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Activation of outward K^+ currents: effect of VIP in oesophagus

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1 Electrical field stimulations (EFS) of the opossum and canine lower oesophageal sphincters (OLOS and CLOS respectively) and opossum oesophageal body circular muscle (OOBCM) induce non-adrenergic, non-cholinergic (NANC) relaxations of any active tension and NO-mediated hyperpolarization. VIP relaxes the OLOS and CLOS and any tone in OOBCM without major electrophysiological effects. These relaxations are not blocked by NOS inhibitors. Using isolated smooth muscle cells, we tested whether VIP acted through myogenic NO production.

2 Outward currents were similar in OOBCM and OLOS and NO increased them regardless of pipette Ca^{2+}_i, from 50–8000 nM. L-NAME or L-NOARG did not block outward currents in OLOS at 200 nM pipette Ca^{2+}.

3 Outward currents in CLOS cells decreased at 200 nM pipette Ca^{2+} or less but NO donors still increased them. VIP had no effect on outward currents in cells from OOBCM, OLOS or CLOS under conditions of pipette Ca^{2+} at which NO donors increased outward K^+ currents.

4 We conclude, VIP does not mimic electrophysiological effects of NO donors on isolated cells of OOBCM, OLOS or CLOS. VIP relaxes the OLOS and CLOS and inhibits contraction of OOBCM by a mechanism unrelated to release of myogenic NO or an increase in outward current.

5 Also, the different dependence of outward currents of OOBCM and OLOS on pipette Ca^{2+} from those of CLOS suggests that different K^+ channels are involved and that myogenic NO production contributes to K^+ channel activity in CLOS but not in OLOS or OOBCM.

Keywords: Vasoactive intestinal peptide (VIP); lower oesophageal sphincter; nitric oxide; K^+ channels; smooth muscle relaxation; mediators of NANC nerves

Abbreviations: CLOS, canine lower oesophageal sphincter; EFS, electrical field stimulation; ICC, interstitial cell of Cajal; L-NAME, N^G-nitro-L-arginine methyl ester; L-NOARG, N^G-nitro-L-arginine; OOBCM, opossum oesophageal body circular muscle; OLOS, opossum lower oesophageal sphincter; NOS, nitric oxide synthase; SNP, sodium nitroprusside; VIP, vasoactive intestinal peptide

Introduction

Activation of the intrinsic nerves of the opossum or the canine lower oesophageal sphincter (OLOS & CLOS respectively) produces a non-adrenergic, non-cholinergic (NANC) hyperpolarization and relaxation (Allescher *et al*., 1988; Daniel *et al*., 1989). Also in opossum oesophageal body circular muscle (OOBCM) similar hyperpolarization and relaxation of any active tension occurs in response to NANC nerve stimulation (Daniel *et al*., 1982; Christinck *et al*., 1991; Murray *et al*., 1991; Jury *et al*., 1992). These responses are blocked by NOS inhibitors such as L-NAME or L-NOARG (Christinck *et al*., 1991; Cayabyab & Daniel, 1995; Jury *et al*., 1992). The nitric oxide donors sodium nitroprusside (SNP) and sydnondimine (Sin-1) also hyperpolarize and relax the OOBCM or OLOS (Cayabyab & Daniel, 1995; 1996; Gaumnitz *et al*., 1997; De Man *et al*., 1991) and the CLOS and can increase outward potassium currents (Jury *et al*., 1992; Salapatek *et al*., 1996; 1998b).

VIP, often colocalized in inhibitory nerves with mNOS (Costa *et al*., 1992; Lynn *et al*., 1995; Wang *et al*., 1997), also relaxes the OLOS and CLOS and inhibits activity of OOBCM. VIP may play a key role in the NANC inhibitory response in sphincteric tissue and has been suggested to act by releasing NO from muscle cells (Jin *et al*., 1996; Grider & Murthy, 1996; Murthy *et al*., 1993; 1996; Murthy & Makhlouf, 1994). However, relaxation induced by VIP in organ bath studies of the OLOS (Daniel *et al*., 1989) or CLOS (De Man *et al*., 1991) was not blocked by NOS inhibitors L-NAME or L-NOARG. Neither was there any electrophysiological effect of VIP in CLOS when measured using the sucrose gap or microelectrode technique and VIP caused only slight hyperpolarization dependent on external chloride in OOBCM and OLOS (Daniel *et al*., 1987; 1989; Jury *et al*., 1992).

Murthy *et al*. (1993) reported that VIP stimulates both cyclic AMP kinase and cyclic GMP kinase in isolated gastric smooth muscle cells. This group found evidence of a membrane-bound cNOS activated by VIP through release of intracellular Ca^{2+} (Murthy & Makhlouf, 1994; Murthy *et al*., 1996). They postulated that VIP activates cNOS *via* a receptor, G-protein mechanism and release of intracellular Ca^{2+} to stimulate NO production from a myogenic cNOS. In their model, both VIP, acting to raise cyclic AMP, and NO, produced in muscle in response to VIP and acting to raise cyclic GMP, contribute to relaxation. Tottrup *et al*. (1993) has shown that in the human LOS the inner layers of the LOS circular smooth muscle stained positively for NADPH diaphorase. Gaumnitz *et al*. (1997) have recently shown that a few bundles within the inner circular muscle layer of the 'true OLOS', the thickened muscle band composed of clasp fibres, but avoiding adjacent sling fibres, possessed NADPH-diaphorase reactivity. In CLOS, muscle cells stain uniformly for NADPH-diaphorase and there is cNOS activity in isolated plasma membrane-enriched fractions free of neural contamination (Salapatek & Daniel, 1995; Salapatek *et al*., 1998a). In addition to histochemical and biochemical evidence, Salapatek *et al*. (1998b) found functional evidence of a membrane bound cNOS in canine

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LOS which was continuously activated by Ca^{2+} entry to release NO to activate K^+ channels. Konturek *et al.* (1997) have also shown that in the human LOS, studied *in vivo*, the arginine/NO pathway is involved in the maintenance of LOS pressure.

