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VIP-induced relaxation of guinea-pig intestinal smooth muscle cells: sequential involvement of cyclic AMP and nitric oxide

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1 A possible interaction between cyclic AMP and nitric oxide (NO) in mediating the relaxant effect of vasoactive intestinal polypeptide (VIP) on intestinal smooth muscle cells has been investigated. The effects of the inhibitor of NO synthesis, N^G-nitro-L-arginine methyl ester (L-NAME), have been studied on VIP-, forskolin-, and 8 bromo-cyclic AMP- induced relaxation of cells, dispersed by enzymatic digestion of muscle strips from the circular layer of guinea-pig ileum.

2 VIP alone did not modify the length of isolated muscle cells. By contrast, when the cells were contracted by cholecystokinin octapeptide, CCK8 (10 nM), VIP inhibited this contraction, inducing a concentration-dependent relaxation of the cells. Maximal relaxation was induced by 1 μM VIP ($EC_{50} = 408.2 \pm 16.7$ pM).

3 N-ethylmaleimide, inhibitors of adenylate cyclase or somatostatin, abolished the relaxing effect of VIP. (R)-*p*-cAMPs, an antagonist of cyclic AMP on protein kinase A also inhibited the VIP-induced relaxation by $92.1 \pm 6.3\%$. Inhibitors of nitric oxide synthase (NOS), L-NAME and L-NMMA, partially inhibited VIP-induced relaxation. The effect of L-NAME was reversed by L-arginine but not by D-arginine.

4 (R)-*p*-cAMPS and L-NAME also inhibited the cell relaxation induced either by forskolin which directly stimulates adenylate cyclase activity or 8-bromo-cyclic AMP, an analogue of cyclic AMP.

5 When cells were incubated for 30 min with dexamethasone 10 μM, a glucocorticoid known to decrease the synthesis of iNOS, the relaxing effect of a maximal concentration of VIP was decreased by $52 \pm 4\%$ and L-NMMA had no further effect on this residual VIP-induced relaxation. Milrinone, a phosphodiesterase type III inhibitor, potentiated the relaxant effect of VIP.

6 These data demonstrate that the intracellular pathway mediating the relaxant effect of VIP in intestinal smooth muscle cells includes the sequential activation of adenylate cyclase, protein kinase A, activation of NOS and finally production of NO and cyclic GMP. NO could in turn regulate the cyclic AMP-dependent pathway of cell relaxation.

Keywords: Vasoactive intestinal polypeptide; cyclic AMP; cyclic GMP; nitric oxide; phosphodiesterase; intestinal smooth muscle

Introduction

Vasoactive intestinal polypeptide (VIP), a 28 amino acid peptide which belongs to a family of regulatory peptides including secretin, PHI (peptide histidine-isoleucine) and glucagon, is present in the central and peripheral nervous system (Larson *et al.*, 1976). Among peripheral actions of VIP, smooth muscle cell relaxation has been recognized as being mediated through the stimulation of adenylate cyclase (Bitar & Makhlouf, 1982a). At the level of the myenteric plexus, VIP plays the role of an inhibitory neurotransmitter, released by non-adrenergic, non-cholinergic (NANC) neurones (Goyal *et al.*, 1980).

Recently, nitric oxide (NO), was recognized as a neurotransmitter of NANC nerves in the myenteric plexus throughout the gastrointestinal tract (Bult *et al.*, 1990) being able to relax stomach, intestine, colon and sphincters (Allescher *et al.*, 1992; Jin *et al.*, 1993; Gustafsson & Delbro, 1993; Maggi *et al.*, 1994). NO is synthesized from L-arginine by the action of NO synthase (NOS), which has been found in many isoforms (Forstermann *et al.*, 1991). Initially, two forms of NOS were distinguished: a constitutive Ca^{2+}, calmodulin-dependent form, present in the brain and the endothelial cells and an inducible calmodulin- and Ca^{2+}-independent form, which is induced in macrophages, hepatocytes and smooth muscle by cytokines and *Escherichia coli* lipopolysaccharide (LPS). So far, the cDNAs for three different forms of NOS (brain, endothelial and macrophage) have been cloned and characterized (Bredt *et al.*, 1991; Lamas *et al.*, 1992; Xie *et al.*, 1992). In the myenteric plexus, NOS and VIP are colocalized in many neurones (Furness *et al.*, 1992). NO relaxes smooth muscle cells through stimulation of soluble guanylate cyclase (Furchgott & Zawadzki, 1980; Wolin *et al.*, 1982) and an increase in intracellular guanosine 3',5'-cyclic monophosphate (cyclic GMP) (Murad *et al.*, 1978; Gruetter *et al.*, 1981). In gastric muscle strips, the relaxation caused by VIP is suppressed by NOS inhibitors (Grider *et al.*, 1992). Using dispersed gastric muscle cells, Jin *et al.* (1993) have shown that VIP-induced relaxation is mediated through an increase in cyclic GMP. They suggested that VIP could be the primary NANC relaxing transmitter and that VIP stimulates NO production in gastric smooth muscle cells (Grider *et al.*, 1992; Jin *et al.*, 1993).

It is also understood that VIP induces cell relaxation by increasing adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels in smooth muscle cells (Bitar & Makhlouf, 1982a). However, the exact relationship between cyclic AMP and NO is not fully understood. The time course of activation of adenylate cyclase and NOS has not been defined.

