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Interplay between nitric oxide and vasoactive intestinal polypeptide in the pig gastric fundus smooth muscle

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Abstract

The aim of this study was to investigate the exact mechanism of interaction between nitric oxide (NO) and vasoactive intestinal polypeptide (VIP) as inhibitory non-adrenergic non-cholinergic (NANC) neurotransmitters in isolated smooth muscle cells and smooth muscle strips of the pig gastric fundus. In isolated smooth muscle cells, the maximal relaxant effect of VIP (10^{-9} M) was inhibited by 94% by the NO synthase (NOS) inhibitor N^G-nitro-L-arginine (L-NA, 10^{-4} M) and by 85% by the inducible NOS (iNOS)-selective inhibitor *N*-(3-(aminomethyl)-benzyl)acetamide (1400W; 10^{-6} M). The relaxant effect of VIP was reduced by more than 70% by the guanylyl cyclase inhibitor 1*H*-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ; 10^{-6} M), the glucocorticoid dexamethasone (10^{-5} M) and three protein kinase A inhibitors: (*R*)-*p*-cyclic adenosine-3′,5′-monophosphothioate ((*R*)-*p*-cAMPS; 10^{-6} M), {(8*R*,9*S*,11*S*)-(−)-9-hydroxy-9-*n*-hexylester-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7*b*,11*a*-triazadibenzo[*a*,*g*]cycloocta[*cde*]-trin-den-1-one} (KT5720; 10^{-6} M) and *N*-(2-(*p*-bromo-cinnamylamino)ethyl))-5-isoquinoline sulfonamide dihydrochloride (H-89; 10^{-5} M). In contrast, no influence of the NOS inhibitors, ODQ, dexamethasone, nor the protein kinase A inhibitors could be observed on the relaxant effect of VIP in smooth muscle strips. These data demonstrate that the experimental method completely changes the influence of NOS inhibitors on the relaxant effect of VIP in the pig gastric fundus. The isolation procedure of the smooth muscle cells might induce iNOS that can be activated by VIP.

Keywords: Nitric oxide (NO); VIP (vasoactive intestinal polypeptide); Gastric fundus, Pig

1. Introduction

It has been well recognized that both nitric oxide (NO) and vasoactive intestinal polypeptide (VIP) serve as inhibitory non-adrenergic non-cholinergic (NANC) mediators in the gastrointestinal tract (D'Amato et al., 1992; Sanders and Ward, 1992; Brookes, 1993; Shuttleworth and Keef, 1995; Rand and Li, 1995). Still, there is a lot of controversy about the interaction between NO and VIP in the gastrointestinal tract. In many neurons of the myenteric plexus from different gastrointestinal tissues, including the pig gastric fundus, immunoreactivity revealed colocalization of VIP and neuronal NO synthase (nNOS, Furness et al., 1992; Berezin et al., 1994; Lefebvre et al., 1995) suggesting cotransmission of VIP and NO. Hereby NO is thought to induce relaxation via a guanosine 3′5′ cyclic monophosphate (cyclic GMP)-dependent pathway and VIP via an adenosine 3′5′ cyclic monophosphate (cyclic AMP)-dependent pathway. In line with the model of cotransmission of VIP and NO is the observation that the relaxation by VIP in many gastrointestinal tissues is not influenced by NOS inhibitors (Tøttrup et al., 1991; Boeckxstaens et al., 1992; Barbier and Lefebvre, 1993; Keef et al., 1994). In contrast, the group of Makhlouf reported that in both isolated smooth muscle cells and smooth muscle strips of the guinea-pig gastric fundus and rat colon, the VIP-induced relaxation is inhibited by NOS inhibitors. A sequential link between NO and VIP is proposed whereby VIP seems to be the primary neurotransmitter, inducing relaxation partially via activation of adenylate cyclase and partially via stimulation of NO production in the smooth muscle cells (Grider et al., 1992; Grider, 1993; Jin et al., 1993). The enzyme involved in the muscular production of

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NO would be endothelial NO synthase (eNOS) as measured by reverse transcription-polymerase chain reaction and Southern blot analysis in rabbit gastric and human intestinal smooth muscle cells (Teng et al., 1998).

Recent results obtained in our laboratory showed a completely different influence of NOS inhibitors in smooth muscle cells versus smooth muscle strips of the guinea-pig gastric fundus (Dick et al., 2000). In isolated smooth muscle cells, the relaxation by VIP is inhibited by NOS inhibitors, including the inducible NO synthase (iNOS)-selective inhibitor 1400W, whereas in isolated smooth muscle strips it is not. These results suggest that the experimental method determines the influence of NOS inhibitors on the relaxant effect of VIP in the guinea-pig gastric fundus. An NOS isoform with properties of iNOS, probably induced by the isolation procedure of the smooth muscle cells, might be involved in the relaxation induced by VIP in the isolated smooth muscle cells.