Thus it is unclear whether the inhibitory action of VIP is, in part, by activation of cNOS. This is more likely in CLOS cells which uniformly appear to possess this enzyme. In CLOS cells, cNOS does not require activation by nerve stimulation (Salapatek *et al.*, 1998a). Moreover, responses to electrical field stimulation (EFS) of NANC nerves in strips of OLOS or CLOS were abolished by L-NOARG or L-NAME but relaxations by VIP were unaffected by the NOS synthase inhibitors (Jury *et al.*, 1992). Neither slow nor fast relaxations of OLOS to EFS were inhibited by VIP antagonists (Oh *et al.*, 1997). EFS of NANC nerves or exposure to NO donors increased the accumulation cyclic GMP in the opossum LOS, while exposure of the opossum LOS to VIP increased cyclic AMP, not cyclic GMP (Torphy *et al.*, 1986; Miller *et al.*, 1986; Murray *et al.*, 1991). Different results were reported by Grider & Murthy, (1996), who reported that VIP activated NO formation from opossum gut tissues including OLOS.

If the Makhlouf-Grider-Murthy model applies and VIP acts to release myogenic NO, in CLOS, OLOS and OOBCM, then; (A) in canine LOS cells which have myogenic NOS, VIP should enhance outward current whenever large conductance, Ca^{2+} dependent K^+ ($BK^+/_{Ca^{2+}}$) channels are not maximally activated by endogenous NO release so that additional endogenous as well as exogenous NO could increase outward currents; (B) in opossum LOS and OOBCM cells, in which there is no evidence of spontaneous myogenic NOS activity but some cells may have a NOS enzyme, VIP should also increase outward currents under conditions when NO donors increase outward currents. Furthermore, any effects of VIP on outward currents should be attenuated by NOS inhibitors. These hypotheses were tested in patch clamp studies using isolated cells from OLOS, CLOS and OOBCM.

A portion of this work was presented in abstract form at Digestive Diseases Week, Washington D.C. May 1997 (Jury & Daniel, 1997).

Methods

Cell isolation

Mongrel dogs of either sex, weighing 15–25 kg, were euthanized by an intravenous injection of sodium pentobarbitone (100 mg kg^{-1}. Opossums, obtained from a licensed dealer and weighing 2–4 kg, were euthanized *via* an intra thoracic injection of sodium pentobarbitone (50 mg kg^{-1}). These procedures were approved by The Animal Care Committee of McMaster University and conform with the guidelines of the Canadian Council on Animal Care.

Canine LOS, opossum LOS and OOBCM were dissected as previously described. Care was taken to obtain the true sphincter in each species, avoiding nearby sling muscle (Daniel *et al.*, 1982; Jury *et al.*, 1992; Salapatek *et al.*, 1998a). Circular smooth muscle strips were cut into 1–2 mm^2 square pieces and placed in a dissociation solution containing (mM) EDTA 0.25, NaCl 125, KCl 4.8, glucose 10, $CaCl_2$ 1, $MgCl_2$ 1, HEPES 10 for 30 min. An enzyme solution containing papain (130 mg ml^{-1}), (−)-1,4-dithio-L-threitol (L-DTT, 15.4 mg ml^{-1}), BSA (100 mg ml^{-1}) and one of the Sigma collagenase blends L, H, or F (130 mg ml^{-1}) was added to the tissue pieces incubated at 37°C for 30–60 min. After incubation the enzyme solution was decanted off and the tissue pieces were rinsed in enzyme

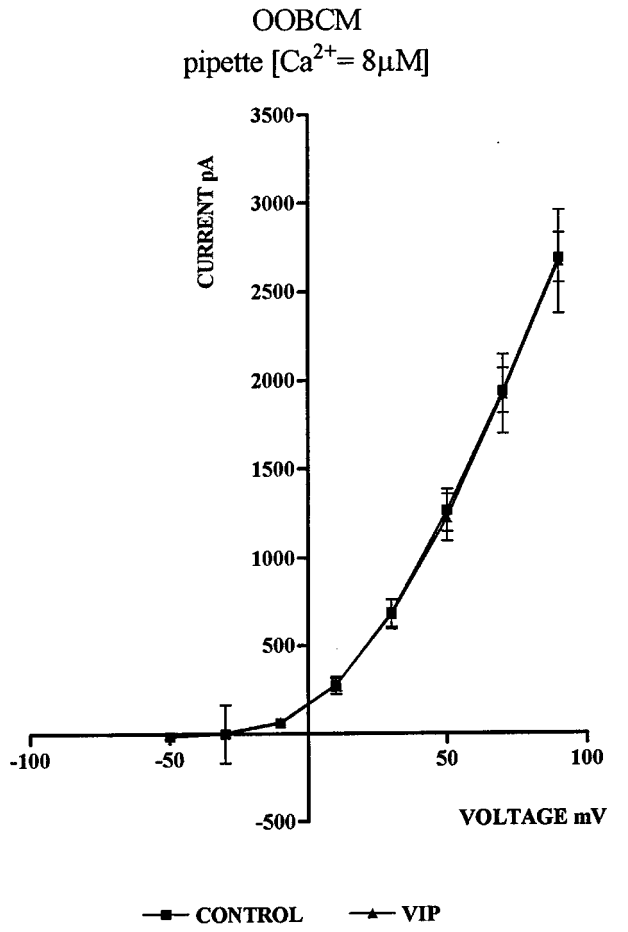

Figure 1 Current/voltage relationships showing the lack of effect of 1 μM VIP on the outward currents measured from isolated opossum OBCM cells. $Ca^{2+}{}_{pipette} = 8$ μM. Under these conditions NO donors significantly increased outward currents (Jury *et al.*, 1992). $n = 8$ when each value was from a cell from a different animal.