Consequently, in the present study, the respective role of NO and cyclic AMP in mediating the relaxant effect of VIP in intestinal smooth muscle cells have been examined. Cell relaxation was induced by VIP, by direct stimulation of adenylate cyclase with forskolin or by increasing the intracellular

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level of cyclic AMP by incubating cells with 8-bromo-cyclic AMP, an analogue of cyclic AMP, in the presence or absence of NOS inhibitors. Then, we evaluated the influence of an inhibition of NOS expression and activity on VIP- and cyclic AMP-induced relaxation of the cells. We completed this study by measuring the level of intracellular cyclic GMP under these various conditions.

Methods

Cell dispersion

Cell dispersion was achieved as previously described (Bitar & Makhlouf, 1982b; Botella *et al.*, 1992; 1994). Briefly, albino male guinea-pigs (250–300 g) were killed by cervical dislocation and the circular muscle layer of the ileum was removed. Small muscle strips (10–15 cm) collected from the oral side of the ileo-caecal junction, were incubated for 2 successive periods of 30 min at 31°C in a medium with the following composition (mM): (NaCl 132, KCl 5.4, Na_2HPO_4 5, NaH_2PO_4 1, $MgSO_4$ 1.2, $CaCl_2$ 1, HEPES 25, glucose 0.2% (w/v), bovine serum albumin 0.2% (w/v); pH 7.4, bubbled with 95% O_2: 5% CO_2 and supplemented with antibiotics (penicillin G 100 iu ml^{-1}, streptomycin 50 $\mu g\ ml^{-1}$), in the presence of 0.25 iu ml^{-1} collagenase, 0.2 mg ml^{-1} pronase and 0.2 mg ml^{-1} soybean trypsin inhibitor. At the end of the second incubation, the medium was filtered and the partly digested muscle strips were washed four times with enzyme-free medium. These strips were then transferred into fresh, enzyme-free medium and left to stand for 20 min to allow the muscle cells to disperse spontaneously under very slow mechanical agitation. Cells were harvested through a 500 μm nylon filter. Only those cells that had dissociated spontaneously in enzyme-free medium were used for functional measurements. Viability tests (exclusion of trypan blue) showed that more than 85% of cells in suspension were viable at the time of contraction experiments.

Measurement of contractile response

Smooth muscle contractile responses of isolated cells were usually assayed within 30 min of dispersion. Cell density of the suspension was adjusted to 250,000 cells ml^{-1}; 250 μl aliquots of cell suspension were added to 250 μl of a solution containing the agent to be tested or control medium, thereby ensuring rapid mixing, and incubated for 30 s at 31°C. The reaction was interrupted by addition of glutaraldehyde to a final concentration of 2.5%.

To measure cell length, an aliquot of cells fixed with glutaraldehyde was placed on a Malassez slide and the length of the first 50 cells randomly encountered in successive microscopic fields was measured. Only cells appearing entire at microscopic examination were measured. Cell length measurements were performed with a scale mask placed on a video screen. Magnification due to the video recording had first been calculated by comparison with length measurement obtained through the image splitting eye piece connected to a micrometer.

Experiments of inhibition or relaxation

For relaxation experiments, cells were preincubated for 1 min in the presence of various concentrations of the inhibitory or relaxing agents to be tested. Then the contracting agent (CCK8) was added and the reaction stopped after 30 s as described above.

Expression of results

The decrease in cell length was calculated using the following formula: $[[L_0\text{-}L_X]/L_0] \times 100$ where L_0 is the mean length of cells in resting state and L_X, the mean length of treated cells. In relaxation experiments, the degree of inhibition was expressed as the percentage decrease in the contractile response from the maximal response observed in the absence of inhibitors, taken as 100%.

Measurements of cyclic GMP levels

Cyclic GMP was assayed, by an enzyme immunoassay method, according to the original procedure proposed by Cayman Chemical Company, (Ann Arbor, MI, U.S.A.). VIP, 8-bromo cyclic GMP or forskolin was added to 0.5 ml of cell suspension (10^6 cells ml^{-1}). Relaxing agents and inhibitors were added to the medium in the same time sequence as for relaxation experiments. The reaction was then stopped by immersion of samples into 2 ml of a cold 95% methyl alcohol and 5% formic acid solution. The mixture was then centrifuged at 4000 *g* for 10 min at 4°C, lyophilized to dryness and resuspended in 50 mM potassium phosphate buffer (pH 7.4) before distribution to duplicate microtiter plate wells. The cyclic GMP level in standard and experimental solutions was determined following competition between cyclic GMP and the acetylcholinesterase-linked cyclic GMP tracer for specific antiserum binding sites. The antiserum complex, linked to acetylcholinesterase, was used to cleave Ellman's Reagent (which contains the substrate for acetylcholinesterase), and absorbance was measured at 412 nm. Cyclic GMP content of samples was determined from a standard curve constructed by measuring the amount of cyclic GMP contained in standard solutions. The level of cyclic GMP was expressed in fmol cyclic GMP/10^6 cells. To evaluate the loss of cyclic GMP during this procedure, some samples were processed in the presence of [^{32}P]-cyclic GMP at a concentration corresponding to 10% of the expected amount of cyclic GMP in the sample. The amount of [^{32}P]-cyclic GMP recovered at the end of the procedure was 94.3±7.1%.

Chemicals

Collagenase and soybean trypsin inhibitor were purchased from Worthington Biochemical Corporation (Freehold, New Jersey, U.S.A.). Pronase was purchased from Boehringer Mannheim Ltd (Meylan, France). Penicillin G and streptomycin G were obtained from Specia (Paris, France). Sulphated C-terminal octapeptide of cholecystokinin (CCK8), VIP, forskolin, 8-bromoadenosine-3′:5′-cyclic monophosphate (8-bromo-cyclic AMP), somatostatin, N-ethylmaleimide, N^{ω}-nitro-L-arginine methyl ester (L-NAME), N^{ω}-monomethyl-L-arginine (L-NMMA), L-arginine, D-arginine, dexamethasone and milrinone were obtained from Sigma (St Louis, MO, U.S.A.). (**R**)-*p*-cyclic adenosine-3′,5′-monophosphothioate ((**R**)-*p*-cAMPs) was obtained from Biolog (Life Science Institute, Bremen, Germany).