Among the non-primate mammalian species, the pig has been proposed as one of the best models for the study of nutritional issues in man, due to the similarity of the morphology and physiology of the gastrointestinal tracts (Miller and Ullrey, 1987). It has been shown before in the pig gastric fundus that NO and to a smaller extent VIP are involved in NANC relaxation (Lefebvre et al., 1995). The aim of this study was to investigate whether the differential effect of NOS inhibitors in gastric smooth muscle cells and strips, as observed in a rodent, could be reproduced in a non-rodent higher mammalian species, the pig.

2. Methods

2.1. Preparation of isolated smooth muscle cells

Circular smooth muscle cells were isolated from the pig gastric fundus by collagenase digestion as previously described (Bitar and Makhlouf, 1982; Botella et al., 1992). Briefly, the stomach was removed from healthy 6-months-old male castrated pigs, slaughtered at a local abattoir and transported to the laboratory in ice-chilled physiological salt solution. After removal of the mucosa and submucosa, the circular muscle layer was carefully dissected from the rest of the stomach wall. Small sheets from the circular muscle layer were incubated for two successive periods of 30 min at 31°C, in 15 ml of *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-buffered medium (25 mM), containing 250 U ml^{-1} collagenase (Type II) and 0.01% soybean trypsin inhibitor and gassed with a mixture of 95% O_2 and 5% CO_2. The medium consisted of NaCl, 95.5 mM; KCl, 12.5 mM; NaH_2PO_4, 2.5 mM; $CaCl_2$, 2.4 mM; $MgCl_2$, 1.2; D(+)-glucose, 11.5 mM; bovine serum albumin, 0.2% (w v^{-1}) and was supplemented with sodium pyruvate, 5 mM; sodium fumarate, 5 mM; sodium glutamate, 5 mM; glutamine, 2 mM; amino acid mixture, 1% (vol vol^{-1}); vitamin mixture, 1% (vol vol^{-1}); penicillin G, 50 μg ml^{-1} and streptomycin, 50 μg ml^{-1}. The pH of the buffered medium was adjusted to 7.4. At the end of the second incubation, the medium was filtered through a 500-μm Nitex filter and the partly digested tissues were washed with 30 ml enzyme-free medium, whereafter they were allowed to disperse spontaneously in enzyme-free medium for 30 min. Finally, the spontaneously dissociated muscle cells were harvested by filtration and used for functional measurements.

Viability tests by exclusion of trypan blue (Collins and Gardner, 1992) showed that 87.6 ± 1.4% (mean ± S.E.M., $n = 6$) of the cells in suspension were viable at the time of contraction experiments. Cell suspensions were studied usually within 30 min at 31°C.

The length of the isolated smooth muscle cells was determined by Image Splitting after fixation with glutaraldehyde. An aliquot of 50 μl treated cell suspension was placed on a Malassez slide. The first 50 randomly encountered and morphologically intact cells were measured using a Carl Zeiss eyepiece at a magnification of at least 200 times. For the vials with control cells and carbachol-treated cells, two different aliquots were taken and 2 times 50 cells were measured. The absolute cell length measurement was performed with a scale mask placed on a video screen, connected to a video camera. Magnification due to the video camera had been first calculated by use of a micrometer.

2.2. Measurement of relaxation (inhibition of contraction) in isolated smooth muscle cells

Untreated cells served as controls. Cells can be maximally contracted by incubation with 10^{-8} M carbachol for 30 s, followed by fixation of the cells with glutaraldehyde (pH 7.4) to a final concentration of 2.5%. In relaxation experiments, the relaxant agent VIP (10^{-14}–10^{-6} M) or forskolin (10^{-6} M) was added 60 s before carbachol. The inhibition of the carbachol-induced contraction was considered as relaxation as previously described (Grider et al., 1992; Jin et al., 1993; Rekik et al., 1996). The term relaxation will be used throughout the manuscript. To study the mechanism of relaxation, the cells were incubated before addition of the relaxant agents with: the NOS inhibitors N^G-nitro-L-arginine (L-NA) and *N*-(3-(aminomethyl)-benzyl)acetamide (1400W), with or without L-arginine or D-arginine (incubation time 5 min), the protein kinase A inhibitors (*R*)-*p*-cyclic adenosine-3′, 5′-monophosphothioate ((*R*)-*p*-cAMPS; 5 min), *N*-(2-(*p*-bromocinnamylamino)ethyl)-5-isoquinoline sulfonamide dihydrochloride(H-89, 5 min) and {(8*R*,9*S*,11*S*)-(−)-9-hydroxy-9-*n*-hexylester-8-methyl-2, 3, 9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2, 7*b*, 11*a*-triazadibenzo[*a*,*g*]cyclo-

octa[*cde*]-trin-den-1-one} (KT5720, 5 min), the guanylyl cyclase inhibitor 1*H*-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ; 20 min) and the glucocorticoid dexamethasone (30 min). In parallel control vials, the cells were incubated with the solvent of these agents.