Table 1 Ca^{2+} dependence of outward currents in opossum LOS and $OBCM^{-1}$

$[Ca^{2+}{}_{pipette}]$	8 nM	50 nM	200 nM	8000 nM
OLOS	663±121 (4)	2016±340 (4)	2340±448 (3)	2637.5±414 (3)
OOBCM	952±177 (5)	2164.5±60 (4)	1817±127 (9)	1908±191 (5)
CLOS	577±15 (4)*	1330±174 (4)	1835±131 (5)	3667±48 (6)*

The effects of $[Ca^{2+}{}_{pipette}]$ on outward currents (pA) were measured at 70 mV. Numbers in parenthesis are *n* values. There were no significant differences except that values at 8 nM were significantly less than others for each tissues. *Values taken from Salapatek *et al.*, (1998b).

free dissociation solution. Single cells were gently mechanically agitated with siliconized Pasteur pipettes to disperse tissue and isolate single smooth muscle cells. Cells used in this study were patch-clamped at room temperature (22–24°C) usually within 8 h of isolation.

Patch clamp

The cell suspension was placed in a glass bottomed dish. Within 30 min cells adhered to the dish. The cells were then washed by perfusion with Ca^{2+}-containing external solution (in mM: NaCl 140.0, KCl 4.5, $CaCl_2$ 2.5, $MgCl_2$ 1.0, HEPES 10.0, glucose 5.5, ph adjusted to 7.35 with NaOH). Patch electrodes were made using borosilicate glass capillary tubes using a Flame Brown micropipette puller (Sutter Instruments Inc., CA, U.S.A.). After polishing using a microforge (Narishige MF-83) and filling pipettes had resistances of 3–5 Mohms. High Ca^{2+} pipette solution contained (in mM) $CaCl_2$ 2.5, KCL 140, $MgCl_2$ 1, HEPES 10, Na-ATP 4, EGTA 0.3, to obtain an intracellular free calcium of 8 μM. $CaCl_2$, KCl and EGTA levels were adjusted to obtain 50 nM and 200 nM free calcium levels in pipettes as calculated using MAX Chelator software (version 6.72) by Bers *et al.* (1994).

A standardized stimulation protocol was used to evoke currents from isolated smooth muscle cells, which were studied without leak subtraction. Cell resistances were not measured in the earliest work. Later all cells used had access resistance of <25 mohms. Cell capicitances were not measured in all cells,

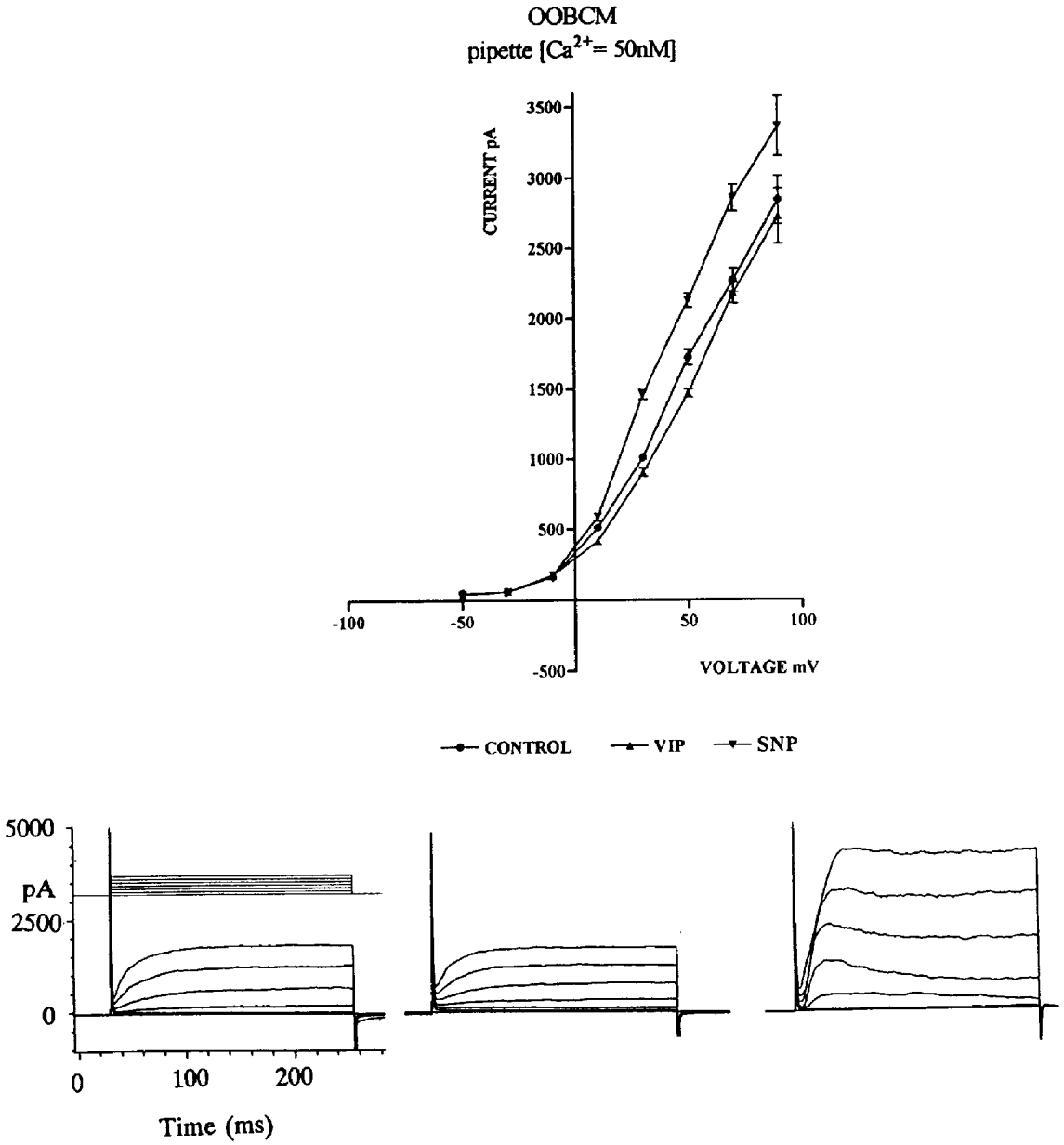

Figure 2 Current/voltage relationships evoked from OOBCM cells, recorded with a pipette containing only 50 nM free calcium. Bottom panel shows actual current traces from a representative experiment. At left current traces from the standard protocol (holding potential −50 mV and depolarization in 20 mV steps of 250 ms duration to +90 mV). The middle trace shows traces from the same cell 6 min after 1 μM VIP was added and the right hand trace shows traces 4 min after 0.1 mM SNP. At this lower level of intracellular calcium compared to earlier studies, 1 μM VIP had no significant effect on the maximum outward currents, but 0.1 mM Na nitroprusside (SNP) increased them. Outward currents were as large as in Figure 1 despite lower pipette free Ca^{2+}. *$P \leqslant 0.05$ that difference in outward currents after SNP were due to chance alone. $n=3$. n values determined as in Figure 1.