Statistical analysis

Throughout this paper, the data are expressed as the mean±s.e.mean, *n* refers to the number of experiments, each of those performed on samples from different animals. EC_{50} and IC_{50} values were determined by linear regression analysis. Statistical evaluation was carried out by Student's *t* test, and the normality of the cell samples was assessed by the normal law test of Kolmogoroff as previously described (Berry *et al.*, 1991). Values of $P<0.05$ were considered to be statistically different.

Results

Relaxant effect of VIP

When dispersed cells were incubated with increasing concentrations of VIP (10 fM to 1 μM), cell length was unaffected. CCK8 contracted isolated smooth muscle cells from the circular layer of guinea-pig ileum in a concentration-dependent manner, with a maximal effect at 10 nM ($n=8$, results not

shown). The maximal contraction induced by CCK8 corresponded to a 23.3±2.20% ($n=8$) decrease in cell length from controls (Table 1). When cells were preincubated for 1 min with increasing concentrations of VIP ranging from 10 fm to 1 μM, the CCK8-induced contraction was inhibited in a concentration-dependent manner (Figure 1). The concentration of VIP inducing a half-maximal relaxation (EC_{50}) was 408±16.7 pM ($n=7$) and the CCK8-induced contraction was abolished at 1 μM VIP.

Effect of N-ethylmaleimide (NEM) and somatostatin (SS) on VIP-induced relaxation SS inhibits cyclic AMP production by activation of the guanine nucleotide binding protein G_i which is negatively coupled to adenylate cyclase (Gu *et al.*, 1992b). NEM has been shown to inhibit adenylate cyclase in a number of experiments in various systems (Ashkenazi *et al.*, 1989) including smooth muscle cells (Moummi & Rattan, 1988; Botella *et al.*, 1994). Incubation of the cells (20 min) in the presence of NEM or SS each 1 μM, did not alter the mean cell length of resting circular smooth muscle cells. When cells were incubated in the presence of 1 μM NEM or SS, followed by addition of CCK8 (10 nM), the CCK8-induced contraction was not altered. By contrast, when cells were incubated in the presence of 1 μM NEM or SS, followed by addition of a maximal relaxing concentration of VIP (1 μM), the relaxant response to VIP was abolished. NEM and SS decreased the cell length by 20.9±2.30% ($n=5$) and 22.3±3.91% ($n=5$) respectively from control and the CCK8-induced contraction was fully restored (Table 1).

Table 1 Effect of CCK8, VIP, N-ethylmaleimide and somatostatin on muscle cell length and contraction

	Muscle cell length (μm)	*Contraction* (% decrease in cell length)
Controls	104±7.4 3 ($n=8$)	
CCK8 10 nM	80.2*±9.12 ($n=8$)	23.3±2.28
CCK8 10 nM + VIP 1 μM	102±7.82 ($n=7$)	1.96±4.44
CCK8 10 nM + VIP 1 μM + N-ethylmaleimide 1 μM	82.7*±9.43 ($n=5$)	20.9±2.34
CCK8 10 nM + VIP 1 μM + somatostatin 1 μM	81.3*±7.58 ($n=5$)	22.3±3.98

Values are mean±s.e.mean from $n=5$ to 8 experiments. *$P<0.05$, from controls.

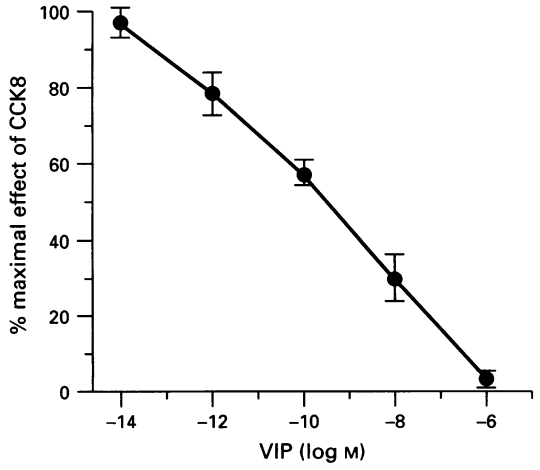

Figure 1 Concentration-response curve to VIP in dispersed intestinal smooth muscle cells from the circular layer of guinea-pig ileum. Cell were preincubated at 31°C in the presence of increasing concentrations of VIP for 1 min. Then, CCK8 (10 nM) was added for 30 s and cells were fixed with 2.5% glutaraldehyde. Relaxation was expressed as a percentage decrease of the maximal contraction induced by CCK8 (10 nM). Values are mean±s.e.mean of 7 separate experiments on cells from different animals.

Effect of (R)-p-cAMPs on cell relaxation induced by VIP Preincubation of dispersed cells for 5 min in the presence of 1 μM **(R)**-*p*-cAMPs, an antagonist of cyclic AMP at its binding site on cyclic AMP-dependent protein kinase (protein kinase A), did not alter the mean cell length of resting smooth muscle cells nor the contraction induced by 10 nM CCK8. By contrast, when cells were incubated in the presence of increasing concentrations of **(R)**-*p*-cAMPs for 5 min, followed by addition of a maximal relaxant concentration of VIP (1 μM), the relaxing effect of VIP was inhibited by **(R)**-*p*-cAMPs in a concentration-dependent manner (Figure 2a). The cell relaxation induced by VIP was inhibited by 92.1±6.33% ($n=6$) at 10 μM **(R)**-*p*-cAMPs and the concentration of **(R)**-*p*-cAMPs inducing to half-maximal inhibition (IC_{50}) of the relaxant effect of VIP was 0.27±0.05 nM ($n=6$).