2.3. Preparation of smooth muscle strips

After removal of the mucosa, strips (15×3 mm) were prepared from the gastric fundus by cutting in the direction of the circular muscle layer. The strips were suspended between two platinum plate electrodes under a load of 2 g in 5 ml or 20 ml organ baths containing physiological salt solution, maintained at 37°C and gassed with a mixture of 95% O_2 and 5% CO_2. The physiological salt solution had the following composition: NaCl, 137 mM; KCl, 5.9 mM; KH_2PO_4, 1.2 mM; $MgCl_2$, 1.2 mM; $CaCl_2$, 2.5 mM; $NaHCO_3$, 25.0 mM and D(+)-glucose, 11.5 mM. The Krebs solution always contained 10^{-6} M atropine and 4×10^{-6} M guanethidine to inhibit cholinergic and noradrenergic responses. Changes in length were recorded isotonically via Hugo Sachs B40 Lever transducers type 373 on a Graphtec Linearcorder 8 WR 3500 in the 5-ml baths and via Palmer Bioscience T3 transducers on a Graphtec Linearcorder WR 3701 F in the 20-ml baths. Electrical field stimulation was performed by means of a Hugo Sachs Stimulator I type 215/I in the 5-ml baths and by a Grass S88 Stimulator in the 20-ml baths. The tissues were equilibrated for 1 h 30 min with rinsing every 15 min.

2.4. Measurement of relaxation in muscle strips

After the equilibration period, tone was raised by the administration of 3×10^{-7} M 5-HT. Once a stable contraction was obtained, electrical field stimulation was performed or relaxant agents were administered. Frequency–response curves to electrical field stimulation (40 V, 0.1 ms, 0.25–16 Hz) were obtained by stimulating the tissues with 10 s trains at 5 min intervals. VIP (10^{-8}–3×10^{-7} M) and forskolin (10^{-7}–10^{-4} M) were administered in a cumulative way. Because of the slow development of the relaxations induced by VIP and forskolin, the increasing concentrations of VIP and forskolin were added approximately every 15 min. After obtaining the first frequency– or concentration–response curve, the tissues were regularly rinsed for 30 min. L-NA, 1400W, ODQ, (*R*)-*p*-cAMPS, H-89, KT5720, the adenylyl cyclase inhibitor 9-(tetrahydro-2-furanyl)-9*H*-purin-6-amine (SQ22536) or dexamethasone were then added and incubated for 30 min. Tone was then again raised by adding 3×10^{-7} M 5-HT and a second frequency– or concentration–response curve was obtained. In parallel control strips, only the solvent of the tested drug was incubated. None of the solvents influenced the tone of the tissues. At the end of the experiments, tissues were rinsed for 30 min; tone was again induced with 3×10^{-7} M 5-HT and relaxation was obtained with 10^{-5} M sodium nitroprusside (reference relaxation).

2.5. Data analysis

The contraction of the isolated smooth muscle cells was expressed as the percentage decrease in cell length from untreated controls, using the following formula: $((L_0 - L_x)L_0^{-1}) \times 100$ where L_0 is the mean length of cells in control state and L_x the mean length of carbachol-treated cells. In relaxation experiments, the degree of inhibition of contraction was expressed as the percentage decrease in maximal contractile response, as observed in carbachol-treated cells in the absence of relaxant agent.

Relaxations in the smooth muscle strips were expressed as percentage of the sodium nitroprusside-induced relaxation at the end of the experiment.

Results are given as means ± standard error of the mean (S.E.M.) and *n* refers to material from different animals. Responses in parallel vials with isolated smooth muscle cells were compared by analysis of variance (ANOVA) and the *t*-test corrected for multiple comparisons (Bonferroni procedure). Responses during the first and second curves in the strips were compared by a paired *t*-test. If a statistically significant difference was reached in the control strips, these differences were compared with the differences obtained in the strips treated with the tested drug by an unpaired *t*-test. *P*-values of less than 0.05 were considered statistically significant.