but mean whole capacitances of 58 ± 5, 56 ± 4 and 68 ± 7 pF ($n = 6$) for CLOS, OLOS and OOBCM were found. Cells were held at -50 mV and subsequently depolarized in seven cumulative steps of 20 mV. Each step was 250 ms in duration. Currents were measured every 30–90 s after breakthrough and drug addition. Current/voltage curves were constructed using the maximum current values measured at t = 200 ms of each step. Membrane currents were measured with an Axopatch 1C voltage clamp amplifier, filtered with a 3 db Bessel filter at 1 Khz and recorded online using pclamp 5.5 software.

VIP was dissolved in a buffer containing BSA, NaH_2PO_4 and NaCl. L-NOARG or L-NAME stock solutions were dissolved in 2 N HCL and subsequently diluted to 0.02 N in Krebs solution. At 10^{-6} M, VIP produces maximum relaxation in the CLOS and OLOS as well as maximal inhibitory and electrophysiological effects in OOBCM (Daniel *et al*., 1982; 1989; Christinck *et al*., 1991; Jury *et al*., 1992; De Man *et al*., 1991) and this concentration was chosen to test its effects. VIP was added directly to the bath and current measurements were made intermittently until 10 min later. The last set of measurements was taken for analysis. SNP was added after VIP without washout to ensure that the effects of the two compounds were compared on the same cells and under the same conditions. Its effects on currents were determined to be maximal in 3–4 min and currents measured at that time were analysed. All drugs used were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.) and other chemicals were all reagent quality.

Data are expressed as mean ± s.e.mean. Statistical significance of differences of results were determined by paired or unpaired Student's *t*-test, as appropriate. A $P \leqslant 0.05$ was considered significant.

Results

Initial experiments were carried out using a pipette which contained a high (8000 nM) free calcium concentration in order to stimulate the conditions in our earlier work which showed that nitric oxide donors increase outward current in OOBCM cells (Jury *et al*., 1996). Control current- voltage curves were similar to those obtained previously with maximum current measured at the 90 mV step of the protocol; 2574 ± 265 pA in the previous study and 2691 ± 140 pA ($n = 5$) in this study. L-NAME or L-NOARG at 0.1 mM had no effect on these outward currents (data not shown). After control curves were established, VIP (1 μM) was added to the bath and currents were measured every 30–90 s for 15 min. There was no significant change in the current voltage relationships at any time after exposure to VIP (Figure 1). Maximum current measured when the cells were depolarized to +90 mV was 2668 ± 292 pA ($n = 5$).

Jury *et al*. (1985; 1996) found that the hyperpolarizing effects of endogenous and exogenous NO in OOBCM were mediated in part by Ca^{2+}-dependent K^+ channels, which were unusual in that they were not blocked by apamin or TEA, but were blocked by quinine. They were also unaffected by charbydotoxin or iberiotoxin (Jury & Daniel, unpublished). Salapatek *et al*. (1996; 1998b) found that canine LOS cells had iberiotoxin-sensitive, Ca^{2+} dependent K^+ channels activated by NO donors or release, observable when the membrane bound cNOS in these cells was inhibited by L-NOARG or reduced in activity by lowering pipette free Ca^{2+} to $\leqslant 200$ nM. Free $[Ca^{2+}_{pipette}]$ was varied and the effect on outward currents and the lack of VIP effect on OOBCM cells re-examined. Unlike the CLOS, in which currents measured with pipette free $[Ca^{2+}]$ of 50 nM or 200 nM were reduced compared to those measured at 1000 nM (Salapatek *et al*., 1998b), those in OOBCM cells were not significantly different than those measured with the high calcium pipette (Table 1). They were reduced at a pipette free Ca^{2+} of 8 nM.

When recording from OBCM cells with a free $[Ca^{2+}_{pipette}]$ of 50 nM the addition of VIP 1 μM to the bath did not significantly change the I/V curve over 10 min (Figure 2). Outward currents measured at +90 mV were 2851 ± 175 pA before and were not significantly changed at 2734 ± 198 pA, 10 min after VIP was added ($n = 3$). SNP (0.1 mM) increased the outward currents ($P \leqslant 0.05$) in these cells to 3378 ± 213 pA as shown in the Figure. There was also no significant change in outward currents 10 min after VIP was added to the bath when the free $[Ca^{2+}_{pipette}]$ was 200 nM. Control values in three cells (at +90 mV) were 2419 ± 75 pA compared to 2269 ± 85 pA after the addition of VIP 1 μM (Figure 3).

