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α-Trinositol: A Functional (Non-receptor) Neuropeptide Y Antagonist in Vasculature

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Abstract

Neuropeptide Y is a sympathetic co-neurotransmitter released with noradrenaline upon sympathetic nerve stimulation. This study describes the ability of a synthetic inositol phosphate, α-trinositol (D-*myo*-inositol 1,2,6-triphosphate; PP 56) to antagonize vasoconstrictor responses to neuropeptide Y in-vitro as well as in-vivo.

In human and guinea-pig isolated arteries α-trinositol potently (10 nM to 1 μM extracellular concentration) suppressed the constriction evoked by neuropeptide Y alone, the potentiation by neuropeptide Y of noradrenaline-evoked constriction, and the neuropeptide Y-induced inhibition of relaxation. Moreover, in the pithed (areflexive) rat, a non-adrenergic portion of the pressor response to preganglionic sympathetic nerve stimulation was sensitive to α-trinositol. As studied in the recently cloned human (vascular-type) Y1 receptor, the action of α-trinositol does not occur through antagonism at the neuropeptide Y recognition site nor does it induce allosteric changes of this receptor. However, we found α-trinositol to inhibit the rise in intracellular Ca^{2+} as well as inositol triphosphate concentrations induced by neuropeptide Y.

It is, therefore, proposed that α-trinositol represents a non-receptor, but yet selective antagonist of neuropeptide Y in vasculature, opening up the possibility to investigate involvement of neuropeptide Y in sympathetic blood pressure control and in cardiovascular disorders.

It has been proposed that the putative sympathetic co-transmitter, neuropeptide Y may co-operate with the other neurotransmitters, noradrenaline and adenosine triphosphate (ATP) to stimulate vascular smooth muscle and certain other sympathetic effector systems. The support for this, however, is pharmacologically indirect and has been based on the presence in and release from sympathetic nerves of neuropeptide Y, the approximate mimicry of exogenous neuropeptide Y of certain components of responses to sympathetic nerve stimulation; the disappearance of such components following desensitization procedures, and the demonstration of certain adrenoceptor-independent sympathetic responses (Edvinsson et al 1987; Lundberg et al 1990; Wahlestedt et al 1990a). Thus, biologically useful antagonists of neuropeptide Y have not been available for studies on the vasculature, limiting our understanding of the role of this peptide in sympathetic vasoconstrictor co-transmission and its possible involvement in cardiovascular disorders. In a recent in-vitro study (Edvinsson et al 1990), we found that a novel inositol phosphate, α-trinositol (d-*myo*-inositol 1,2,6-triphosphate) antagonized neuropeptide Y-evoked vasoconstriction of the guinea-pig basilar artery without affecting the primary sympathetic neurotransmitter, noradrenaline, nor a range of other vasoconstrictors or vasodilators. α-Trinositol, whose chemical structure is shown in Fig. 1, is an isomer of the frequently studied intracellular second messenger, Ins[1,4,5]P_3 (IP_3), which is generated by agonist-stimulated hydrolysis of phosphatidylinositol 4,5-biphosphate by phosphoinositidase C, and whose ability to mobilize Ca^{2+} from intracellular, non-mitochondrial stores is well-documented. In addition, there appears to exist an array of other inositol phosphates, the levels of many of which are sensitive to agonist stimulation (Berridge & Irvine 1989; Shears 1989; Majerus et al 1990; Stephens & Irvine 1990). α-Trinositol is a synthetic inositol phosphate and its natural occurrence has not been demonstrated. The present report demonstrates

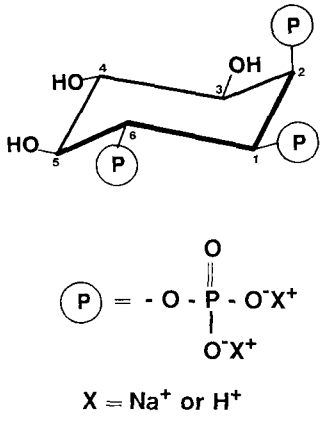

FIG. 1. Chemical structure of D-*myo*-inositol 1,2,6 triphosphate (α-trinositol). The penta sodium hydrogen salt of α-trinositol was used in the present study.

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that α-trinositol is indeed a useful neuropeptide Y antagonist in-vitro as well as in-vivo, and investigates the mode of action of the novel inositol phosphate.