Effect of NOS inhibitors on relaxation induced by VIP When cells were incubated with increasing concentrations of L-NAME or L-NMMA, ranging from 10 fM to 1 μM, the relaxation induced by 1 μM VIP was inhibited. The effects of L-NAME and L-NMMA were concentration-dependent with an IC_{50} of 1.10±0.52 pM ($n=6$) and 8.30±4.85 pM ($n=6$) respectively (Figure 2b). The maximal inhibition of VIP-induced relaxation was obtained at 1 μM L-NAME and L-NMMA and corresponded to a decrease of 87.8±8.25% and 89.3±10.5% respectively in the relaxant response to VIP. Inhibition of VIP-induced relaxation by L-NAME was fully reversed by L-arginine (100 μM) but not by D-arginine.

Cyclic GMP production in response to stimulation by VIP Preincubation of dispersed cells with increasing concentrations of VIP for 1 min stimulated cyclic GMP production in intestinal smooth muscle cells in a concentration-dependent manner (Figure 2c). Basal cyclic GMP concentration was 377±11.4 fmol/10^6 cells ($n=5$). VIP (1 μM) raised intracellular cyclic GMP to 514±45.7 fmol/10^6 cells ($n=5$, $P<0.05$). The stimulation of cyclic GMP production by VIP was inhibited by 10 μM L-NAME.

Relationship between cyclic AMP and NO

In order to determine the sequence of the intracellular cascade triggered by VIP in inducing smooth muscle cell relaxation, the effect of NOS inhibitors on cell relaxation triggered by forskolin or by an analogue of cyclic AMP was evaluated. When dispersed cells were incubated for 15 min with increasing concentrations (10 fM to 1 μM) of either forskolin or 8-bromo-cyclic AMP alone, cell length was not affected. However, the contraction induced by CCK8 (10 nM) was inhibited by forskolin, and by 8-bromo-cyclic AMP each in a concentration-dependent manner. The cell relaxation induced by forskolin (1 μM) or 8-bromo-cyclic AMP (1 μM) was inhibited in a concentration-dependent manner by **(R)**-*p*-cAMPs and abolished at 10 μM **(R)**-*p*-cAMPs (Figure 3a, 4a).

When cells were incubated with the NOS inhibitor L-NAME for 5 min, the relaxation induced by either forskolin or 8-bromo-cyclic AMP was markedly reduced but not abolished (78.8±9.40% ($n=6$) and 76.4±6.10% ($n=5$) respectively at 1 μM L-NAME) (Figures 3b,4b). As for VIP-induced relaxation, the inhibitory effect of L-NAME was fully reversed by preincubation of cells with L-arginine (100 μM) (data not shown).

Cyclic GMP production in response to stimulation by forskolin and 8-bromo-cyclic AMP When dispersed cells were incubated with forskolin (1 μM) or 8-bromo-cyclic AMP (1 μM),

cyclic GMP rose to 432±31.5 and 464±24.5 fmol/10^6 cells respectively (Figures 3c and 4c) levels which were significantly higher than those measured in controls (334±19.9 and 342±32.7 fmol/10^6 cells, ($P<0.05$). Blocking NO production by L-NAME (10 μM), abolished the increase in intracellular cyclic GMP triggered by forskolin or 8-bromo-cyclic AMP.