The effect of 1 μM VIP on freshly isolated opossum LOS cells was also tested. As in OOBCM cells VIP had no effect on outward currents regardless of the pipette free [Ca]. Figure 4 shows current voltage relationships (free $Ca^{2+}_{pipette} = 200$ nM) of control currents and currents recorded 10 min after the addition of VIP 1 μM to the bathing solution. NO donors did increase outward currents under these conditions. Currents

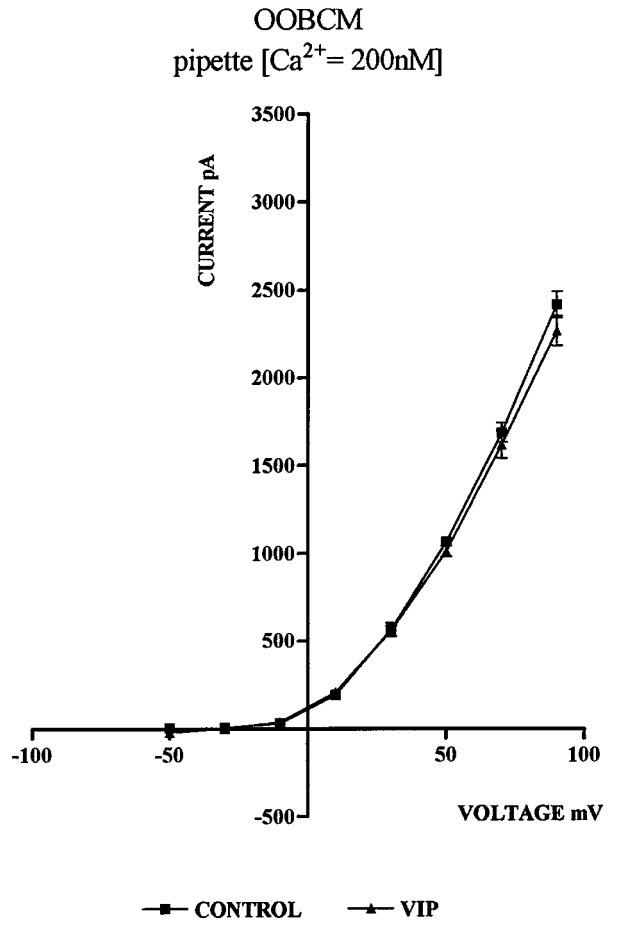

Figure 3 Current/voltage relationships evoked from OOBCM cells, recorded with a pipette containing 200 nM free calcium. At this level of intracellular calcium, lower than in earlier studies, 1 μM VIP had no effect on the maximum outward currents. Outward currents were as large as in Figure 1 despite lower pipette free Ca^{2+}. $n = 4$, values determined as in Figure 1.

measured in three cells at +90 mV before and after 1 μM VIP were not significantly changed at 2339±228 and 2402±198 respectively, but increased ($P \leqslant 0.01$) to 3304±201 after 0.1 mM SNP.

In cells of the opossum LOS outward currents were similar from 50 to 8000 nM free Ca^{2+} levels in the pipette (Table 1), and fell only when pipette free Ca^{2+} was calculated to be $\leqslant$8 nM. In contrast, outward currents recorded from isolated canine LOS cells depended differently on pipette free calcium; the EC_{50} for increasing pipette Ca^{2+} concentration to increase outward currents was 108 nM Ca^{2+} (Salapatek *et al.*, 1998b). In this study also, the maximum currents recorded with a 50 nM or 200 nM free calcium pipette were significantly lower than those measured with a 8000 nM pipette (Table 1).

The effects of 1 μM VIP were tested on CLOS cells at three different levels of pipette free [Ca^{2+}]. Figure 5 shows current voltage relationships, recorded using a [$Ca_{pipette}$] of 200 nM, obtained from control cells, then exposed to VIP and SNP. VIP had no effect after 10 min, whereas subsequent addition of SNP significantly increased outward current. At 200 nM the values for outward currents measured at +90 mV in four cells before and after VIP and then after SNP were 2192±89, 2402±89 and 3290±97 pA respectively. Lowering the pipette free calcium to 50 nM did not unmask an effect of VIP and SNP still caused an increase in outward currents (Figure 6). At 50 nM, outward currents measured in four cells at +90 mV before and after VIP and after SNP (0.1 mM) were 2345±62, 2236±100 and 3290±206 pA respectively. Only the increases after SNP were significant ($P \leqslant 0.01$ in both cases).

Our previous study showed that at free Ca^{2+} concentrations in the pipette of 8 nM, NO donors still increased outward currents (Salapatek *et al.*, 1998b). We repeated these experiments with the addition of SNP after the addition of VIP. There was no change in maximum current

