Vasoactive intestinal polypeptide and non-adrenergic, non-cholinergic inhibition in lower oesophageal sphincter of opossum

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1 Field stimulation or vasoactive intestinal polypeptide (VIP) relaxed lower oesophageal sphincter (LOS) from North American opossum. Pretreatment with carbachol in Cl⁻-ion-containing or Cl⁻-ion-free Krebs solution or with 10^{-3} M 9-aminoacridine abolished or markedly reduced relaxation due to VIP applied exogenously but not that elicited by field stimulation of non-adrenergic, non-cholinergic nerves.

2 Inhibitory junction potentials (7.5 ± 1.2 mV, $n = 5$) could be recorded in LOS strips with the sucrose gap technique. They lacked significant after-depolarizations but were accompanied by decreased membrane resistance ($61 \pm 6\%$, $n = 3$). In these strips, VIP (10^{-6} M) produced small hyperpolarizations (2.1 ± 1.1 mV, $n = 5$) sometimes followed by membrane potential oscillations but no change in conductance.

3 Removal of external chloride depolarized the membranes (7.6 ± 1.7 mV) but did not prevent the hyperpolarization to VIP or the occurrence of inhibitory junction potentials. Restoration of external chloride repolarized the cells. It appears that an appreciable chloride conductance may be present in sphincter muscle cells and this may cause them to be more depolarized than non-sphincter muscle.

4 We conclude that it is very unlikely that VIP is the inhibitory NANC neurotransmitter since it does not mimic the inhibitory junction potential.

Introduction

Previous studies of opossum lower oesophageal sphincter (LOS) *in vivo* revealed the existence of relaxation which could be activated by stimulation of vagal preganglionic nerves, by field stimulation of post-ganglionic non-adrenergic, non-cholinergic nerves (NANC), by swallows or by proximal balloon distention (Rattan & Goyal, 1974; Sarna *et al.*, 1977; Daniel *et al.*, 1979; Goyal & Rattan, 1978). Further studies suggested that the mediator might be vasoactive intestinal polypeptide (VIP) since VIP given intra-arterially relaxed the LOS by a direct action and VIP-antiserum reduced the relaxation to vagal stimulation but not that to isoprenaline (Goyal *et al.*, 1980; Rattan *et al.*, 1982).

However, studies of the circular muscle just proximal to the LOS revealed that VIP could not be the mediator of NANC-inhibition since its electrophysiological actions did not mimic those of the mediator.

In particular, the mediators released by field stimulation caused an inhibitory junction potential (i.j.p.) by increasing the K⁺-conductance of the smooth muscle cell membrane (Daniel *et al.*, 1983; Jury *et al.*, 1985). In contrast, VIP caused slight or no membrane hyperpolarization accompanied by a decreased membrane conductance, followed by membrane repolarization and membrane oscillations, which triggered action potentials on the depolarizing phase (Daniel *et al.*, 1983; 1987). Further, a material similar to VIP (in its electrophysiological effects) was shown to be released by dopamine acting via dopamine (D₁-like) receptors on nerve endings (Daniel *et al.*, 1987). This release was tetrodotoxin-insensitive but sensitive to scorpion venom and solutions containing low Ca and high Mg. Both the effects of dopamine and VIP were abolished by removal of external chloride (Daniel *et al.*, 1987). Dopamine-induced hyperpolarization, caused by the release of an endogenous VIP-like

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material, showed a reversal potential near the chloride-equilibrium potential. Thus, it was not possible that VIP was the transmitter released by NANC inhibitory nerves acting directly on circular muscle of the body of the oesophagus (BCM) since the actions of exogenous VIP and endogenous VIP-like material were similar to each other but differed from the actions of the mediator.

The purpose of the present study was to determine whether VIP could be the mediator of the NANC-relaxation in the LOS itself. Since LOS preparations, which have fewer gap junctions than proximal muscle (Daniel & Posey-Daniel, 1984), often fail to show nerve-mediated responses in the sucrose gap, we also studied VIP- and electrically-evoked relaxations of the LOS in an *in vitro* muscle chamber. We examined whether removal of external chloride or application of 9-aminoacridine (9-AA), a putative potassium-conductance blocking agent (Volle, 1971; Volle & Henderson, 1972) affected relaxations to these two stimuli differently. In addition, experiments were carried out with the double sucrose gap technique to characterize the response of the muscle to VIP and to the nerve mediator.

