smooth muscle is mediated by nitric oxide (NO) or a NO-like substance which acts through the activation of soluble guanylate cyclase in the smooth muscle cell (Kim *et al.*, 1991; Pickard *et al.*, 1991;

Rajfer et al., 1992; Holmquist et al., 1992). The implication of this knowledge is that for VIP to serve as the main NANC neurotransmitter in human cavernosal smooth muscle it must act predominately through the release of NO which in its turn activates guanylate cyclase leading to formation of cyclic GMP and consequent smooth muscle relaxation. In the second set of experiments we have tested this hypothesis by comparing the effect of inhibitors of NO-synthase and guanylate cyclase on neurogenic and VIP-evoked relaxations of human corpus cavernosum and our findings indicate that it should be rejected. The NO-synthase inhibitor, L-NOARG virtually abolished the neurogenic relaxant responses of cavernosal smooth muscle but did not affect the relaxant response to VIP, thus implying that functional integrity of NO-synthase is an essential prerequisite for evoking relaxant responses to nerve stimulation alone. In this respect human cavernosal smooth muscle activity is similar to that of the rabbit (Holmquist et al., 1992). These results are in conformity with the view that NO is the mediator of nerve-evoked relaxation whilst VIP does not require NO to exert its relaxant effect. Additional evidence for this contention comes from our finding that methylene blue, an inhibitor of guanylate cyclase activation also failed to reduce significantly VIP-evoked relaxation in this tissue, thereby indicating that VIP-evoked relaxation, unlike neurogenic relaxation is independent of the NO-cyclic GMP pathway.

Our exclusion of VIP as a major inhibitory neurotransmitter in this tissue is in keeping with the recent immunocytochemical localization of NO-synthase in nerves supplying erectile tissue of the bull and rat indicating that NO acts as a true neurotransmitter in these tissues (Sheng et al., 1992; Burnett et al., 1992). Although the site of NO-synthesis has yet to be determined in human corpus cavernosum it seems likely that NO is also neuronal in origin in man.

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