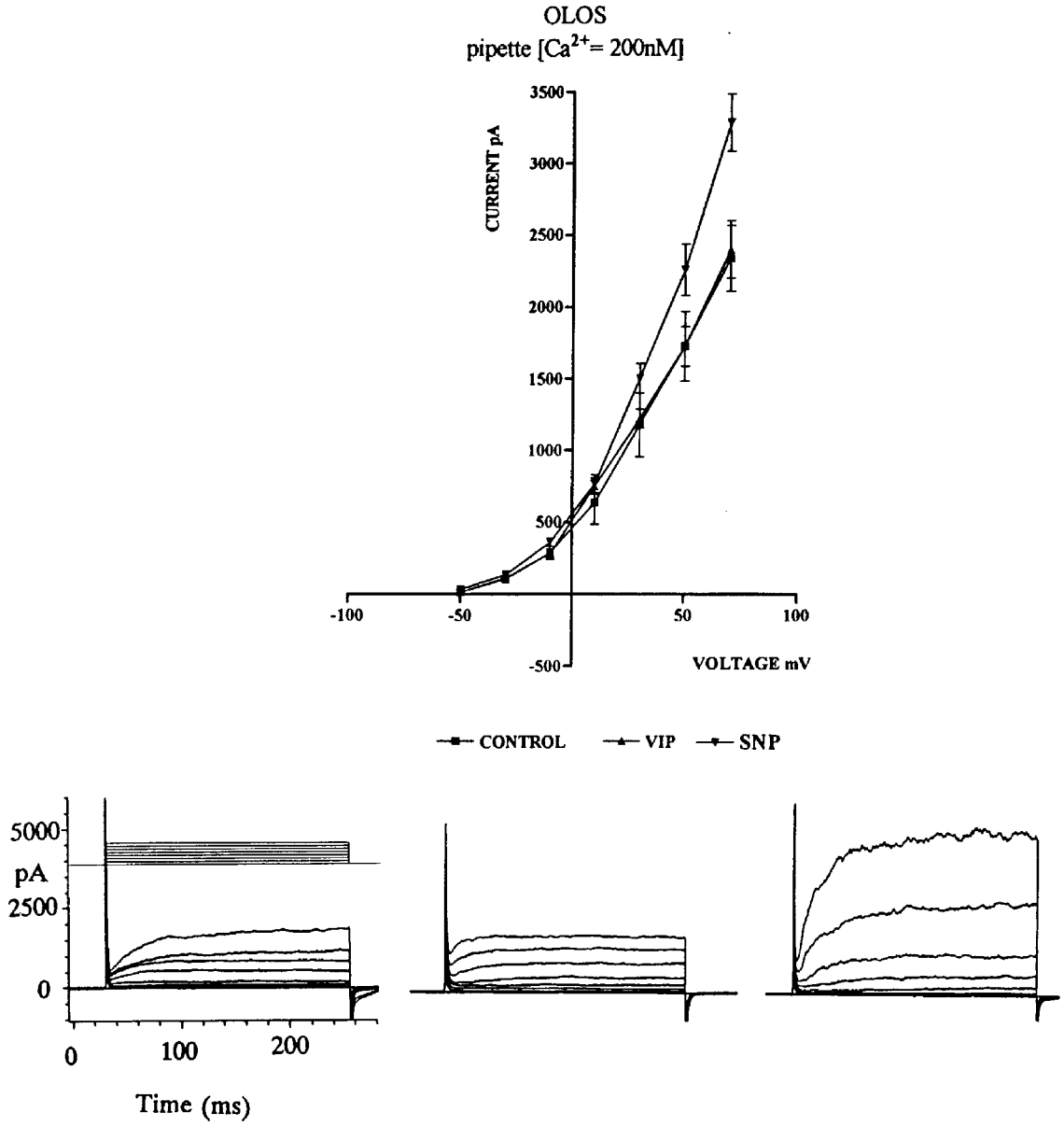

Figure 4 Current/voltage relationships evoked from OLOS cells, recorded with a pipette containing 200 nM free calcium. The bottom panel shows examples of current traces in a cell: control (left), 10 min after VIP (middle) and 4 min after SNP (right) using a standard protocol (holding potential −50 mV and depolarizing in steps of 20 mV and 250 ms duration to +70 mV). 1 μM VIP did not affect the current/voltage relationship but SNP (0.1 mM) increased them. $n=4$; values determined as in Figure 1.

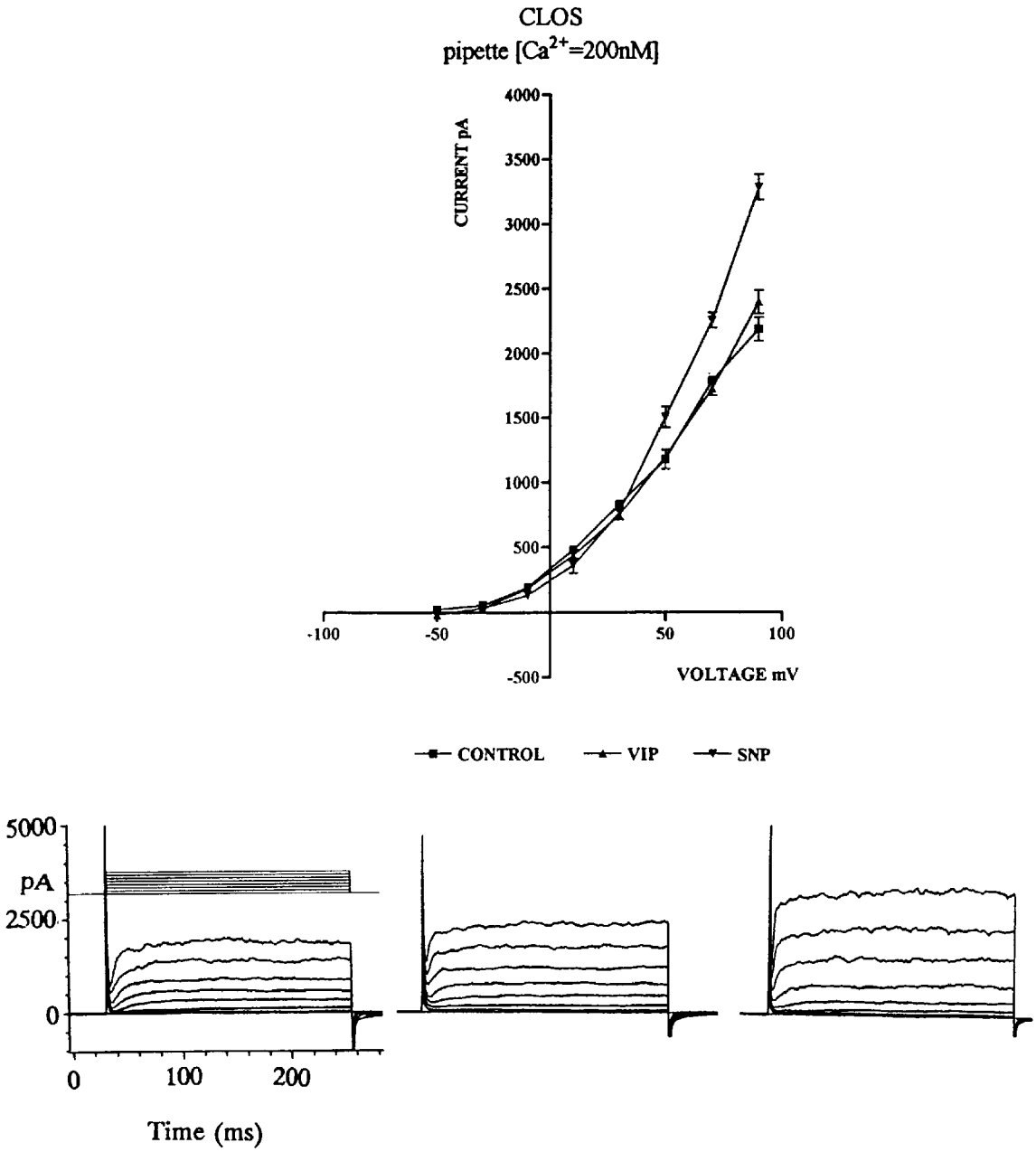

Figure 5 Current/voltage relationships evoked from CLOS cells when $[Ca_{pipette}] = 200$ nM. The bottom panel shows current traces in a cell: control (left), 10 min after VIP (middle) and 4 min after SNP (right) using a standard protocol (holding potential -50 mV and depolarizing in steps of 20 mV and 250 ms duration to $+90$ mV). VIP (1 μM) had no effect on outward currents, but SNP (0.1 mM) increased them. $*P \leqslant 0.05$ that difference due to chance alone. $n = 5$; values determined as in Figure 1.

from control (1064 ± 78) to VIP (1186 ± 146); after the addition of SNP, currents were increased significantly ($P \leqslant 0.05$) to (2299 ± 124) $n = 3$. Thus each cell type studied had outward currents increased by SNP in conditions under which VIP did not.