2.6. Chemicals

Collagenase was purchased from Worthington Biochemical (Freehold, New Jersey, USA). VIP was obtained from Bachem (Bubendorf, Switzerland) and carbamoylcholine chloride (carbachol) from Fluka (Switzerland). 1400W, SQ22536, H-89 and KT5720 were obtained from Alexis (Nottingham, UK) and ODQ from Tocris Cookson (Bristol, UK). D-arginine hydrochloride, L-arginine hydrochloride, atropine sulphate, bovine serum albumin, dexamethasone, essential amino acid mixture, forskolin, glutamine, glutaraldehyde, guanethidine sulphate, L-NA, penicillin G, (*R*)-*p*-cAMPS, sodium nitroprusside, sodium fumarate, sodium glutamate, sodium pyruvate, streptomycin, trypan blue and vitamin mixture were from Sigma Chemicals (St. Louis, MO, USA). HEPES and soybean trypsin inhibitor were from Boehringer Mannheim Biochemicals (Indianapolis, IN, USA).

All drugs were dissolved in deionized water, except for sodium nitroprusside which was dissolved in 0.9% NaCl solution, forskolin and ODQ which were dissolved in pure ethanol up to 10^{-2} M and KT5720 which was dissolved in dimethylsulfoxide up to 10^{-3} M; further dilutions were

Table 1
Effect of L-NA on VIP-induced relaxation in isolated smooth muscle cells
Values are mean ± S.E.M. from $n = 6$.

	Muscle cell length (μm)	Contraction (percent decreasein cell length)	Relaxation (percent inhibition of carbachol-induced contraction)	Inhibition (percent inhibition of VIP-induced relaxation)
Controls	118.2 ± 5.1			
Carbachol (10^{-8} M)	92.3 ± 3.2[a]	21.6 ± 2.3		
Carbachol (10^{-8} M) + VIP (10^{-9} M)	117.9 ± 4.3	−0.2 ± 1.6	102.4 ± 7.4	
Carbachol (10^{-8} M) + VIP (10^{-9} M) + L-NA (10^{-4} M)	94.0 ± 3.5[a]	20.2 ± 2.5	6.3 ± 8.0	94.3 ± 7.6

[a] $P < 0.001$ significantly different from control cells.

made in physiological salt solution. The solvents, diluted in physiological salt solution till the final concentration given to the strips or the cells, had no effect per se on the tone of the strips or on control isolated smooth muscle cells. Stock solutions of 1400W, SQ22536, (*R*)-*p*-cAMPS, H-89, all up to 10^{-2} M, and VIP up to 10^{-4} M, were prepared in deionized water and stored at −20°C. All other solutions were prepared on the day of the experiment.

3. Results

3.1. Isolated smooth muscle cells

Untreated control cells, obtained after dispersion of the circular muscle layer of the pig gastric fundus, had a mean cell length of 118.2 ± 5.1 μm ($n = 6$, Table 1). Carbachol incubated for 30 s contracted the cells in a concentration-dependent manner with a maximal effect at 10^{-8} M (results not shown). Carbachol (10^{-8} M) produced 21.6 ± 2.3% shortening of the cells to 92.3 ± 3.2 μm ($n = 6$, Table 1). When cells were preincubated for 60 s with increasing concentrations of VIP (10^{-14}–10^{-6} M), the contraction was inhibited in a concentration-dependent manner although the concentration–response curve was not smooth (Fig. 1). Full relaxation was obtained at 10^{-9} M VIP and this concentration was selected for further

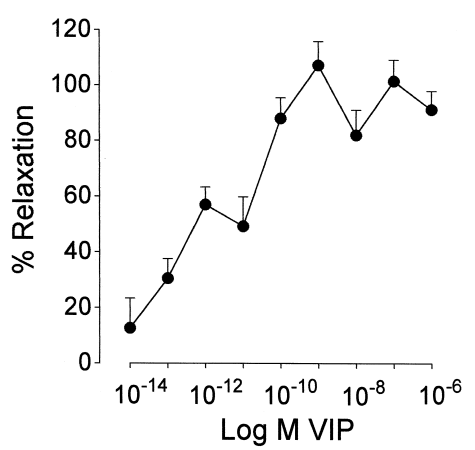

Fig. 1. Concentration–response curve to VIP (10^{-14}–10^{-6} M) in isolated smooth muscle cells from the circular muscle layer of the pig gastric fundus. Values are mean ± S.E.M. from $n = 6$.