Methods

North American opossums (*Didelphis marsupialis*) were obtained (Singleton, FL, U.S.A.), handled as previously described (Daniel *et al.*, 1983) and killed by intraperitoneal pentobarbitone (100 mg kg⁻¹). The oesophagus and proximal stomach were removed and dissected as previously described (Daniel *et al.*, 1983). After removal of the mucosa, the LOS was identified by its thickened structure and locus and divided into 2 mm wide strips. Strips from the centre of this thickened muscle were included in the results presented. These were mounted through concentric platinum electrodes 1 cm apart in 4 ml muscle baths containing a modified Krebs solution, warmed to 37°C and bubbled with 95% O₂ and 5% CO₂. This solution contained (mM): NaCl 115, KCl 4.5, NaHCO₃ 21.9, NaH₂PO₄ 1.16, CaCl₂ 2.5, MgSO₄ 1.16 and glucose 11. Cl-free solution was made by substituting NaCl and KCl with isethionate salts and Calevalinate for CaCl₂. The pH of all solutions used was 7.4 at 37°C.

The tissue was tied at one end to the electrode holder and at the other to a strain gauge transducer (Grass FT03C). Tension was recorded on a Beckman R611-8 channel oscillograph. 9-Aminoacridine was obtained from Fluka. Since chloride-free solutions first increased but subsequently abolished the basal active tension of LOS strips, carbachol (10⁻⁶ M) was added after chloride removal to restore tone. We

have shown previously (Daniel *et al.*, 1979) that this does not impair relaxation to field stimulation. Field stimulation was carried out for 10 s at 40 V, 0.5 ms, 5 Hz, parameters shown previously (Daniel *et al.*, 1979) to cause maximal and complete relaxation. We also studied inhibitory junction potentials (i.j.p.) in response to field stimulation (30 Hz, 0.3 ms duration, with supramaximal field strength 20 V mm⁻¹ for 300 ms trains) by the double sucrose gap technique as previously described (Jury *et al.*, 1985; Daniel *et al.*, 1987). These were compared to the effects of VIP (10⁻⁶ M). Also, changes in conductance during the i.j.p. and the responses to VIP were compared by applying brief pulses of hyperpolarizing currents. The effects of chloride-free solutions were also studied. Data were expressed as means ± standard error of mean (s.e. mean) and statistical significance was determined using Student's *t* test for paired or unpaired values as appropriate (*P* < 0.05 was taken to be significant).

Results

After the strips of LOS were placed in the muscle bath under 10 mN tension, they rapidly acquired additional tone of about 10 to 15 mN. After 1 h equilibration, field stimulation (FS) caused maximal relaxation, with an amplitude of 13.6 ± 1.2 mN (*n* = 49). This represented the active tension and left a residual passive tension of 5 to 10 mN. Subsequently, VIP in a concentration (10⁻⁶ M) found in preliminary studies to be maximal in effect, was tested in the same strips. It caused a gradual relaxation equal to 107 ± 11% (*n* = 49) of the rapid relaxation produced by FS in the same tissues. After washout of VIP, the preparations regained active tension. Then Cl-free solution followed by carbachol was used and effects of FS and VIP retested. Substitution of isethionate for chloride resulted in loss of active tension; carbachol-induced contraction was, therefore, induced to allow relaxation to be observed. Figure 1 shows typical results of experiments which are summarized in Table 1. Cl-free medium abolished or nearly abolished the relaxation to VIP (compare traces (i) and (v) in Figure 1a) but had little effect on relaxation to FS (compare traces (i) and (iii) in Figure 1b).

