British Journal of Pharmacology (2001) 134, 425–433

www.nature.com/bjp

Antisense knockdown of inducible nitric oxide synthase inhibits the relaxant effect of VIP in isolated smooth muscle cells of the mouse gastric fundus

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1 Our previous results showed that the non-selective nitric oxide synthase (NOS) inhibitor L-N^G-nitroarginine (L-NOARG) and the selective inducible NOS (iNOS) inhibitor N-(3-(acetaminomethyl)-benzyl)acetamidine (1400W) inhibited the relaxant effect of vasoactive intestinal polypeptide (VIP) in isolated smooth muscle cells of the mouse gastric fundus, suggesting the involvement of iNOS. The identity of the NOS isoform involved in the VIP-induced relaxation in isolated smooth muscle cells of the mouse gastric fundus was now further investigated by use of antisense oligodeoxynucleotides (aODNs) to iNOS.

2 Incubation of isolated smooth muscle cells with fluorescein isothiocyanate (FITC)-labelled aODNs showed that nuclear accumulation occurs quickly and reaches saturation after 60 min. The *in vivo* intravenous administration of aODNs to iNOS, 24 and 12 h before murine tumour necrosis factor alpha (mTNFα) challenge, significantly reduced the nitrite levels induced by the mTNFα challenge.

3 Intravenous administration of aODNs to iNOS in mice, 24 and 12 h before isolation of the gastric smooth muscle cells, decreased the inhibitory effect of the NOS inhibitors L-NOARG and 1400W on the relaxant effect of VIP, whereas neither saline nor sODNs had any influence.

4 Preincubation of the isolated smooth muscle cells with aODNs almost abolished the inhibitory effect of L-NOARG and 1400W on the VIP-induced relaxation, whereas sODNs failed.

5 These results illustrate that the inhibitory effect of NOS inhibitors in isolated smooth muscle cells of the mouse gastric fundus is due to inactivation of iNOS. iNOS, probably induced by the isolation procedure of the smooth muscle cells, seems involved in the relaxant effect of VIP in isolated gastric smooth muscle cells.

British Journal of Pharmacology (2001) **134,** 425–433

Keywords: Mice; gastric fundus; nitric oxide; vasoactive intestinal polypeptide; antisense oligodeoxynucleotide; inducible nitric oxide synthase

Abbreviations: aODNs, antisense oligodeoxynucleotides; DAPI, 4′,6-diamino-2-phenylindole; D-ARG, D-arginine; eNOS, endothelial nitric oxide synthase; FITC, fluorescein isothiocyanate; iNOS, inducible nitric oxide synthase; i.v., intravenous; L-ARG, L-arginine; L-NOARG, L-N^G-nitroarginine; mTNFα, murine tumour necrosis factor alpha; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; s.e.mean, standard error of the mean; sODNs, sense oligodeoxynucleotides; VIP, vasoactive intestinal polypeptide; 1400W, N-(3-(acetaminomethyl)-benzyl)acetamidine

Introduction

Several lines of evidence favour an important role for nitric oxide (NO) and vasoactive intestinal polypeptide (VIP) as inhibitory non adrenergic non cholinergic neurotransmitters in the regulation of gastrointestinal motility (Brookes, 1993; Lefebvre, 1993; Shuttleworth & Keef, 1995). However, the mode of interaction between NO and VIP remains controversial. Most investigators believe that NO and VIP are released in parallel. Indeed, immunofluorescence studies revealed colocalization of VIP and neuronal NO synthase (nNOS) in many neurons of the myenteric plexus from different gastrointestinal tissues (see e.g. Lefebvre *et al*., 1995). NO, which is generated from L-arginine by the action of NO synthases, activates hereby soluble guanylyl cyclase and induces smooth muscle relaxation *via* elevation of guanosine 3′5′ cyclic monophosphate (cyclic GMP; Moncada *et al*., 1991). VIP acts *via* activation of the adenylyl cyclase/adenosine 3′5′ cyclic monophosphate (cyclic AMP) signal transduction pathway (Bitar & Makhlouf, 1982b). The notion of this parallel mode of action between NO and VIP is supported by the observation that the relaxation by VIP is not blocked by NOS inhibitors in smooth muscle strips from different gastrointestinal tissues such as the opossum lower esophageal sphincter (Tøttrup *et al*., 1991), rat gastric fundus (Boeckxstaens *et al*., 1992), cat gastric fundus (Barbier & Lefebvre, 1993), pig gastric fundus (Lefebvre *et al*., 1995) and human gastric fundus (Tonini *et al*., 2000).