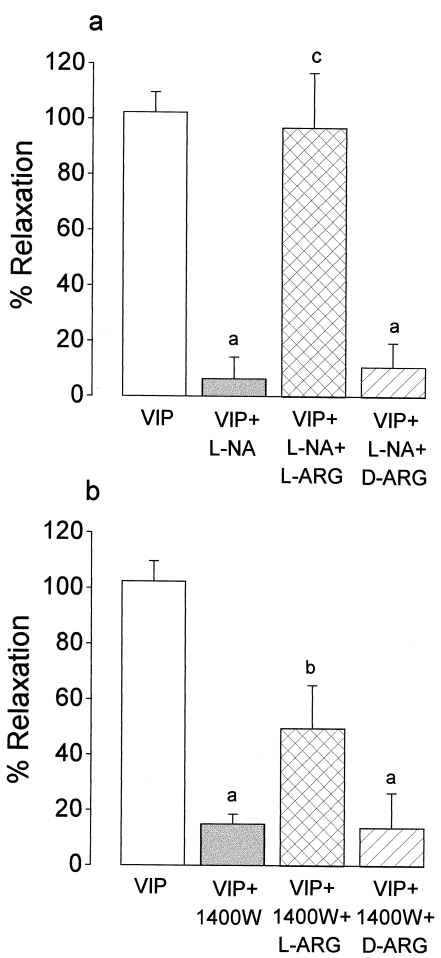

Fig. 2. Effect of L-NA (10^{-4} M, a) and 1400W (10^{-6} M, b) with or without L-arginine (L-ARG, 10^{-4} M) and D-arginine (D-ARG, 10^{-4} M) on VIP-induced relaxation in isolated smooth muscle cells of the pig gastric fundus. Values are mean ± S.E.M. from $n = 6$. [a] $P < 0.001$ significantly different from the cells treated with VIP. [b] $P < 0.05$, [c] $P < 0.001$ significantly different from cells treated with VIP and NOS inhibitor.

Table 2
Inhibition (%) of the relaxation induced by the relaxant agents VIP and forskolin and by electrical field stimulation (EFS; for the strips) under the influence of NOS inhibitors, ODQ, protein kinase A inhibitors, dexamethasone and SQ22536 in isolated smooth muscle cells and isolated smooth muscle strips NT means not tested; = means not inhibited. Values are mean ± S.E.M. from $n = 6$ except for SQ22536 versus VIP in the strips ($n = 4$). Inhibition of the relaxation by various agents was expressed as percent decrease in relaxation induced by the relaxant agent.

	Isolated smooth muscle cells		Isolated smooth muscle strips		
	VIP (10^{-9} M)	Forskolin (10^{-6} M)	VIP (10^{-8}–3×10^{-7} M)	Forskolin (10^{-7}–10^{-4} M)	EFS (0.5–16 Hz)
L-NA (10^{-4} M)	94.3 ± 7.6[a]	NT	=	NT	↓[b]
1400W (10^{-6} M)	85.0 ± 3.6[a]	NT	=	NT	=
ODQ (10^{-6} M)	73.9 ± 9.0[a]	NT	=	NT	NT
(*R*)-*p*-cAMPS (10^{-6}, 10^{-4} M)[c]	73.7 ± 6.0[a]	84.1 ± 12.7[a]	=	=	NT
KT5720 (10^{-6} M)	87.0 ± 4.6[a]	89.9 ± 11.7[a]	=	=	NT
H-89 (10^{-5} M)	94.4 ± 7.2[a]	93.5 ± 10.0[a]	=	=	NT
Dexamethasone (10^{-5} M)	68.6 ± 8.1[a]	NT	=	NT	NT
SQ22536 (10^{-6} M)	NT	NT	=	NT	NT

[a] $P < 0.001$ significantly different from cells treated with the relaxant agent alone.
[b] $P < 0.05$ at 0.5 and 16 Hz, $P < 0.01$ at 4 and 8 Hz, $P < 0.001$ at 1 and 2 Hz significantly different from strips treated with L-NA.
[c] 10^{-6} M (*R*)-*p*-cAMPS was tested on the VIP- and forskolin-induced relaxation in cells, 10^{-4} M (*R*)-*p*-cAMPS was tested on the VIP- and forskolin-induced relaxations in strips.

investigation. In the absence of carbachol, VIP did not affect the cell length of the isolated smooth muscle cells ($n = 6$).

When cells were incubated with 10^{-4} M of the NOS inhibitor L-NA, the relaxant effect of 10^{-9} M VIP was inhibited by 94.3 ± 7.6% ($n = 6$, Table 1). The inhibition of the VIP-induced relaxation was reversed by preincubation for 5 min with 10^{-4} M L-arginine, but not by 10^{-4} M D-arginine (Fig. 2a). At 10^{-6} M, the selective iNOS inhibitor 1400W antagonized the VIP-induced relaxation by 85.0 ± 3.6% ($n = 6$, Table 2, Fig. 2b). In contrast to L-NA, the inhibitory effect of 10^{-6} M 1400W on the VIP-induced relaxation was only partially reversed by 10^{-4} M L-arginine.