Materials and Methods

Inositol phosphate preparation

D-*myo*-Inositol[1,2,6]triphosphate (α-trinositol) (Perstorp Pharma AB, Lund, Sweden) is a new chemical entity which is being developed for therapeutic use and is currently in Phase II clinical trials. Its chemical structure is shown in Fig. 1.

Vasomotor studies

Patients. After obtaining informed consent from the Human Ethics Committee of Lund University, arterial specimens were obtained from patients undergoing surgery for non-vascular reasons. Anaesthesia included a combination of thiopentone, nitrous oxide, fentanyl and a muscle relaxant. Subcutaneous arteries were dissected out from the lower abdominal region and immersed in a cold oxygenated buffer solution at the beginning of the operation.

Animals. Young guinea-pigs, 200–250 g, of either sex were decapitated under pentobarbitone anaesthesia, and uterine and basilar arteries were dissected free under an operation microscope.

The vessels were cut into cylindrical segments (2–3 mm long) with intact functional endothelium and were used for experimentation immediately. Each segment was mounted on two L-shaped metal prongs, one of which was connected to a force displacement transducer (FT03C; Grass Inc., Quincy, MA, USA) attached to a Grass polygraph (Grass Inc.) for continuous recording of the isometric tension and the other to a displacement device. The position of the other holder could be changed by means of a movable unit allowing fine adjustments of the vascular tension by varying the distance between the metal prongs. The mounted specimens were immersed in temperature-controlled (37°C) tissue baths containing a buffer solution of the following composition (mM): NaCl, 119; $NaHCO_3$, 15; KCl, 4·6; $MgCl_2$, 1·2; NaH_2PO_4, 1·2; $CaCl_2$, 1·5 and glucose 11. The solution was continuously gassed with 5% CO_2 in O_2 giving a pH of 7·4. A tension of 4 mN was applied to the arterial segments and they were allowed to stabilize at this level for 1·5 h. The contractile capacity of the vessel segments was examined by exposure to a potassium-rich (60 mM) buffer solution which had the same composition as the standard buffer solution except that some of the NaCl was exchanged for an equimolar concentration of KCl. Only after two reproducible contractions had been achieved were the vessels used for further studies (variation less than 10%).

The contractile effects of neuropeptide Y or noradrenaline (Sigma; USA) were tested upon cumulative applications of the agonists. α-Trinositol was administered 20 min before the agonist. In potentiation experiments, neuropeptide Y (10^{-8} mol) was given 3 min before noradrenaline cumulatively.

In some experiments on the guinea-pig uterine artery, precontraction was obtained by giving 3×10^{-6} mol $PGF_{2\alpha}$ (Sigma, St Louis, MO, USA). This caused a stable level of contraction during which acetylcholine (Sigma) was given in increasing concentrations resulting in relaxation. Administration of neuropeptide Y (10^{-8} mol Auspep Pty, Parkville, Australia) 3 min before acetylcholine caused maximal inhibition of the acetylcholine-induced relaxation. α-Trinositol (Perstorp Pharma, Lund, Sweden) was given 20 min before neuropeptide Y.

Isolated vas deferens

The prostatic part (approx. 10 mm) of Sprague-Dawley rat vas deferens was mounted vertically on Perspex holders in a 15 mL-tissue bath maintained at 24°C. The mechanical activity was recorded isometrically using a Grass FT03 force displacement transducer and a Grass model 7 polygraph (Grass Inc., Quincy, MA, USA). Before starting the experiments, the preparations were allowed to equilibrate for 60–90 min at a tension of about 10 mN. Electrical field stimulation with square wave pulses (0·15 Hz, 25 V, 1 ms duration) was applied continuously. As soon as consecutive pulses gave stable twitch responses, the effects of the compounds were tested. Neuropeptide Y (procine) (Auspep Pty, Parkville, Australia) and clonidine both concentration-dependently inhibited the twitches and concentration-response curves were constructed following cumulative application of the agonists. When tested on preparations from the contralateral side, α-trinositol (up to 100 μM neither affected the twitches per se nor did the drug affect neuropeptide Y- or clonidine-evoked twitch inhibition. A linear regression of the data in the 10–90% response interval allowed the calculation of pD_2 values (i.e. the negative logarithm of the IC50 values) for neuropeptide Y and clonidine.

Pithed rats

Male Sprague-Dawley rats (Möllegaard Hansen Avelslaboratorie A/S, Denmark), 250–350 g, were maintained on a diet of standard rat pellets and tap water ad libitum and housed in cages in groups of 5, at 26°C, with 60% humidity and 05.00 to 19.00 h light regime.