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In contrast, Makhlouf and colleagues propose a sequential model, whereby VIP is the predominant enteric inhibitory neurotransmitter and NO is largely produced in post-junctional muscle cells in response to VIP stimulation (Grider *et al*., 1992; Grider, 1993; Jin *et al*., 1993). Indeed, studies in isolated smooth muscle strips and smooth muscle cells of the guinea-pig gastric fundus and rat colon, demonstrated that NOS inhibitors were able to inhibit the relaxant effect of exogenous VIP, and that VIP induced NO release as assessed by measuring ^{3}H-L-citrulline after loading the strips or cells with ^{3}H-L-arginine. Teng *et al*. (1998) reported, having used reverse transcription-polymerase chain reaction and Southern Blot Analysis, that eNOS would be responsible for the muscular production of NO in response to VIP in rabbit gastric and human intestinal smooth muscle cells, although Western and immunoblot analysis of these tissues did not reveal the presence of eNOS. Correspondingly, Börjesson *et al*. (1999) reported that the relaxant effect of VIP in segments of the rat distal colon was partially reduced by both the protein kinase A inhibitor H-89 and the guanylate cyclase inhibitor ODQ. Still, careful analysis of a possible sequential link between VIP and NO in canine gastric fundus and colon was negative (Keef *et al*., 1994; Bayguinov *et al*., 1999).

Recent results obtained in our laboratory in the guinea-pig, pig and mouse gastric fundus demonstrate a clearcut discrepancy in the mechanism of VIP when studied in isolated smooth muscle cells versus smooth muscle strips. In the smooth muscle strips, the relaxation by exogenous VIP was not influenced by NOS inhibitors, but in isolated smooth muscle cells it clearly was (Dick *et al*., 2000; Dick & Lefebvre, 2000; Dick *et al*., submitted). The active NOS inhibitors included the selective inducible NO synthase (iNOS) inhibitor 1400W, which suggests that an NOS with properties of iNOS is involved in the relaxation induced by VIP in the isolated smooth muscle cells. Further evidence for iNOS involvement was obtained by use of iNOS and eNOS knockout mice. The inhibitory effect of the NOS inhibitors was still present in isolated smooth muscle cells from eNOS knockout mice but not in isolated smooth muscle cells from iNOS knockout mice, suggesting the involvement of iNOS, and not eNOS, in the relaxation by VIP in isolated smooth muscle cells (Dick *et al*., submitted). All these results thus suggest that the isolation procedure of isolated smooth muscle cells leads to the induction of iNOS that can be activated by VIP.

Antisense oligodeoxynucleotides (aODNs) are short nucleic acid fragments, generally 15–25 bases in length, designed to interfere with gene function through the hybridization with their specific mRNAs (Crooke, 1992, 1995; Stein & Cheng, 1993). Knockdown strategy with aODNs offers the opportunity to block the gene of interest with high selectivity. The aim of the present study was therefore to further investigate the identity of the NO synthase involved in the VIP-induced relaxation in isolated smooth muscle cells of the mouse gastric fundus by use of aODNs to iNOS.

Methods

Animals

Eight to ten week old C57BL/J6 mice from either sex (25–30 g) were purchased from Iffa Credo (les Oncins, France) and housed in a 12 h light/dark cycle in a temperature-controlled, air conditioned room with food and water *ad libitum*.

Antisense oligonucleotides

The 18-base phosphorothioated antisense oligodeoxynucleotides (aODNs) 5′-CACCTCCAACACAAGATC-3′, complementary to the bases 1 through 18 of the mouse iNOS cDNA sequence (Genbank accession number M87039) were purchased from Biognostik (Göttingen, Germany). The corresponding sense ODNs (sODNs) sequence with the base composition 5′-CCTTCGTACCCTTTTTCC-3′, was used as control ODNs.

In vivo *administration of ODNs*

Mice were randomly divided into three groups receiving 2 nmol aODNs, 2 nmol sODNs or 200 μl saline intravenously (i.v.) 24 and 12 h before the isolation procedure of the gastric smooth muscle cells. The aODNs and sODNs were dissolved in a total volume of 200 μl saline and injected with a 26 gauge needle in the vein of the mouse tail, warmed up under infrared light for a few minutes. The project was approved by the Ethics Committee for Experimental Animals of the Faculty of Medecine and Health Sciences, Ghent University.

In vitro *administration of ODNs*

After enzymatic dissociation of the smooth muscle cells (see below), 4 nmol aODNs or 4 nmol sODNs both dissolved in 40 μl saline were added to the enzyme-free medium (final concentration 1 nmol ml^{-1}) in which the cells are allowed to disperse spontaneously for 60 min.

In vitro *cellular uptake study with FITC-labelled aODNs*

Once the smooth muscle cells were completely dissociated, fluorescein isothiocyanate (FITC)-labelled aODNs were added to the medium. Samples of these cells were viewed after an interval of 0, 7.5, 22.5, 37.5, 52.5 and 67.5 min with an inverted Nikon Eclipse TE300 epifluorescence microscope using a 40 × oil-immersion lens. FITC fluorescence images were obtained by excitation at 480 nm, reflection off a dichroic mirror with a cut-off wavelength at 510 nm, and bandpass emission filtering at 535 nm. Images were captured with an intensified CCD (Extended Isis camera, Photonic Science, East Sussex, U.K.) and were stored in a PC equipped with an image acquisition and processing board (Data Translation, type DT3155, Marlboro, MA, U.S.A.). Nuclei were counterstained with 4′,6-diamino-2-phenylindole (DAPI), 0.5 μg ml^{-1} in 0.01 M PBS for 1 min. The intensity of the highest signal obtained in the nucleus of the first 8–10 randomly encountered and morphologically intact cells was measured.