Discussion

Salapatek & Daniel (1995) and Salapatek *et al.* (1998a) showed that canine LOS cells contain a membrane bound cNOS (NADPH diaphorase positive) capable of transforming L-arginine to L-citrulline in a Ca-calmodulin dependent fashion. Blockade of this activity in the absence of nerve function increased tone in a $[Ca^{2+}]_e$ dependent manner. Also nifedipine abolished both basal tone as well as the increased tone after inhibition of cNOS activity. Whole cell outward currents recorded from these cells were large, abolished when Cs^+ replaced K^+, inhibited by TEA, iberiotoxin, nifedipine and block of cNOS, and restored by NO donors after cNOS inhibition but not after iberiotoxin inhibition (Salapatek *et al.*, 1998b). Procedures that inhibited outward currents also depolarized cells recorded in current clamp mode. We concluded that the outward currents contributing to resting membrane potential were carried by K^+ through BK_{Ca} channels and were dependent on spontaneous cNOS activity and L-Ca^{2+} channel activity. Only when not maximally activated by endogenous NO production could outward K^+ currents in canine LOS cells be significantly increased by NO donors (Salapatek *et al.*, 1998b) while those in opossum

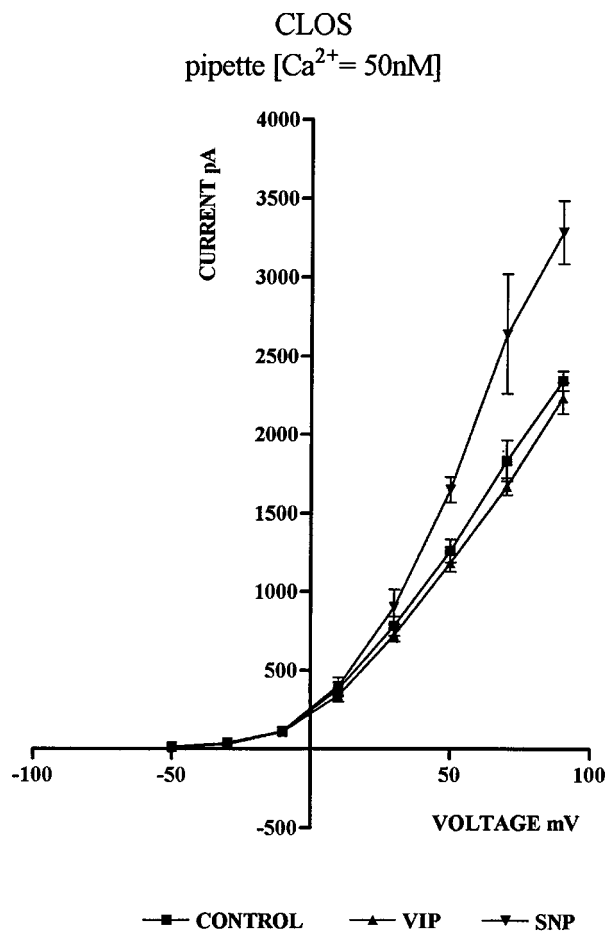

Figure 6 In CLOS cells, when cNOS was not maximally activated, i.e. using pipette calcium concentrations of 50 nM, VIP had no effect on the current voltage relationships. Under the same conditions SNP increased outward currents. $^{**}P \leqslant 0.01$ that difference due to chance alone. $n = 3$; values determined as in Figure 1.

OOBCM (Jury *et al*., 1996) were increased even when pipette Ca^{2+} was 8000 nM.

In canine LOS cells, when cNOS was inhibited by L-NOARG or by low pipette Ca^{2+}, the outward currents were reduced by up to 80%, but NO donors restored outward currents to control levels (Salapatek *et al*., 1998b). Therefore in this study we used pipettes with 50 nM Ca^{2+} to reduce the cNOS activity of canine LOS cells and unmask a possible VIP effect. Analogous to our previous microelectrode and sucrose gap studies of canine LOS (Jury *et al*., 1992) in which no net effects of VIP were found on membrane potential, VIP did not effect outward current, regardless of pipette free calcium concentration.

Gaumnitz *et al*. (1997) reported that a few bundles within the inner circular muscle layer of the 'true OLOS' showed NADPH-diaphorase reactivity. In this study, in which cells were isolated from the 'true OLOS' (i.e. those strips that would show only relaxation on electrical field stimulation), we found that pipette free Ca^{2+} concentrations from 50–200–8000 nM did not affect the maximum outward currents (Table 1) and that NO donors increased outward current at all, even very high, pipette free Ca^{2+}, as previously described (Jury *et al*., 1996). It is possible that all the cells recorded from in this study were not from NADPH-diaphorase positive bundles. In any case none of the cells studied showed any change in outward current with the addition of VIP.