Preincubation of the cells with the guanylyl cyclase inhibitor ODQ (10^{-6} M) for 20 min decreased the relaxant effect of VIP by 73.9 ± 9.0% ($n = 6$, Table 2). When the cells were incubated for 5 min with one of the protein kinase A inhibitors (*R*)-*p*-cAMPS (10^{-6} M), KT5720 (10^{-6} M) or H-89 (10^{-5} M), the effect of VIP was reduced by 75% to 95% (Table 2). Also, the glucocorticoid dexamethasone (10^{-5} M, 30 min) reduced the relaxant effect of VIP by approximately 70% (Table 2). None of these NOS inhibitors or protein kinase A inhibitors nor ODQ altered the mean cell length of the control resting circular muscle cells, nor the degree of the carbachol-induced contraction ($n = 6$).

The adenylyl cyclase stimulant forskolin elicited relaxation in dispersed smooth muscle cells with full inhibition of carbachol-induced contraction at 10^{-6} M. This maximally effective concentration was selected for further investigation. Forskolin did not affect the mean length of

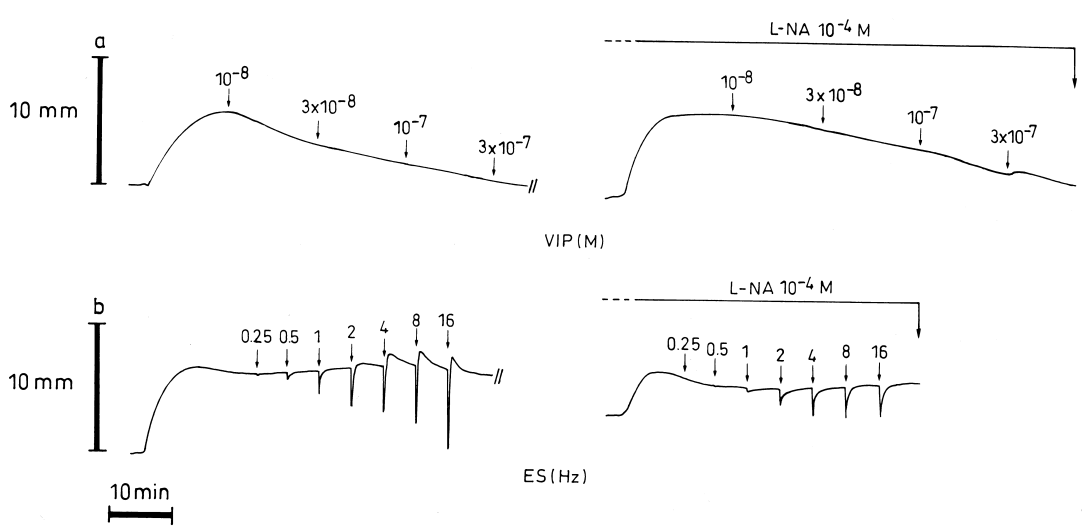

Fig. 3. Representative traces from circular smooth muscle strips of the pig gastric fundus showing the responses to VIP (a) and to electrical field stimulation (40 V, 0.1 ms, 0.25–16 Hz) with 10-s trains (b) before and after addition of L-NA (10^{-4} M). Tone was raised by the administration of 3×10^{-7} M 5-HT.

control resting smooth muscle cells ($n = 6$). When the cells were incubated for 5 min with 10^{-6} M *(R)-p*-cAMPS, 10^{-6} M KT5720 or 10^{-5} M H-89, the relaxant effect of 10^{-6} M forskolin was antagonized by approximately 85% to 95% (Table 2).