Evaluation of the iNOS aODNs efficiency in vivo *by nitrite assay*

To evaluate the efficacy of the aODNs to block the expression of iNOS, mice received 24 and 12 h before a

challenge with mTNFα randomly 200 μl saline, 2 nmol aODNs or 2 nmol sODNs i.v. NOS activity in response to mTNFα was assessed by measurement of nitrite/nitrate production using the Griess reaction. Blood samples from the saline-, sODNs- or aODNs-treated mice challenged i.v. with 20 μg mTNFα were collected from the retro-orbital plexus under ether anaesthesia 3, 6 and 9 h after mTNFα challenge. The NO_x^- level in serum was determined by measuring the levels of nitrite and nitrate, following the procedure of Granger *et al.* (1991) in a slightly modified form. Thirty microliters of nitrite and nitrate standards, prepared in pooled normal murine serum, or of samples was transferred to a V- or U-shaped microtiter plate. *Pseudomonas oleovorans* bacteria were quickly thawed and diluted in TC-100 medium to a concentration of 5×10^9 Colony Forming Units (CFU) ml^{-1}. Thirty microliters of this bacterial suspension was then added to the samples and to the nitrate standard, which were incubated for at least 2 h at 37°C. Thirty microliters of TC100 medium was added to the nitrite standard. Then the plate was centrifuged at $1300 \times g$ for 5 min to remove the bacterial pellet. Forty microliters of supernatant was transferred to a second V- or U-shaped 96-well microtiter plate to which 80 μl of Griess reagent was added (Griess, 1879). After thorough mixing, 80 μl of 10% Trichloroacetic Acid (TCA) was added to every well, and the plate was centrifuged at $1300 \times g$ for 15 min to remove the protein precipitate. Finally, 120 μl of supernatant was transferred to a flat-bottom 96-well microtiter plate; absorbance was determined at 540 (test) and 620 nm (ref).

Preparation of isolated smooth muscle cells

Circular smooth muscle cells were isolated from the gastric fundus of mice by collagenase digestion as previously described (Bitar & Makhlouf, 1982a; Botella *et al.*, 1994). Briefly, 3–4 mice of either sex (25–30 g) were killed by cervical dislocation. The gastric fundus was isolated immediately and the circular muscle layer was separated from the rest of the stomach wall by careful dissection under the microscope. Small sheets from the circular muscle layer were incubated for 15 min at 31°C, in 15 ml of N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES)-buffered medium (25 mM), containing 150 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 (mM): NaCl 98, KCl 6, NaH_2PO_4 2.5, $CaCl_2$ 1.8, D(+)-glucose 11.5, bovine serum albumin 0.2% (w v^{-1}) and was supplemented with (mM): sodium pyruvate 5, sodium fumarate 5, sodium glutamate 5, glutamin 2, amino acid mixture, 1% (v v^{-1}); vitamin mixture, 1% (v v^{-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 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 60 min. Finally the spontaneously dissociated muscle cells were harvested by filtration and used for functional measurements.

Viability tests by exclusion of Trypan blue (Collins & Gardner, 1982) showed that 86.6±1.2% (mean±s.e.mean, $n=6$) of the cells in suspension obtained from control mice 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 25 or 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 two times 25 or 50 cells were measured. The averaged value accounted for $n=1$ from one animal. 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.

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

Untreated cells served as controls. Cells were contracted by incubation with 10 nM 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 (1 nM) or pinacidil (10 μM) 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 study. To study the mechanism of relaxation, the cells were incubated before addition of relaxant agents with the NOS inhibitors L-N^G-nitroarginine (L-NOARG, 100 μM; incubation time 5 min) and N-(3-(aminomethyl)-benzyl)acetamidine (1400W, 1 μM, 5 min), with or without L-arginine (100 μM, 5 min) or D-arginine (100 μM, 5 min) and glibenclamide (100 μM, 5 min). In parallel control vials, the cells were incubated with the solvent of these agents. The influence of pinacidil, that relaxes smooth muscle by opening of ATP-sensitive K^+-channels (Richer *et al.*, 1990), and the blocker of ATP-sensitive K^+-channels glibenclamide (Schmid-Antomarchi *et al.*, 1987) was studied to evaluate possible non-specific effects of the treatment with aODNs on relaxant agents and their antagonists.

Data analysis

The measured fluorescence intensity of the cells was expressed as the percentage of the highest fluorescence intensity obtained among all the viewed cells taken as 100%. The contraction of the isolated smooth muscle cells was expressed as the percentage decrease in cell length from untreated controls, using the following formula: $((L_0\text{-}L_x)\ L_0^{-1}) \times 100$ 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.

Results are given as means±s.e.mean and n refers to material from different animals. Responses in parallel vials with isolated smooth muscle cells and nitrite levels from mice treated with aODNs versus those from mice treated with saline or sODNs were compared by analysis of variance (ANOVA) and the t-test corrected for multiple comparisons

(Bonferroni procedure). The level of significance was set at $P<0.05$.