Further analysis showed that the VIP-induced relaxation was already slowed and impaired in magnitude (compare traces (i) and (iii) in Figure 1a) or abolished by the use of carbachol in normal medium to cause contraction. Table 1 shows that on average, VIP-induced relaxation was nearly complete within 2 min in the absence of carbachol but attained a smaller and slower relaxation only after 4 min when carbachol was present. A stability control for

Figure 1 Comparison of relaxant responses to field stimulation (FS) and VIP in carbachol (CCh) and chloride-free media. (a) All tracings from the same strip: (i) control responses to VIP 10^{-6} M; (ii) and (iii) show the response of the tissue to carbachol (10^{-6} M) without or with the addition of VIP (10^{-6} M) at the arrow. The dashed lines show the starting tension in each case. Note that there appears to be a definite but smaller relaxation to VIP. This was not always found. (iv) and (v) show responses to carbachol (10^{-6} M) in chloride-free bathing solution. There was no increase in the decay of tension with time when VIP was added at the arrow. (b) (i) Control relaxation response to FS (10 s, 5 Hz, 0.5 ms, 40 V); (ii) relaxation to FS in CCh 10^{-6} M; (iii) relaxation to FS in CCh added to chloride-free bathing solution. The degree of relaxation is similar in all cases but recovery of tension was faster after CCh.

carbachol-induced tension in the same strips showed that there was usually a significant spontaneous decay of tension induced by carbachol after 4 min (not evident in the second trace in the figure). Removal of chloride from the medium sometimes (as in trace (iv) in Figure 1a) increased the rate of decay of the carbachol-induced tension but did not affect the magnitude of the relaxation which had occurred 4 min after addition of VIP. Thus, the difference between the normal relaxation with time and the VIP-induced relaxation was markedly less in this medium. However, field stimulated relaxations per-

Figure 2 Comparison of the effects of 9-aminoacridine (9-AA) on the relaxant responses to field stimulation (FS) and VIP. (a) Control response to FS (10 s, 5 Hz, 0.5 ms, 40 V), and VIP (10^{-6} M); (b) responses to FS and VIP evoked in the presence of 9-AA 5×10^{-4} M. Note that FS evoked a more complete relaxation without an 'off-response' above basal tension in the presence of VIP. However, VIP failed to relax the preparation; the decay in the tension after VIP is similar to that in strips treated with 9-AA but not VIP.

sisted in normal Krebs medium or chloride-free medium with carbachol as a nearly constant percentage of the total active tension (Table 1). Figure 1b shows an example in which FS relaxed the LOS after carbachol in either normal or chloride-free medium. The recovery of active tension after the stimulus had stopped was faster in the presence of this agonist in this and most strips.

The effects of a putative selective potassium channel blocker 9-AA were also tested in the organ bath studies. As illustrated in Figure 2, 9-AA increased the tone of the preparations, did not reduce the relaxation induced by field stimulation, but abolished the VIP-induced relaxation. In 11 LOS strips, which relaxed 14.0 ± 1.2 mN in control tests and $124 \pm 20\%$ of those values to 10^{-6} M VIP, 9-AA (10^{-3} M) reduced the VIP-induced relaxation to $19 \pm 8\%$ but not FS-relaxation, which remained at $149 \pm 29\%$ of control. After 10 to 15 min in 9-AA, all tone gradually disappeared; then, of course, FS had no further relaxant effect. However, in many cases spontaneous phasic contractions occurred and these were transiently abolished after field stimulation (Figure 3), suggesting that inhibitory mediator

Figure 3 Effects of field stimulation and VIP on contractions induced by 9-aminoacridine (9-AA). In tissue treated with 9-AA (10^{-3} M) for 40 min, persistent active tension disappeared but spontaneous contractions developed in the presence of the putative K-channel blocker. These were not affected by VIP but were inhibited by FS.

Table 1 Effects of carbachol (CCh) and chloride-free medium on relaxation responses to field stimulation and VIP (10^{-6} M)