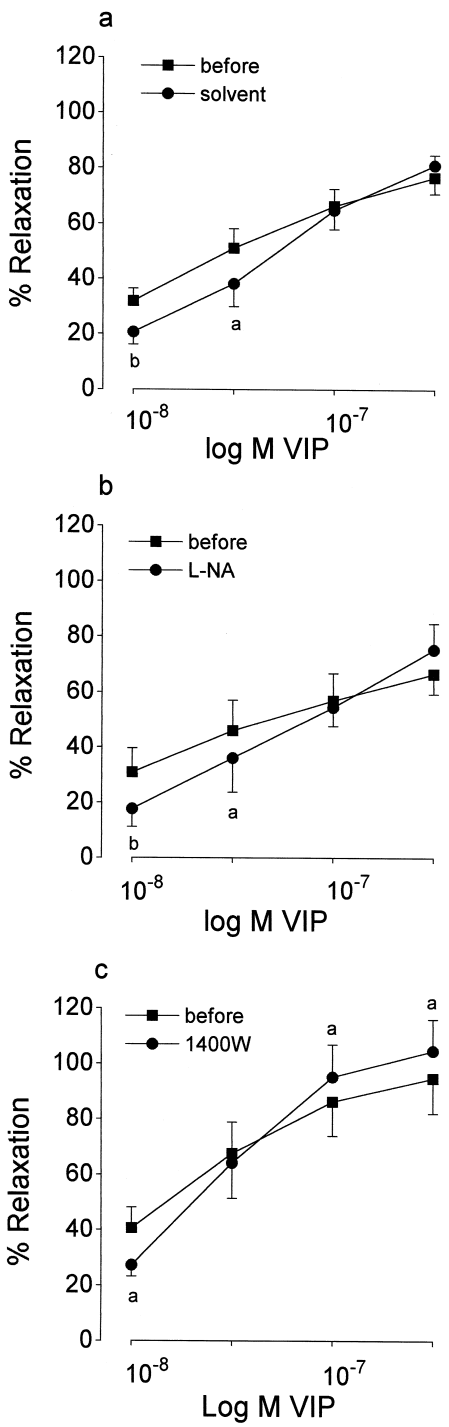

Fig. 4. Concentration–response curves to VIP (a,b,c) in circular smooth muscle strips of the pig gastric fundus in the absence and presence of solvent (a), L-NA (10^{-4} M, b) and 1400W (10^{-6} M, c). Values are mean ± S.E.M. from $n = 6$. $^{a}P < 0.05$, $^{b}P < 0.01$ significantly different from the response before the addition of solvent, L-NA or 1400W.

3.2. Isolated smooth muscle strips

VIP (10^{-8} M–3×10^{-7} M) induced concentration-dependent relaxations of circular smooth muscle strips of the pig gastric fundus (Fig. 3a). In the presence of 10^{-4} M L-NA, the relaxations induced by 10^{-8} M and 3×10^{-8} M VIP were significantly reduced (Fig. 4b), but this inhibitory effect was not significantly different from that observed in the parallel control tissues (Fig. 4a). In the presence of 10^{-6} M 1400W (Fig. 4c), the relaxations induced by 10^{-8} M VIP were significantly decreased, while those induced by 10^{-7} and 3×10^{-7} M VIP were increased, but these changes were not significantly different from those found in the parallel control strips. Neither dexamethasone (10^{-5} M) nor ODQ (10^{-6} M) had any influence on the relaxant effect of VIP in gastric smooth muscle strips (Table 2). Also, when the strips were incubated with *(R)-p*-cAMPS (10^{-4} M), H-89 (10^{-5} M), KT5720 (10^{-6} M) or SQ22536 (10^{-6} M) no influence was observed on the relaxations induced by VIP (Table 2). Forskolin (10^{-7} –10^{-4} M) caused concentration-dependent relaxations of circular smooth muscle strips that were not influenced by 10^{-4} M *(R)-p*-cAMPS, 10^{-5} M H-89 and 10^{-6} M KT5720 (Table 2). Ten-second trains of electrical field stimulation (40 V, 0.1 ms, 4 Hz) in the presence of atropine and guanethidine induced frequency-dependent short-lasting relaxations (Fig. 3b) as previously described (Lefebvre et al., 1995). These relaxations were clearly reduced by 10^{-4} L-NA but were not influenced by 10^{-6} M 1400W (Table 2), illustrating that the latter agent has no influence on nNOS. None of the tested agents (L-NA, 1400W, ODQ, (*R*)-*p*-cAMPS, H-89, KT5720 and SQ22536) had a systematic influence on the tone of the muscle strips.

4. Discussion

The aim of the present study was to investigate the interaction between NO and VIP in smooth muscle cells and smooth muscle strips of the pig gastric fundus. Similarly to what we recently observed in the guinea-pig gastric fundus (Dick et al., 2000), the relaxant effect of VIP was antagonized by the non-specific NOS inhibitor L-NA, the selective iNOS inhibitor 1400W and the selective guanylate cyclase inhibitor ODQ in smooth muscle cells but not in smooth muscle strips. The non-influence of NOS inhibitors on the relaxant effect of VIP in the strips corresponds to data obtained in gastric fundus strips of the rat (Boeckxstaens et al., 1992), cat (Barbier and Lefebvre, 1993) and dog (Bayguinov et al., 1999), suggesting that NO and VIP induce gastric fundus relaxation via parallel pathways. However, the observations in the cells suggest that the relaxation induced by VIP is at least partially due