Chemicals

Collagenase was purchased from Worthington Biochemical Corporation (Freehold, NJ, U.S.A.) and carbamoylcholine chloride (carbachol) from Fluka (Switzerland). N-(3-(aminomethyl)-benzyl)acetamidine (1400W) was obtained from Alexis Corporation (Nottingham, U.K.) and essential amino acid mixture from ICN (Costa Mesa, U.S.A.). D-arginine hydrochloride, L-arginine hydrochloride, glutamin, glutaraldehyde, Griess reagent, L-N^G-nitroarginine (L-NOARG), penicillin G, sodium fumarate, sodium glutamate, sodium pyruvate, streptomycin, Trypan blue, vasoactive intestinal polypeptide (VIP) and vitamin mixture were from Sigma Chemicals (St. Louis, MO, U.S.A.). N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES) and soybean trypsin inhibitor were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.). Merck (Darmstadt, Germany) provided trichloroacetic acid (TCA) and Gibco BRL (Paisley, U.K.) TC-medium. Recombinant murine tumour necrosis factor alpha (mTNFα) was produced and purified at the Department of Molecular Biology. It has a specific activity of 1.9×10^{-8} IU mg^{-1} and contained less than 10 U of endotoxin mg^{-1} protein, as assessed by a chromogenic substrate test (Chromogenix, Stockholm; Sweden). Pinacidil monohydrate was from Leo Pharmaceuticals (Ballerup, Denmark) and glibenclamide from Hoechst (Brussels, Belgium). Antisense oligodeoxynucleotide (aODNs), sense ODNs (sODNs) and fluorescein isothiocyanate (FITC) labelled aODNs directed to iNOS have been designed and manufactered by Biognostik (Göttingen, Germany).

All drugs were dissolved in deionized water, except for pinacidil which was dissolved in pure ethanol and glibenclamide in DMSO (dimethylsulphoxide). Further dilutions were made in physiological salt solution. The solvents, diluted in physiological salt solution until the final concentration given to the cells had no effect *per se* on control isolated smooth muscle cells. For dissolving L-NOARG, powerful vortexing during at least 5 min was required. Stock solutions of 1400W up to 10 mM and VIP up to 100 μM were prepared in deionized water and stored at −20°C. ODNs were dissolved up to 1 nmol 10 μl^{-1} in saline and stored at −20°C. All other solutions were prepared on the day of the experiment.

Results

Control experiments

In vitro *uptake study with FITC-labelled aODNs* To investigate the intracellular incorporation of ODNs, isolated smooth muscle cells were exposed to fluorescein isothiocyanate (FITC)-labelled aODNs and the fluorescence intensity after 0, 7.5, 22.5, 37.5, 52.5, 67.5 min was measured. The nuclei of the circular smooth muscle cells, as confirmed by counterstaining with DAPI, became brightly fluorescent. The nuclear signal was much more intense than the nearly homogeneous, cytoplasmatic fluorescence. The cellular uptake of the FITC-labelled aODNs was visible from 7.5 min after the administration and reached a plateau after 60 min incubation time (Figure 1a).

Efficacy of iNOS aODNs in vivo The effects of *in vivo* administration of aODNs, targeted to iNOS mRNA, and the corresponding sODN were examined on mTNFα-induced NO production. A time dependent increase of the serum nitrite/nitrate level was observed after i.v. challenge with mTNFα in mice treated with saline. When animals were treated *in vivo* with aODNs before mTNFα challenge, the nitrite/nitrate level was significantly decreased at 6 and 9 h after mTNFα challenge, but this was not the case in animals treated with sODNs (Figure 1b).

Isolated smooth muscle cells

In vivo *effect of aODNs* Untreated control cells, obtained after dispersion of the circular muscle layer of the gastric fundus of mice treated with saline, had a mean cell length of 118.7±2.4 μm. Carbachol (10 nM), incubated for 30 s,

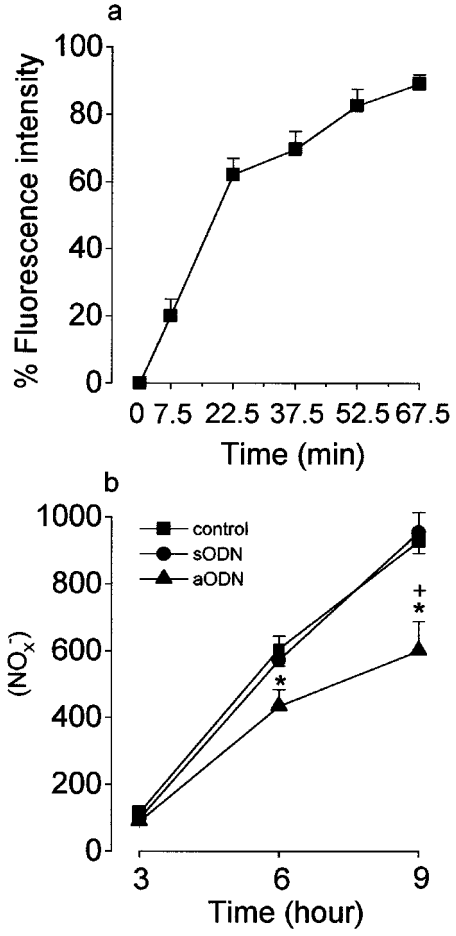

Figure 1 (a) Nuclear fluorescence intensity of isolated smooth muscle cells measured by epifluorescence microscopy at 0, 7.5, 22.5, 37.5, 52.5 and 67.5 min after addition of FITC labelled aODNs. Values are mean±s.e.mean from 8–10 different cells. (b) Concentration of nitrite, after reduction of nitrate to nitrite, detected in serum of mice treated with saline, sODNs or aODNs, 3, 6 and 9 h after mTNFα challenge. Values are mean±s.e.mean from $n=3-6$. *$P<0.05$ significantly different from mice treated with saline, +$P<0.05$ significantly different from mice treated with sODNs.