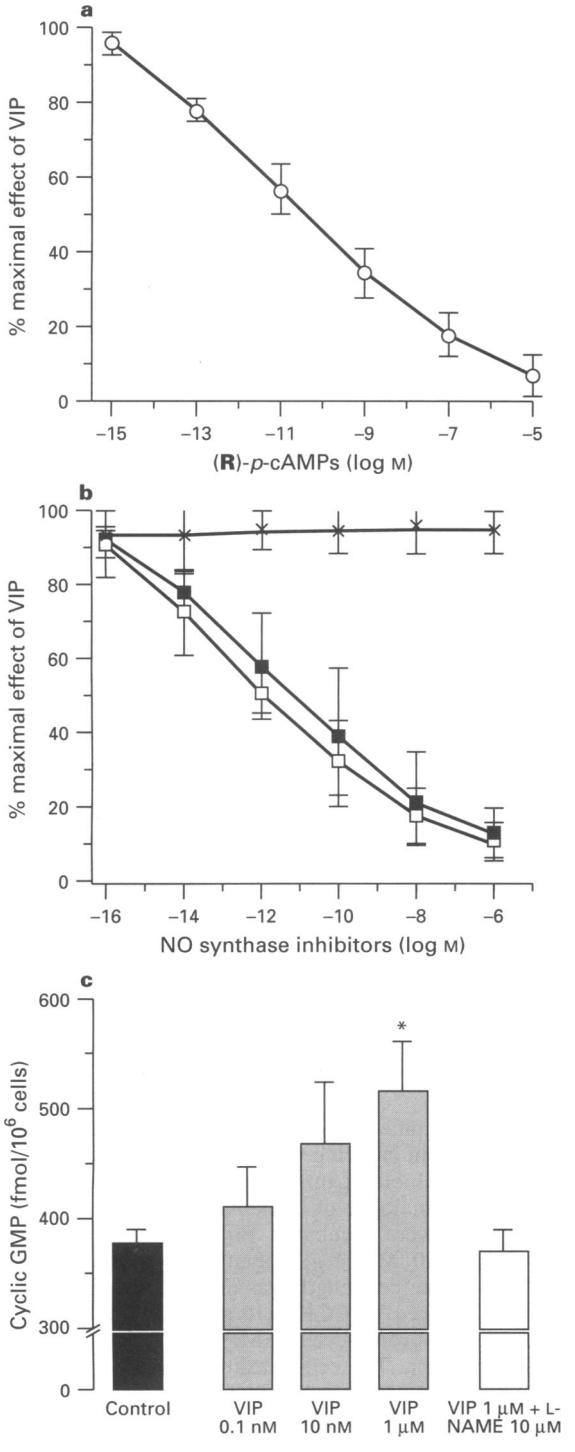

Figure 2 (a and b) Effects of (R)-*p*-cAMPs (a), L-NAME with or without 100 μM L-arginine and L-NMMA (b) on VIP-induced relaxation. Dispersed cells were incubated at 31°C in the presence of (R)-*p*-cAMPs (○), L-NAME (□), L-NAME and 100 μM L-arginine (x) or L-NMMA (■) for 5 min before addition of VIP (1 μM) for 1 min. Then, CCK8 (10 nM) was added for 30 s and cells were fixed with 2.5% glutaraldehyde. Results are expressed as the percentage of the effect of VIP observed in the absence of inhibitors, taken as 100%. (c) Stimulation of cyclic GMP production by VIP. Isolated intestinal smooth muscle cells were incubated with various concentrations (0.1 nM to 1 μM) of VIP for 1 min. Basal cyclic GMP level was 377±11.4 fmol/10^6 cells. Values are means±s.e.mean of 4 to 6 separate experiments on cells from different animals. *$P<0.05$.

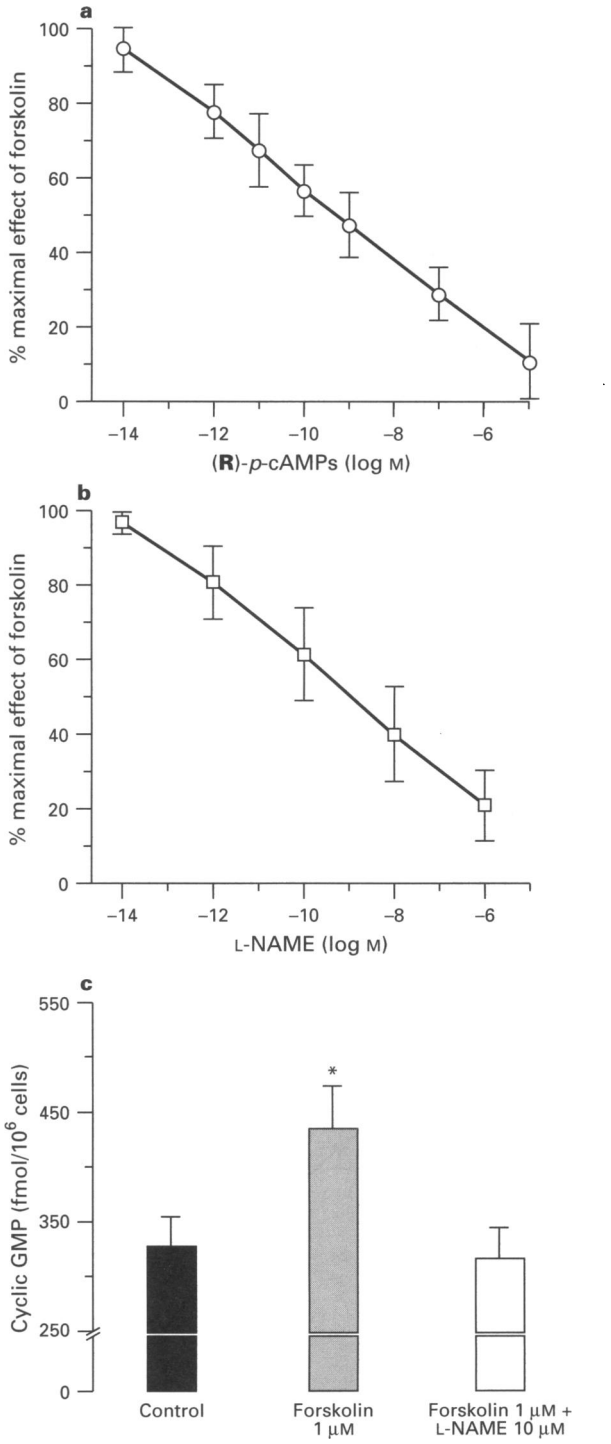

Figure 3 (a and b) Effect of (R)-*p*-cAMPs and of L-NAME on forskolin-induced relaxation of isolated smooth muscle cells from the circular layer of guinea-pig ileum. Cells were incubated at 31°C in the presence of (R)-*p*-cAMPs (a) or L-NAME (b) for 5 min before addition of forskolin (1 μM) for 15 min. Results are expressed as a percentage of cell contraction observed in the absence of (R)-*p*-cAMPs or L-NAME, taken as 100%. (c) Stimulation of cyclic GMP production by forskolin alone or in the presence of 10 μM L-NAME. Results are expressed as fmol cyclic GMP/10^6 cells. Values are mean±s.e.mean of six separate experiments on cells from different animals. *$P<0.05$.