Normal Krebs		Tension		ΔVIP^*		Tension		$\Delta No VIP^\dagger$		No FS	
Tension	$\Delta FS^\dagger$		2 min	4 min			2 min		2 min	4 min	
11.4 ± 1.9 (n = 8)	-7.8 ± 1.9 (-68%)	10.8 ± 2.2	$-5.3 \pm 1.4^{***}$ (-49%)	$-6.4 \pm 1.7^{***}$ (-59%)		12.3 ± 4.5	-0.5 ± 2.9 (4%)		-0.2 ± 2.5 (2%)		
CCh											
			2 min	4 min			2 min		4 min		
27.5 ± 3.6 (n = 8)	$-18.3 \pm 1.9^*$ (-67%)	23.0 ± 2.5	$-1.9 \pm 0.6^{*,**}$ (-8%)	$-4.4 \pm 1.4^{**}$ (-19%)		25.6 ± 1.9	-0.1 ± 0.1 (0.4%)		-1.7 ± 0.8 (7%)		
Chloride free + CCh											
			2 min	4 min			2 min		4 min		
13.3 ± 2.5 (n = 8)	-8.9 ± 1.9 (-67%)	15.3 ± 2.8	$-1.4 \pm 0.6^{*,**}$ (-9%)	$-5.0 \pm 1.8^{**}$ (-32%)		14.4 ± 2.8	-0.3 ± 0.3 (2%)		-2.7 ± 1.1 (19%)		

Values in mN tension mean $\pm$ s.e.mean; values in parentheses; % change in initial tension.

$^\dagger \Delta$ refers in all cases to the net change in tension; this was measured at the maximum change after field stimulation (FS) and at the times indicated in other cases.

* Significantly different by paired *t* test ($P < 0.05$) from values in normal Krebs without carbachol.

** Significantly different by paired *t* test ($P < 0.05$) from magnitude of field-stimulated relaxation.

*** Significantly different from the tensions observed with time in the absence of VIP (right-hand columns); measured at the same time interval after carbachol as when VIP added.

was still active and being released. Effects of 9-AA were not fully reversible on washing out this substance.

Inhibitory junction potentials (i.j.ps) with a peak amplitude of about 8 mV were obtained in 5 strips of LOS (Table 2). These differed from the i.j.ps recorded from body circular muscle (Daniel *et al.*, 1983; 1987; Jury *et al.*, 1985) in being smaller (cf: Table 2 and see Daniel *et al.*, 1983; 1987; Jury *et al.*, 1985) and lacking detectable after-depolarization (Figures 4 and 5). They were accompanied by a significant reduction in the magnitude of electrotonic potentials

evoked by hyperpolarizing constant current pulses (Table 2 and Figure 4), indicating that there was a conductance increase. In the same strips, VIP (10^{-6} M) caused a small (about 2 mV) hyperpolarization that was not accompanied by an increase in the membrane conductance (Table 2 and Figure 5). This hyperpolarization induced by VIP did not alter the value of peak negativity of hyperpolarization associated with the i.j.p. (Figure 5), although it did reduce the amplitude of the i.j.p. to $58 \pm 12\%$ of control ($n = 3$). The rate of repolarization of the i.j.p. was, however, usually slowed (not evident at the

Table 2 Electrophysiological effects of field stimulation and VIP (10^{-6} M)

	i.j.p. amplitude (mV)	Electrotonic potential during i.j.p. (% control)	Hyperpolarizing effect of VIP (mV)	Electrotonic potential in VIP (% control)	i.j.p. amplitude in VIP (% control)
Normal Krebs	8.9 ± 0.8 (5) [8.2 ± 0.9] (3)	[61 ± 6 (3)]*	2.2 ± 1.1 (5)* [3.7 ± 1.6] (3) NS	105 ± 4 (5) [107 ± 4] (3) NS	74 ± 11 (5) [58 ± 12] (3)
Cl-free	7.3 ± 2.0 (3)	—	2.7 ± 1.2 (3) NS*	97 ± 3 (3) NS	89 ± 20 (3)
▲ mV Depol. 7.6 ± 1.7 (3)*					

Mean $\pm$ s.e.mean (number of preparations in parentheses).

* Significantly different ($P < 0.05$) from 0 mV or 100%. Value in bottom line (▲) shows membrane potential change due to removal of external Cl ion.

NS not significantly different ($P > 0.05$) by paired comparison; same three strips compared in normal Krebs (values in []) and in Cl-free Krebs.