produced 23.1±0.9% shortening of the cells to 91.4±2.7 μm. When cells were preincubated for 60 s with 1 nM VIP, the contraction was inhibited and full relaxation was obtained (Table 1). The values for the cell length, the per cent contraction by carbachol and the per cent relaxation by VIP in smooth muscle cells obtained from mice treated with aODNs or sODNs were not significantly different from those in cells of saline-treated animals (data not shown).

The relaxant effect of VIP in gastric smooth muscle cells obtained from saline- and sODNs-treated animals was inhibited by the non selective NOS inhibitor L-NOARG by 79.7±10.9%, respectively 74.2±16.1% (Figure 2). The inhibitory effect of L-NOARG was reversed by 100 μM L-arginine, but not by 100 μM D-arginine. When the cells obtained from saline and sODNs-treated animals were incubated with the iNOS selective inhibitor 1400W, the relaxant effect of VIP was inhibited by 80.5±9.4%, respectively 77.5±11.4% (Figure 2). The inhibitory effect of 1400W on VIP-induced relaxation was not reversed by L-arginine. In gastric smooth muscle cells of the mice treated with aODNs, the relaxant effect of VIP seemed partially inhibited by L-NOARG (49.1±11.0%) and by 1400W (38.5±11.0%) but this effect was not significant (Figure 2). None of the NOS inhibitors altered *per se* the mean cell length of the circular smooth muscle cells obtained from saline-, sODNs- or aODNs-treated animals, nor the degree of the carbachol-induced contraction ($n=4$; data not shown).

In vitro *effect of aODNs* Incubation of the partially digested tissues with sODNs or aODNs revealed no difference in cell length, per cent contraction by carbachol or per cent relaxation by VIP of the isolated smooth muscle cells in comparison to untreated isolated smooth muscle cells (data not shown). When untreated cells or cells treated with sODNs were incubated with 100 μM L-NOARG, the relaxant effect of VIP was significantly inhibited to the same extent (Figure 3). This inhibitory effect of L-NOARG was reversed by 100 μM L-arginine, but not by 100 μM D-arginine ($n=6$; data not shown). Preincubation of the untreated cells or cells treated with sODNs with 1 μM 1400W significantly decreased the relaxant effect of VIP (Figure 3). This inhibitory effect of 1400W on the VIP-induced relaxation was not reversed by 100 μM L-arginine ($n=6$; data not shown). Contrary to these results obtained in untreated cells and cells treated with sODNs, the relaxant effect of VIP in cells treated with aODNs was not significantly inhibited by the NOS inhibitors L-NOARG and 1400W (Figure 3).

The K^+ channel opener pinacidil elicited full relaxation in dispersed smooth muscle cells at a concentration of 10 μM. Both in untreated cells and cells treated with aODNs, 100 μM glibenclamide significantly inhibited the pinacidil-induced relaxation by 87.5±12.6%, respectively 98.4±1.3% (Figure 3).

L-NOARG, 1400W and glibenclamide did not alter the mean cell length or the degree of the carbachol-induced contraction in dispersed circular smooth muscle cells ($n=4$; data not shown).

Discussion

Contrary to the hypothesis of Teng *et al*. (1998) that an eNOS is involved in the relaxant effect of VIP in gastric smooth muscle, our previous results suggested that iNOS, probably induced by the isolation procedure, is responsible for the relaxation by VIP in isolated smooth muscle cells of guinea-pig, pig and mouse gastric fundus (Dick *et al*., 2000; Dick & Lefebvre, 2000; Dick *et al*., submitted). The aim of the present study was therefore to confirm the identity of the NOS isoform involved in the relaxation induced by VIP in smooth muscle cells isolated from the mouse gastric fundus by use of aODNs to iNOS.

To evaluate uptake and distribution of the phosphorothioate ODNs *in vitro*, FITC-labelled aODNs were incubated with freshly isolated smooth muscle cells and the cellular penetration was visualized by fluorescence microscopy. From the beginning of the incubation, FITC-labelled aODNs penetrated into the nuclei of the cells and penetration reached saturation after 67.5 min incubation. Most authors report that aODNs enter cells in culture *via* receptor mediated endocytosis or fluid phase pinocytosis (Loke *et al*., 1989; Bennett *et al.,* 1993) and, in some cases, might become sequestered in intracellular compartments such as lysosomes and endosomes and never gain access to target mRNA (Wagner, 1994). With regard to facilitating the penetration of the ODNs in vascular smooth muscle cell cultures, most authors add transfection reagent to the medium (Itoh *et al*., 1993; Busuttil *et al*., 1996; Marrero *et al*., 1998). However, it is known that cell permeabilization and intracellular delivery of oligonucleotides facilitates the uptake of the aODNs into the nucleus (Wagner, 1994; Lesh *et al*., 1995). Damage inflicted to smooth muscle cells might allow more rapid uptake of aODNs and affect tissue and cellular distribution of oligonucleotides, as Farrell *et al*. (1995) demonstrated that oligonucleotides penetrate more easily into the arterial wall of balloon-injured arteries than in tissue from normal arteries. In our study, enzymatic digestion with collagenase could permeabilize the cell membrane, which