Effect of dexamethasone on 8-bromo-cyclic AMP- and VIP-induced relaxation

Preincubation of isolated cells for 30 min with dexamethasone (10 μM), did not alter the mean length of resting smooth muscle cells, nor the contraction induced by 10 nM CCK8. By contrast, pretreatment of cells with dexamethasone, significantly impaired the relaxant effect induced by VIP or 8-bromo-cyclic AMP. As shown in Figure 5, when relaxation was induced by a maximal concentration of VIP (1 μM) or 8-bromo-cyclic AMP (1 μM) in dexamethasone-treated cells, the amplitude of this relaxation was only 52±4% ($n=6$) and 37±18% ($n=6$) respectively as compared to control cells. After dexamethasone treatment, the NOS inhibitor, L-NMMA (1 μM), no longer had any effect on the remaining VIP- or 8-bromo-cyclic AMP-induced relaxation of the cells.

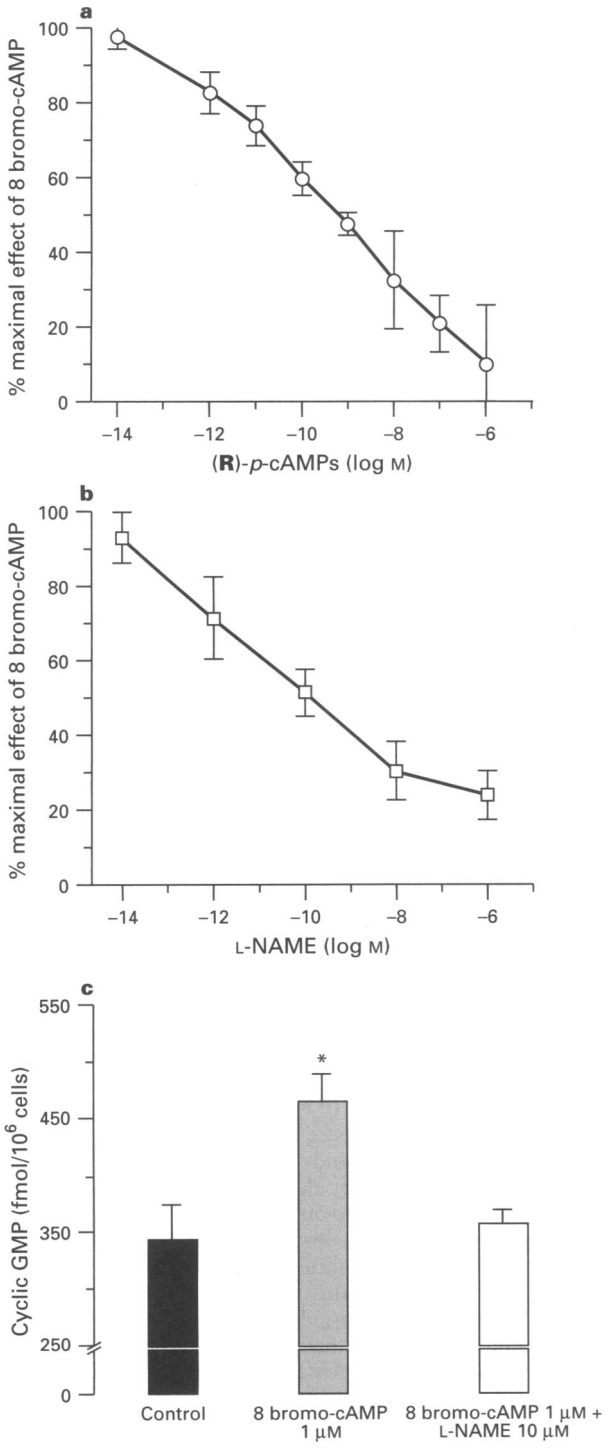

Figure 4 (a and b) Effects of (R)-*p*-cAMPs and of L-NAME on 8-bromo-cyclic AMP-induced relaxation of isolated smooth muscle cells from the circular layer of guinea-pig ileum. Cells were incubated at 31°C in the presence of (R)-*p*-cAMPs (a) and L-NAME (b) for 5 min before addition of 8-bromo-cyclic AMP (1 μM) for 15 min. Results are expressed as the percentage of the cell contraction observed in the absence of (R)-*p*-cAMPs (a) or L-NAME (b), taken as 100%. (c) Stimulation of cyclic GMP production by 8-bromo-cyclic AMP. Results are expressed as fmol cGMP/10^6 cells. Values are means±s.e.mean of six separate experiments on cells from different animals. *$P<0.05$.

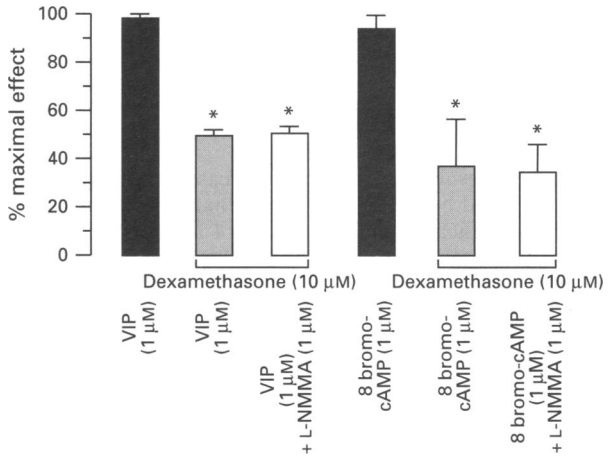

Figure 5 VIP- and 8-bromo-cyclic AMP-induced relaxation in dexamethasone-treated cells. Isolated smooth muscle cells from the circular layer of guinea-pig ileum were incubated at 31°C in the presence of 10 μM dexamethasone for 30 min. VIP or 8-bromo-cyclic AMP (1 μM) was then added, and CCK8 (10 nM) was finally added for 30 s. Then cells were fixed with 2.5% glutaraldehyde. In experiments involving L-NMMA (1 μM), this was added 5 min before addition of VIP or 8-bromo-cyclic AMP. Results are expressed as the percentage of the contraction induced by a maximal concentration of CCK8 in the absence of VIP, taken as 100%. Values are mean± s.e.mean of 4 separate experiments on cells from different animals. *$P<0.05$.