Figure 4 Conductance change associated with inhibitory junction potential (i.j.p.) (sucrose-gap recording). High speed trace of an i.j.p. of opossum oesophagus LOS in response to field stimulation (a) (300 ms, 30 Hz, 0.5 ms, 30 V); initial upward and downward deflections are stimulus artefacts. In (b) repetitive brief (2 s) hyperpolarizing constant current pulses were applied, during which an i.j.p. was elicited. Note that the size of the electrotonic potentials were reduced during the i.j.p. but recovered to control size as the membrane potential recovered. A conditioning hyperpolarization of the same magnitude as the i.j.p. did not affect these brief electrotonic potentials.

chart speed of Figure 5). During prolonged superfusion with VIP the tissue repolarized in its presence, and there were on several occasions persistent membrane oscillations (not shown). Removal of external chloride caused a persistent depolarization of the LOS (about 8 mV). This was sometimes accompanied by a gradual reduction (Figure 5) in the amplitude of the i.j.p. When the i.j.p. was reduced in amplitude (as in Figure 5), the rate of repolarization of the i.j.p. was increased. There was no significant change in the magnitude of the responses to VIP (Table 2 and Figure 5) in chloride-free superfusion medium. Likewise, the magnitude of the i.j.p. in the presence of VIP was not significantly different from the control value in chloride-free medium.

Discussion

These results show that it is possible to abolish or reduce markedly the VIP-induced relaxation or inhibitory response of the LOS by 9-AA or by carbachol in a chloride-free medium without similarly affecting the relaxation response to field stimulation.

This implies that, when VIP is ineffective as a relaxant agent, the mediator of NANC relaxation is still effective. Thus, it is unlikely that VIP is that mediator. It is of interest that the induction of tension by carbachol impairs relaxation to VIP. This impairment was so extensive that removal of chloride, which abolished the effects of VIP on body circular muscle (Daniel *et al.*, 1983; 1987), had no further statistically clear effect. A recent report (Thorpy *et al.*, 1986) shows that VIP-induced relaxation of the LOS is accompanied by an increase in cyclic AMP concentrations, whereas that to field stimulation is accompanied by an increase in cyclic GMP; in several tissues carbachol acting at muscarinic receptors causes a decrease in cyclic AMP concentrations and this may explain its ability to oppose the relaxant actions of VIP but not those of field stimulation (Nathanson *et al.*, 1978; Brown & Goldstein, 1986).

Since VIP-induced relaxation could not be tested in the chloride-free medium unless tone was restored by carbachol, we could not determine whether removal of chloride *per se* selectively inhibited responses to VIP as it did in body circular muscle (BCM) (Daniel *et al.*, 1983; 1987). The observed changes in the time-course of the electrotonic potential consequent upon removal of chloride ions are, however, compatible with the passive membrane properties of smooth muscle cells (Jury *et al.*, 1985). In contrast, removal of external chloride had effects on the LOS different from those induced in BCM; there was a persistent depolarization and transient contraction (not shown) in the LOS rather than a hyperpolarization as observed in BCM. In the LOS the membrane repolarized back to the original membrane potential after re-exposure to a chloride-containing superfusion fluid.

The depolarization of the LOS when chloride was removed may imply that there is an appreciable resting chloride conductance, so that the loss of internal chloride into the chloride-free medium causes depolarization and an increase in the time constant of the electrotonic potential, as observed. This depolarization, however, should be transient, disappearing when internal chloride is lost; however, it persisted for at least 30 min in the absence of chloride. This is so far unexplained. An appreciable resting Cl-conductance, absent in the BCM, could lead to lower resting potentials in the LOS if the chloride equilibrium potential is of the order of 30 mV inside negative, as it appears to be in some smooth muscles (see references in Daniel *et al.*, 1987). This could explain the resting active tension in LOS, absent in BCM.