Table 1 Effect of L-NOARG on VIP-induced relaxation in isolated gastric smooth muscle cells obtained from mice treated with saline *in vivo*

	Cell length (μm)	*Contraction* (% decrease in cell length)	*Relaxation* (% inhibition of carbachol-induced contraction)	*Inhibition* (% inhibition of VIP-induced relaxation)
Controls	118.2±2.4			
Carbachol (10 nM)	91.4±2.7***	23.1±0.9		
Carbachol (10 nM)+VIP (1 nM)	127.0±1.5	−7.2±2.8	129.5±10.2	
Carbachol (10 nM)+VIP (1 nM) +L-NOARG (100 μM)	98.3±4.6***	17.2±3.4	27.4±14.5	79.7±10.9

Values are mean±s.e.mean from $n=6$; ***$P<0.001$, significantly different from control cells.

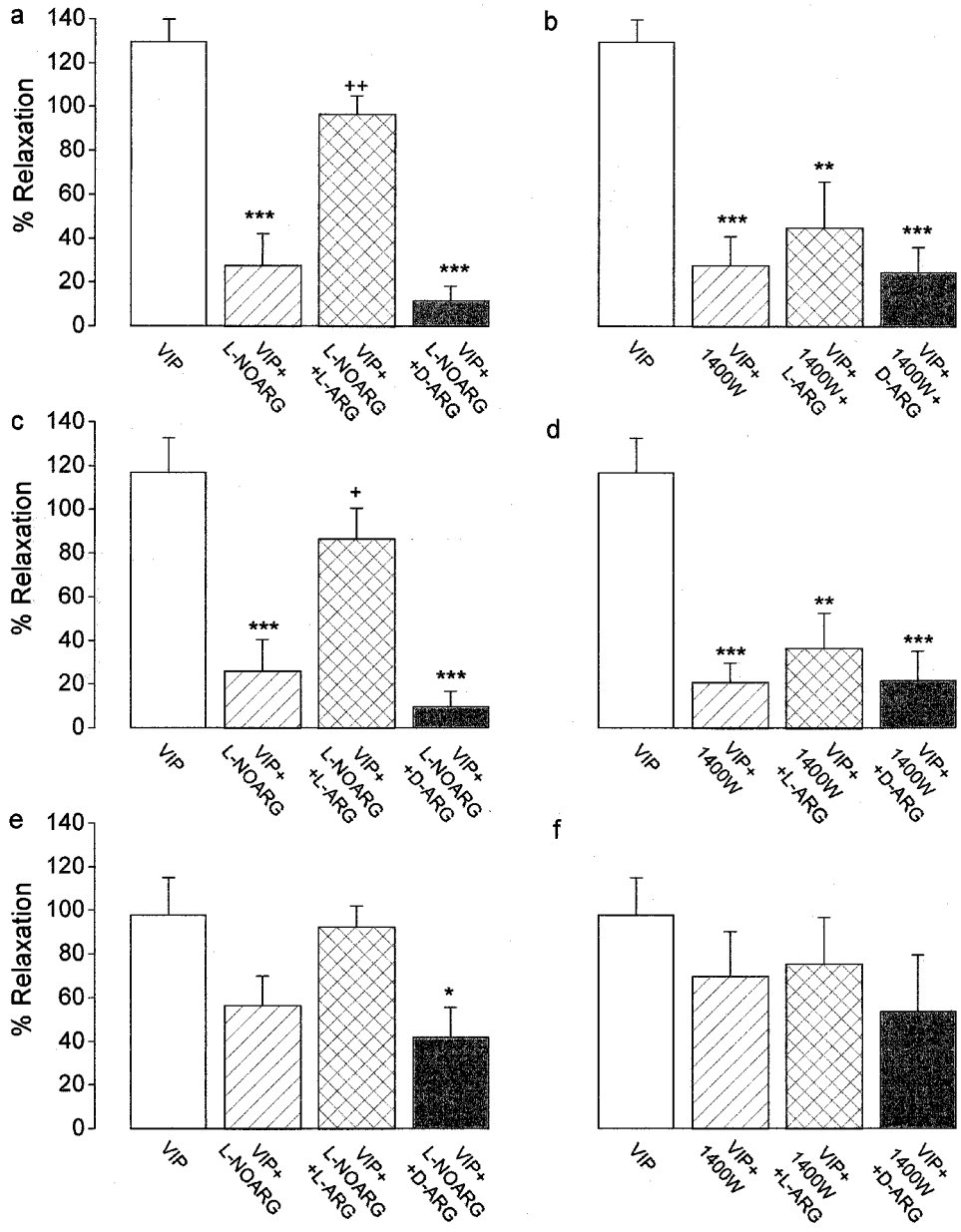

Figure 2 Effect of L-NOARG (100 μM, a,c,e) and 1400W (1 μM, b,d,f) with or without L-arginine (L-ARG; 100 μM) and D-arginine (D-ARG, 100 μM) on VIP-induced relaxation in isolated gastric smooth muscle cells obtained from mice treated i.v. with saline (a,b), sODNs (c,d) or aODNs (e,f). Values are mean$\pm$s.e.mean from $n=6$. ***$P<0.001$, **$P<0.01$, *$P<0.05$ significantly different from cells treated with VIP, ++$P<0.01$, +$P<0.05$, significantly different from cells treated with VIP and NOS inhibitor.