to muscular generation of NO activating cytosolic guanylyl cyclase. This shows that the importance of the experimental procedure on the influence of NOS inhibitors versus VIP is also valid for non-rodent mammalian material. The effect of 1400W in the cells suggests that iNOS is involved in the relaxation of VIP. The partial reversal of the effect of 1400W by L-arginine, in contrast to that of L-NA, might be related to the very tight binding of 1400W to iNOS (Garvey et al., 1997). A possible explanation for the involvement of iNOS in the relaxant effect of VIP in the cells is the induction of iNOS in response to the stress of the dissociation procedure. While iNOS is usually induced by treating tissues with bacterial endotoxin and cytokines (Stuehr and Marletta, 1987; Nathan, 1992; Liu et al., 1993), the induction of iNOS can indeed also result from stress in response to ischemia–reperfusion (Iadecola et al., 1996; Imagawa et al., 1999; Jones et al., 1999). The 3 h of the dissociation procedure in our study are sufficient to induce iNOS in smooth muscle cells (Radomski et al., 1990; Chen et al., 1996; Liu et al., 1997). The involvement of iNOS in the relaxant effect of VIP in the cells is corroborated by the influence of the glucocorticoid dexamethasone versus VIP, as dexamethasone was reported to suppress iNOS gene expression (Di Rosa et al., 1990; Radomski et al., 1990; Imai et al., 1994) but also to lower iNOS mRNA translation and to enhance iNOS protein degradation (Walker et al., 1996), which might explain its effect with an incubation time of only 30 min.

VIP is generally reported to induce smooth muscle relaxation through activation of adenylate cyclase, increase of cAMP and subsequent activation of protein kinase A (Bolton, 1979). In the isolated smooth muscle cells, the protein kinase inhibitors (*R*)-*p*-cAMPS, KT5720 and H-89 (Kase et al., 1987; Botelho et al., 1988; Engh et al., 1996) equally inhibited the relaxant effect of VIP. This corresponds to observations with protein kinase A inhibitors in isolated smooth muscle cells of other gastrointestinal tissues (Rekik et al., 1996) and suggests that VIP activates the iNOS-NO-cGMP pathway in the cells via cAMP and protein kinase A. In isolated smooth muscle cells of the guinea-pig gastric fundus, we have shown that the relaxant effect of VIP and also of two other adenylate cyclase-activating agents, forskolin and isoprenaline, is clearly inhibited by (*R*)-*p*-cAMPS, the NOS inhibitors L-NA and 1400W, and ODQ (Dick et al., 2000).

Although NO is not involved in the effect of VIP in the smooth muscle strips of the pig gastric fundus, one would expect that the relaxant effect is generated via cAMP and protein kinase A. However, none of the protein kinase inhibitors studied influenced the effect of VIP in the smooth muscle strips and this was also the case for the adenylate cyclase blocker SQ22536 (Fabbri et al., 1991). We therefore included forskolin, a direct activator of the catalytic subunit of adenylate cyclase (Laurenza et al., 1989). Similarly to VIP, the relaxant effect of forskolin was reduced to the same extent by the protein kinase A inhibitors in the cells but not in the smooth muscle strips. Possibly, there are diffusion problems for the protein kinase A inhibitors in the thick solid pig gastric fundus strips, although H-89 and (*R*)-*p*-cAMPS have been shown to reduce the effect of VIP in smooth muscle strips of the rat, respectively guinea-pig colon (Maggi and Giuliani, 1996; Börjesson et al., 1999). Alternatively, the VIP- and forskolin-induced relaxation in the strips is not related to cAMP. Indeed, other intracellular mechanisms that can induce smooth muscle relaxation, other than generation of cAMP and activation of protein kinase A, have been reported for VIP such as a decrease of the inositol 1,4,5-trisphosphate concentration (Murray, 1990; Szewczak et al., 1990), and the relaxation by VIP in strips of the pig gastric fundus is not accompanied by an increase in cAMP (Lefebvre et al., 1995). The possibility that forskolin mediates relaxation by activation of cyclic GMP-dependent protein kinase has been reported by Lincoln et al. (1989). If one of these mechanisms would mediate the relaxant effect of VIP and forskolin in the pig gastric fundus strips, it is difficult to understand why the cAMP-protein kinase A pathway shows up in the isolated smooth muscle cells.

In conclusion, in smooth muscle strips of the pig gastric fundus, the relaxation by VIP is not affected by NOS-inhibitors, suggesting a parallel link between NO and VIP. In contrast, in isolated smooth muscle cells of the pig gastric fundus, an iNOS-selective NOS inhibitor inhibits the VIP-induced relaxation, suggesting a serial cascade model in which the muscular synthesis of NO via iNOS occurs upon stimulation of the cells by VIP. As iNOS might be induced by the procedure to prepare the isolated smooth muscle cells, these results illustrate that the experimental procedure determines the influence of NOS inhibitors on the relaxant effect of VIP in pig gastric muscle.

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