Previous studies in BCM (Daniel *et al.*, 1983; 1987) suggested that VIP caused a decrease in chloride conductance. Thus, chloride replacement with an impermeant anion prevented all responses to

Figure 5 Comparison of the effects of chloride-free (OCI^-) bathing medium on inhibitory junction potentials (i.j.ps) and membrane responses to VIP (sucrose-gap recording). The upward deflection and the initial part of the i.j.p. are stimulus artefacts. (a) Shows effects of VIP (10^{-6} M) on the resting potential, on hyperpolarizing electrotonic potentials and on i.j.ps. In (b) the effects of substitution of isethionate for chloride are shown; a large (10 mV) and persistent depolarization leading to a gradual reduction in i.j.p. amplitude, hence in the peak negativity during the i.j.p. The applied electrotonic pulses (8–10 s) did not change in amplitude but had altered time-courses. VIP (10^{-6} M) still caused a similar (5 mV) hyperpolarization compared to control (a) although the i.j.p. was reduced, the peak negativity was the same as that before exposure to VIP, just as when Cl was present (a). In (c) which is continuous with (b), re-exposure to Cl-containing medium led to hyperpolarization to a resting potential slightly greater than the control membrane potential and to restoration of the i.j.p. amplitude without an alteration of the amplitude of electrotonic potentials. Note the varying time scale: the largest deflections show 1 min intervals, the second largest show 5 s intervals and the smallest (sometimes fused) show 1 s intervals.

exogenous VIP or endogenous VIP-like material; there was also a reversal potential near the chloride equilibrium potential for this material. Clearly, a different or additional mechanism must apply in the LOS since chloride replacement did not prevent the hyperpolarization to VIP. It was impossible to determine accurately the reversal potential of the small VIP-induced hyperpolarization in this muscle. Thus, the electrophysiological basis of the action of VIP in the LOS is unclear. If 9-AA is a potassium conductance blocker (Volle 1971; Volle & Henderson, 1972), then its ability to inhibit VIP-mediated relaxation suggests that a potassium conductance change was involved in the mechanism by which VIP acts.

There was also an obvious impairment of contractility in chloride-free media since the LOS relaxed despite persistent depolarization and carbachol con-

tractions were smaller and less well sustained (Figure 1, Tables 1 and 2). This needs further analysis.

NANC-relaxation of gut muscle, whenever it has been analysed (Tomita, 1972; Bywater *et al.*, 1981; Jury *et al.*, 1985) is caused by increased potassium conductance. Our earlier studies (Jury *et al.*, 1985) showed that none of several established K-conductance blockers (apamin, tetraethylammonium, 4-aminopyridine or quinine) abolished the i.j.p. induced by NANC nerve stimulation in BCM, suggesting that a special postjunctional K-channel was involved. Since 9-AA does not block relaxant responses to field stimulation in concentrations effective against VIP-induced relaxation, it too, appears to be unable to affect these channels selectively. Whether VIP actions in other tissues are Cl-dependent or K-dependent remain to be determined.

Apart from the results with 9-AA, there is no evidence that VIP increases K-conductance; in contrast, we observed no change in the membrane conductance in the presence of VIP. An alternative explanation of the selective action of 9-AA on VIP induced relaxations is that 9-AA may have other actions as well as those reported (Volle, 1971; Volle & Henderson, 1972) on K-conductance.

The question arises, can these findings be reconciled with a role of VIP in NANC-mediated relaxation in the LOS. It is clear that the conductance changes underlying VIP-induced relaxation do not mimic those underlying NANC-nerve mediated responses and also their pharmacology differs. It is, therefore, difficult to maintain that the NANC nerve-mediated response in the LOS involves VIP as neurotransmitter acting directly on the smooth muscle cell membrane. Another role of VIP in the process is possible: as a secondary transmitter (Daniel *et al.*, 1983) it may add to the relaxation and lead to the subsequent 'off' contraction, as we have previously suggested. However, 'off' depolarization,

which is thought to lead to 'off' contractions, was very small or absent in LOS. Alternatively, VIP may act mostly on interstitial cells of Cajal, which are major sites of innervation in oesophageal circular muscle (Daniel & Posey-Daniel, 1984) and there initiate events which are transmitted to smooth muscle via gap junction contacts ultimately causing relaxation (Daniel, 1985; Daniel *et al.*, 1985) by increasing potassium conductance. The present type of study did not provide clear evidence for VIP having such an indirect action on LOS. Future studies on this hypothesis will need to establish that VIP is present in the nerves associated with interstitial cells, is released to act on receptors on these cells, and initiates in them a process leading to a potassium conductance increase in smooth muscle cells.

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