allows facilitated uptake of the aODNs in the isolated smooth muscle cells *in vitro*, no transfection reagent seemed required. Our observation regarding nuclear accumulation of aODNs in the smooth muscle cells is significant in that, at least in *in vitro* systems, nuclear accumulation generally correlates with positive antisense oligonucleotide activity (Bennett *et al*., 1992).

The efficacy of *in vivo* administration of aODNs to iNOS mRNA has been reported. Hoque *et al*. (1998) indeed demonstrated the ability of i.v. administration of aODNs to iNOS mRNA to significantly inhibit the LPS-induced increase in NOS activity and iNOS protein expression in rat. To evaluate *in vivo* the efficacy of the aODNs to block the expression of iNOS in mice challenged with mTNFα in our study, nitrite levels were measured in the serum of mice treated with aODNs, sODNs or saline. The aODNs treatment significantly reduced the nitrite/nitrate levels in serum compared with control groups. This was not mimicked by sODNs showing that i.v. administration of aODNs to iNOS mRNA inhibits iNOS expression in mice by an antisense mechanism of action.

To identify the NOS isoform involved in the relaxant effect of VIP, the effect of the non-selective NOS inhibitor L-NOARG and the selective iNOS inhibitor 1400W was investigated on the relaxation by VIP in isolated smooth muscle cells obtained from mice treated intravenously with aODNs, sODNs or saline. As it was already observed in the guinea-pig, the pig and the mouse gastric fundus (Dick *et al*.,

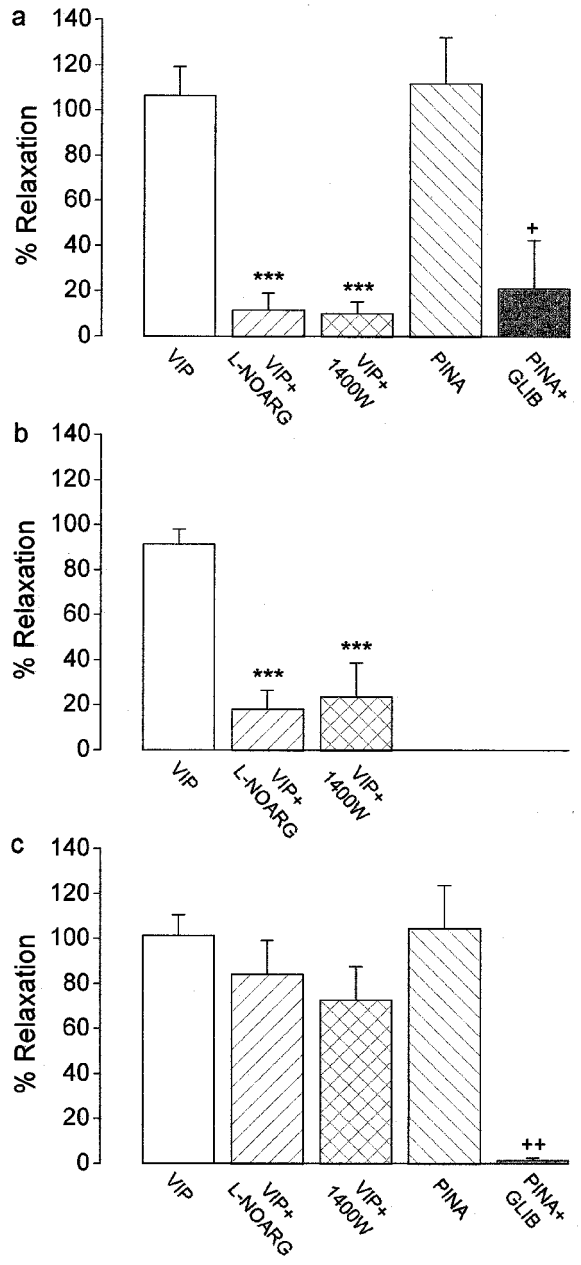

Figure 3 Effect of L-NOARG (100 μM) and 1400W (1 μM) on VIP-induced relaxation and of glibenclamide (GLIB; 100 μM) on pinacidil (PINA; 10 μM)-induced relaxation in mouse isolated gastric smooth muscle cells untreated (a), or treated with sODNs (b) or aODNs (c) *in vitro*. Values are mean$\pm$s.e.mean from $n=4-6$. ***$P<0.001$ significantly different from cells treated with VIP, ++$P<0.01$, +$P<0.05$, significantly different from cells treated with pinacidil.