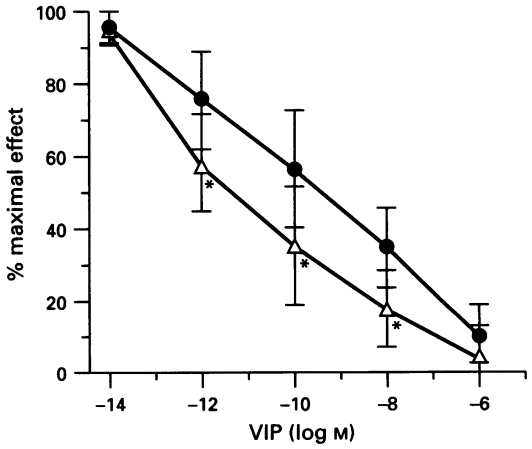

Figure 6 Effect of phosphodiesterases inhibitor, milrinone on VIP-induced relaxation of isolated smooth muscle cells. Cells were incubated with increasing concentrations of VIP (●) (control), or with milrinone (1 μM) (△) for 5 min. Increasing concentrations of VIP were then added for 1 min and finally CCK8 (10 nM) was added for 30 s. Results are expressed as the percentage of contraction induced by CCK8 in the absence of VIP and milrinone, taken as 100%. Values are mean±s.e.mean of six separate experiments on cells from different animals. *$P<0.05$.

Effect of milrinone on VIP-induced relaxation

Incubation of cells in the presence of increasing concentrations of milrinone (10 fM to 1 μM), an inhibitor of phosphodiesterases, did not alter the mean length of resting cells or the CCK8-induced contraction. However, when cells were pre-incubated with 10 μM milrinone for 5 min, followed by addition of increasing concentrations of VIP (10 fM to 1 μM) for 1 min, the CCK8-induced contraction was inhibited by VIP in a concentration-dependent manner, but VIP appeared more effective in inducing cell relaxation in milrinone-pretreated cells than in controls (Figure 6). Indeed, the concentration-response curve for VIP in the presence of milrinone shifted to the left, i.e. to the lower concentrations on the X axis. The EC_{50} of VIP was 0.37 nM in controls cells and 8 pM in milrinone pretreated cells.

Discussion

The present results suggest that the cascade of intracellular events triggered by VIP to relax intestinal smooth muscle cells in guinea-pig includes the activation of adenylate cyclase, an increase in intracellular cyclic AMP, the activation of protein kinase A and subsequently the activation of NOS and the production of cyclic GMP. Moreover, NO could in turn, regulate the cyclic AMP-dependent pathway of VIP-induced cell relaxation.

In the first part of this study, we confirmed that the relaxation induced by VIP in intestinal smooth muscle cells was mediated by cyclic AMP. VIP and other relaxing agents have been shown to induce cell relaxation through activation of the cyclic AMP-dependent pathway in many previous studies on muscle strips (Bolton, 1979) or on dispersed smooth muscle cells (Bitar & Makhlouf, 1982a; Jin *et al.*, 1993; Botella *et al.*, 1994). To assess further that VIP relaxes intestinal smooth muscle cells by activating a cyclic AMP-dependent pathway, we blocked the effect of VIP on guinea-pig intestinal smooth muscle cells by drugs that inhibit specifically the successive steps of cyclic AMP production and stimulation of protein kinase A: (i) VIP-induced relaxation was inhibited by NEM and somatostatin both of which directly inhibit adenylate cyclase. NEM inhibits the activity of the catalytic unit of adenylate cyclase (Smith & Simon, 1980; Moummi & Rattan, 1988; Askhenazi *et al.*, 1989). Intestinal smooth muscle cells express somatostatin receptors, the occupation of which inhibits adenylate cyclase through a G_i protein (Gu *et al.*, 1992b) and thereby cell relaxation. (ii) Cell relaxation induced by VIP was also blocked by (**R**)-*p*-cAMPs, which competes with cyclic AMP on its binding site to the regulatory subunit of protein kinase A, without interaction with protein kinase G (Royhermel *et al.*, 1984; Gu *et al.*, 1992a).

On the other hand, several features support the hypothesis that VIP involves the production of NO by NOS to relax intestinal smooth muscle cells: (i) L-NAME and L-NMMA, inhibitors of NOS, inhibited VIP-induced relaxation of intestinal smooth muscle cells; (ii) VIP stimulated the production of cyclic GMP, at concentrations similar to those inducing cell relaxation. Previous reports have shown that the relaxation induced by VIP in isolated gastric muscle cells is mediated through the stimulation of NOS and NO production (Grider *et al.*, 1992; Jin *et al.*, 1993). L-NAME and L-NMMA are competitive inhibitors of L-arginine, which is the specific substrate used by NOS to produce NO (Bredt & Snyder, 1989; Komori *et al.*, 1994). Indeed, their inhibitory effect was fully reversed by L-arginine in the present experiments, indicating the specificity of their action. Stimulation of cyclic GMP production by VIP has also been observed previously in gastric smooth muscle cells (Jin *et al.*, 1993).