If VIP were acting to release NO in oesophageal muscle cells as claimed by Grider & Murthy (1996) and suggested to be a general phenomenon by Murthy *et al*. (1993; 1996) and Murthy & Makhlouf (1994), VIP should also increase outward current as does exogenous NO. These workers proposed that VIP releases intracellular Ca^{2+} to activate cyclic NOS as well as elevate cyclic AMP, both of which contributed to relaxation. Therefore, we provided conditions in which Ca^{2+} release was possible (Ca^{2+} stores are loaded with Ca^{2+}) and cNOS was not maximally activated. Previously Cayabyab & Daniel (1996) showed that the inhibitory junction potentials of OOBCM were sensitive to cyclopiazonic acid, a selective inhibitor of the sarcoplasmic reticulum calcium pump. Moreover, we have used fura-2 to study Ca^{2+} imaging in CLOS cells isolated exactly as described here and shown that cyclopiazonic acid raises the level of intracellular Ca^{2+} (Lam, Salapatek & Daniel unpublished). Further, Jury *et al*. (1996) showed that pretreatment with cyclopiazonic acid inhibited outward currents in OOBCM. Thus Ca^{2+} stores are likely available in OOBCM and CLOS and this is expected to be the case also in OLOS. As in canine LOS we saw no effect of VIP on outward currents in opossum LOS cells regardless of pipette [Ca^{2+}].

Another possible cause for failure to see increased outward currents after VIP was that the concomitant elevation of cyclic AMP by VIP inhibited the effect of NO on these currents. However, this study showed that even after 10^{-6} M VIP, NO donors still increased outward currents. Moreover, in tissues, EFS, which presumably releases VIP as well as NO, caused large IJPs which were mediated by K^+ channel opening in opossum tissues (Jury *et al*., 1985; 1996). Thus it seems unlikely that VIP elevation of cyclic AMP or another secondary effect acted to inhibit outward currents.

One other possible reason for our failure to see VIP effects mediated by NO release was that the procedure of cell isolation damaged VIP receptors or disrupted their coupling to the G proteins involved in transduction of Ca^{2+} release. This has not been ruled out, but the consistency of the lack of major hyperpolarization in response to VIP in electrophysiological studies with sucrose gap or microelectrodes in OOBCM or OLOS (Daniel *et al*., 1982; 1987; 1989) or in canine LOS (Jury *et al*., 1992) with a lack of increase in outward currents makes this seem unlikely.

Oh *et al*. (1997) also showed in organ bath studies of OLOS that the fast relaxation to EFS was TTX-sensitive and blocked by L-NOARG and neither the fast nor the slow component of EFS was blocked by the VIP antagonists, Peninsula 8076 or VIP 10–28.

This study also revealed (Table 1) that the outward currents in both OLOS and OOBCM had a different dependence on pipette Ca^{2+} levels from that observed in canine LOS by Salapatek *et al*. (1998b). They did not decrease at levels of 200 and 50 nM pipette free Ca^{2+} as did those in CLOS; in all cell types outward currents decreased with 8 nM pipette free Ca^{2+}. This difference between cell types in the dependency of outward currents on pipette Ca^{2+} concentrations may be related to the fact that the Ca^{2+}-dependent K^+ channels were different in opossum oesophagus from those in canine LOS. The latter are TEA and iberiotoxin sensitive and apamin insensitive (Salapatek *et al*., 1998b) while the K^+ channels in opossum were insensitive to TEA, apamin, charybdotoxin and iberiotoxin and were inhibited only by quinine (Jury *et al*., 1985; 1996 and unpublished). In addition, the difference in pipette Ca^{2+} dependence may relate to the fact that the cNOS activity in CLOS was Ca^{2+} dependent (Salapatek *et al*., 1998a)

and NO from this activity clearly influenced K^+ channel activity (Salapatek *et al*., 1998b). We have not evaluated the pipette [Ca^{2+}] dependency of the residual outward currents in CLOS cells after inhibition of NOS.

There has also been controversy over the involvement of interstitial cells of Cajal (ICC) in smooth muscle relaxation, in that ICC within oesophageal circular muscle are close (often <40 nm) to bare nerve endings and connected by gap junctions to muscle cells (Daniel & Posey-Daniel, 1984; Berezin *et al*., 1987; Allescher *et al*., 1988). Thus nerves may release mediators to act on ICC first and their response may be transferred by gap junctions to muscle cells. If ICC have a cNOS enzyme and release in NO in response to EFS, it may diffuse from the ICC to the smooth muscle cell. Torihashi *et al*. (1997) on the other hand showed that, in mouse intestine, the NO related relaxation by intrinsic nerve stimulation was absent in newborn mice as were ICC's (evaluated by c-*kit* staining). A few weeks after birth c-*kit* immunoreactive cells (ICC) appear as do the L-NOARG sensitive relaxations. This suggests that ICC's may play a role in the NO induced NANC relaxation of mouse intestine.

Burns *et al*. (1996) reported that in W/W^v mice lacked the intramuscular ICC near nNOS nerves in the gastric fundus, despite normal NOS innervation. Responses to NO mediated neural inhibition and to NO donors were much attenuated. They suggested that ICCs may transduce the response to NO into electrical responses of muscle. Recently, Ward *et al*. (1998) reported similar findings in the LOS and pyloric sphincter of W/W^v mice. However, ICCs were absent from the cells patched in our study. We always chose cells with the double conical shape of smooth muscle and without the multiple cell processes characteristic of ICCs. One possibility to explain results suggesting that VIP releases NO to cause relaxation is that ICC with VIP receptors may be present and provide the source of NO. Consistent with that possibility, we have found that VIP-containing nerve varicosities are present in canine LOS very close ($\sim$40 nm) to ICC cells which, are in gap junction contact with muscle (Berezin *et al*., 1987).

We conclude that VIP induced inhibition of motility in opossum oesophagus circular muscle or relaxation of opossum LOS and of canine LOS occurs by mechanisms unrelated to release of NO from muscle cells to activate outward currents. These findings are consistent with previous electrophysiological studies which found that NO released from nerves or added exogenously by NO donors caused hyperpolarization toward the K^+ equilibrium potential while inducing relaxation and inhibiting contraction, but VIP relaxed or inhibited contraction without such hyperpolarization. We also conclude that the dependence of outward K^+ currents on pipette free Ca^{2+} levels differs in canine and opossum oesophageal smooth muscles. The mechanisms underlying these differences warrant further analysis.

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