2000; Dick & Lefebvre, 2000; Dick *et al*., submitted), L-NOARG and 1400W inhibited significantly the relaxant effect of VIP in isolated smooth muscle cells obtained from mice treated with saline. The inhibitory effect of L-NOARG was reversed by L-arginine but not by D-arginine, thereby indicating the specificity of the inhibitory effect. The inhibitory effect of 1400W was not reversed by L-arginine confirming data obtained in isolated smooth muscle cells of the guinea-pig, pig and mouse gastric fundus (Dick *et al*., 2000; Dick & Lefebvre, 2000; Dick *et al*., submitted). This might be related to the very tight binding of 1400W to iNOS (Garvey *et al*., 1997). L-NOARG and 1400W did not significantly reduce the VIP-induced relaxation in cells obtained from mice treated with aODNs. The effects of the aODNs was sequence specific, as sODNs failed to reduce the inhibitory effect of the NOS inhibitors on the relaxant effect of VIP. To confirm our results obtained *in vivo*, we also studied the influence of *in vitro* administration of the aODNs. Incubation of the cells with aODNs, almost abolished the effect of the NOS inhibitors on the VIP-induced relaxation, but this effect could not be perceived in untreated cells or cells incubated with sODNs. Glibenclamide is known to inhibit the relaxant effect of the potassium channel opener pinacidil in the rat gastric fundus (Lefebvre & Horacek, 1992). In both untreated cells and cells incubated with aODNs, glibenclamide blocked the relaxation by pinacidil, confirming the specificity of the non-effect of NOS inhibitors on the relaxant effect of VIP in cells incubated with aODNs. The decreased inhibitory effect of L-NOARG and 1400W on the relaxation by VIP in smooth muscle cells, both after *in vivo* and *in vitro* treatment with aODNs to iNOS mRNA, corroborates that iNOS is involved in the relaxation by VIP in isolated smooth muscle cells.

The influence of the aODNs on the inhibitory effect of the NOS inhibitors versus the VIP-induced relaxation tended to be more pronounced in the *in vitro* study compared to the *in vivo* study, although this did not reach statistical significance. Oligonucleotide uptake and distribution in isolated smooth muscle cells *in vitro* are quite different from uptake into cells *in vivo*. Patterns of oligonucleotide distribution to the target tissues *in vivo* might contribute to this difference, as the majority is distributed to the kidney and liver before becoming available to other tissues (Agrawal *et al*., 1991; Cossum *et al*., 1993). Degradation in serum seems unlikely as the phosphorothioated aODNs used in this study have a sulphur atom replacing one of the non bridging oxygen atoms at each interbase phosphorus, creating a phosphorothioate linkage that is nuclease resistant (Tsuneyoshi *et al*., 1996).

As discussed before (Dick *et al*., 2000), the involvement of iNOS in the relaxant effect of VIP in the isolated gastric smooth muscle cells might be related to the induction of iNOS in response to the stress of the dissociation procedure. Induction of iNOS can indeed also result from stress in response to ischaemia-reperfusion (Iadecola *et al*., 1996; Imagawa *et al*., 1999; Jones *et al*., 1999). The whole procedure of cell dissociation takes about 2 h. This seems sufficient for the expression of iNOS as Lui *et al*. (1997) demonstrated a threshold time point for iNOS mRNA induction between 20 and 40 min after lipopolysaccharide administration in rat vascular preparations, and iNOS activity was measurable in vascular endothelial cells activated with Interferon-γ and LPS after a lag period of 2 h (Radomski *et al*., 1990).

In vivo administration of aODNs or incubation of the isolated smooth muscle cells with aODNs *in vitro* did not affect the relaxant effect of VIP. Apparently, when the expression of the overwhelming iNOS-NO pathway is prevented, the classic mechanism of relaxation by VIP *via* cyclic AMP-protein kinase A remains active. Similar results were obtained in isolated smooth muscle cells of iNOS knockout mice where the relaxant effect of VIP was

maintained but it was no longer influenced by NOS inhibitors (Dick *et al*., submitted).

In conclusion, in isolated gastric smooth muscle cells from mice treated with aODNs and in isolated smooth muscle cells incubated with aODNs, the inhibitory effect of the NOS inhibitors L-NOARG and 1400W versus the relaxant effect of VIP was significantly decreased. These results illustrate that the inhibitory effect of the NOS inhibitors on the relaxant effect of VIP in isolated smooth muscle cells of the mouse gastric fundus is due to inactivation of iNOS. iNOS, probably induced by the isolation procedure of the gastric smooth muscle cells, seems involved in the relaxant effect of VIP in isolated gastric smooth muscle cells.

This study was supported by grant No. 3G0031.96 from the Fund for Scientific Research Flanders, grant O11A1696 from the Special Investigation Fund of the Ghent University and by Interuniversity Pole of Attraction Programme P4/16 (Services to the Prime Minister – Federal Services for Scientific, Technical and Cultural Affairs). The authors thank Luc Leybaert for his help with the *in vitro* cellular uptake experiments.

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(Received February 7, 2001
Revised June 1, 2001
Accepted June 26, 2001)