NOS have been characterized in various cell types and was classified into 2 main categories: the constitutive NOS, present in nerve terminals and endothelial cells, the activation of which results in the release of small amounts of NO (pmol) for short periods of time and the inducible NOS, found in macrophages and vascular smooth muscle cells that releases larger amounts of NO (nmol) for long periods of time (for review, see Moncada, 1992). However, the time course of the effect of VIP on intestinal smooth muscle cells (cell relaxation induced in less than 1 min) precludes the induction of NOS, which is a time requiring process involving protein synthesis. More recently, the distinction between these 2 types of NOS was clouded in view of molecular biological results, which described at least 4 isoforms of NOS. Some of these isoforms are constitutively present in cells but share pharmacological properties with inducible forms (Berdeaux, 1993). In smooth muscle cells, it has been shown that a constitutive Ca^{2+}/calmodulin-dependent NOS is present in the plasma membrane of dispersed gastric smooth muscle cells where it could behave like a 'receptor-effector coupling protein', activated by VIP-PACAP II receptors (Murthy & Makhlouf, 1994). Intestinal smooth muscle cells could thus constitutively express an isoform of NOS, the pharmacological properties of which are similar to those previously reported for the inducible form.

Many questions have not been solved, regarding the relationship between the two intracellular pathways triggered by VIP to induce cell relaxation. Indeed, in the present experiments, NOS inhibitors did not fully inhibit VIP-induced cell relaxation, the residual effect, being up to 20% of the maximal effect of VIP. NOS and adenylate cyclase may be activated by VIP sequentially or in a parallel way as previously suggested (Jin *et al.*, 1993).

To investigate further the relationship between NO and cyclic AMP in mediating the effect of VIP, we tested the influence of NOS inhibitors on the relaxant effect of various drugs either directly stimulating adenylate cyclase or increasing the intracellular level of cyclic AMP: forskolin which increases intracellular cyclic AMP by activating the catalytic subunit of adenylate cyclase (Laurenza *et al.*, 1989) and 8-bromo-cyclic AMP, a membrane-permeable analogue of cyclic AMP. Both compounds induced cell relaxation, which was inhibited in a concentration dependent manner by (**R**)-*p*-cAMPs but also by the inhibitor of NOS, L-NAME. Moreover, both forskolin and 8-bromo-cyclic AMP stimulated the production of cyclic GMP in intestinal smooth muscle cells. Therefore, we assume that the intracellular pathway of VIP-induced cell relaxation successively involves activation of adenylate cyclase, production of cyclic AMP, activation of protein kinase A, activation of NOS, production of NO and finally production of cyclic GMP and activation of protein kinase G. However, NOS inhibitors did not abolish VIP-, forskolin-, and 8-bromo-cyclic AMP-induced relaxation of cells whereas (**R**)-*p*-cAMPs, the inhibitor of protein kinase A, abolished this effect, indicating that the main part of the effect of VIP is mediated through cyclic AMP production and protein kinase A activation.

Moreover, the present study has also shown that inhibition of the induction of NOS by dexamethaxsone, impairs the relaxant effect of VIP down to 50% of its maximal value and the remaining relaxation is insensitive to L-NAME. Dexamethasone is known to inhibit the synthesis and the expression of a number of proteins including NOS in macrophages (Di Rosa *et al.*, 1990), in vascular smooth muscle cells (Imai *et al.*, 1994) and porcine endothelial cells (Radomski *et al.*, 1990). Since the remaining cyclic AMP-mediated cell relaxation after dexamethasone treatment is insensitive to NOS inhibitors, we assume that VIP-induced relaxation is mainly mediated through activation of cyclic AMP production but that this cyclic AMP-dependent intracellular pathway requires production of NO and cyclic GMP to relax the cells fully. Thus, it seems that NO acts not only as an intracellular messenger of the relaxant effect of VIP, but modulates the activity of the cyclic AMP-dependent pathway, by maintaining a 'relaxing tone' of the smooth muscle cells. The interactions between NOS and cyclic AMP-dependent pathway could occur at the level of protein kinases. Bredt *et al.* (1992) have observed that the constitutive brain NOS is stoichiometrically phosphorylated by protein kinase A, protein kinase C and Ca^{2+}/calmo-

dulin-dependent protein kinase. Phosphorylation of brain NOS by protein kinase C results in a dramatic decrease of its activity, whereas phosphorylation by protein kinase A has no effect.

Interaction between NO and cyclic AMP could also occur at the level of phosphodiesterases (PDEs). These latter enzymes regulate the intracellular level of cyclic nucleotides in a specific way: various types of phosphodiesterases have a selective effect on the metabolism of cyclic AMP and cyclic GMP. On the other hand, in some cell types, cyclic GMP regulates the activity of phosphodiesterases (Beavo & Reifsnyder, 1990). In rat aortic smooth muscle cells, cyclic GMP enhances cyclic AMP-mediated relaxation through the inhibition of PDE III (Komas *et al.*, 1991; Eckly & Lugnier, 1994). To assess this assumption further, we evaluated the effect of milrinone, an inhibitor of type III PDE on the cell relaxation induced by VIP. We observed that inhibition of PDE resulted in a potentiation of the effect of VIP, marked by a left shift of its concentration-response curve. The intensity of cell relaxation induced by VIP is thus dependent on the activity of PDE which can be regulated by NO and cyclic GMP.

In conclusion, VIP sequentially activates adenylate cyclase and NOS to induce cell relaxation in intestinal circular smooth muscle. However part of the effect of VIP is mediated through a NO-independent pathway. In turn, NO could act in intestinal smooth muscle cells by maintaining a relaxed tone of the cells. Action of NO on intestinal smooth muscle cells could thus be multiple, including transmission of the NANC nerve signal, mediation of the effect of VIP and modulation of various metabolic activities.

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