Anaesthesia and surgical preparation. Anaesthesia was induced with methohexitone sodium (Brietal, Eli Lilly & Co., USA; 70 mg kg^{-1}, i.p.) and the animals were then tracheotomized. The left carotid artery and both jugular veins were cannulated with polyethylene catheters (PE 50; Portex Ltd, Hythe Kent, UK). The rats were then pithed by insertion of a steel rod (1·5 mm diameter) through the orbit and the foramen magnum, down the spinal cord to the level of the first to second sacral vertebrae. Another steel needle was inserted under the skin of the back of the animal to serve as a neutral electrode. The animals were artificially ventilated with room air (4 mL $beat^{-1}$ kg^{-1}; 78 beats min^{-1} and 40/60 inspiration/expiration ratio) using a Carlsson ventilator (No. 8908, Mölndal, Sweden). Arterial blood gases and pH were regularly monitored on an Acid-Base Analyzer (ABL 30, Radiometer, Copenhagen, Denmark). Pancuronium bromide (Pavulon, Organon, The Netherlands, 2 mg kg^{-1}, i.v.) and atropine sulphate (Sigma, 1 mg kg^{-1}, i.v.) were injected 5 min after pithing to prevent muscle contraction and muscarinic receptor activation during spinal cord stimulation, respectively. During the experiment the animals were kept on an electrically-heated operating table (37°C); blood pressure and heart rate were continuously recorded on a Grass model 7D polygraph (Grass Inc., Quincy, MA, USA).

Experimental protocol. About 40 min after the pithing procedure the sympathetic nervous system was stimulated preganglionically (preganglionic nerve stimulation (PNS) 1; 0·8 Hz, 1 ms, 65 V for 20 s) via a Grass model S4 stimulator (Grass Inc.). Ten min after the blood pressure response had stabilized and baseline conditions were present, the first bolus dose of phenylephrine (Sigma, St Louis, MO, USA), 25 nmol kg^{-1} was given. These PNS and phenylephrine injections were regarded as the control values for each animal in the group. Four minutes after the first dose of phenylephrine was given, 0·4 mL 0·9% NaCl (saline) or a dose of α-trinositol was infused over 2 min. At 60 min, the first continuous neuropeptide Y infusion was started and administered for 10 min. At 10 min after the end of the neuropeptide Y or vasopressin (CRB, UK) administration, the second PNS was performed followed by a second phenylephrine injection.

In separate experiments, prazosin (1 mg kg^{-1} was infused 2 min before the second PNS. Porcine neuropeptide Y (Peninsula, CA, USA) was dissolved in saline containing 0·5% bovine serum albumin to yield a solution of 20 μg mL^{-1} and given in a 10 min continuous infusion of 2 μg kg^{-1} min^{-1}. The solution was prepared in fresh saline before the experiment and administered by injection in various doses (panel A of Fig. 2) over 2 min.

Mesangial cell preparation and Ca^{2+} fluorescence microscopy
Mesangial cells were prepared from rat isolated renal glomeruli by a sieving procedure. The outgrowth of collagenase-treated glomerular remnants was performed in RPMI 1640 supplemented with 10% foetal calf serum (FCS). Cell growth was arrested for 24 h before measurements of intracellular calcium, by depleting the medium of FCS. For measurements of ionic cytoplasmic calcium the cells were incubated for 45 min at 37°C in a medium containing 5 μmol fura-2 acetoxymethylester (Calbiochem, La Jolla, CA, USA), whereafter the cell-containing cover glasses were rinsed in the medium subsequently used, containing 20 mmol HEPES (pH 7·4), 0·1% bovine serum albumin, 3 mM glucose, 0·5 mM magnesium, 1·25 mmol calcium, physiologically balanced with Cl^-. Cover glasses were used at the bottom of an open chamber suitable for microscopic work, which was inserted into an inverted microscope (Nikon Diaphoot, Japan). The climate box microscope was equipped for dual wavelength epifluorescence microfluorometry with a Xenon lamp light source. The wavelength was altered by changing between 340 and 380 nm interference filters. Calcium-dependent fluorescence, excited at 340 nm, was recorded continuously with frequent checks of fluorescence excited at 380 nm. The intracellular Ca^{2+} concentration was calculated from the 340/380 nm fluorescence ratio. The incubation with α-trinositol (10^{-5} mol) was started 2 min preceding the addition of 100 pmol neuropeptide Y. The experiments were carried out with 1·25 mmol external calcium in the medium.

Assay for total inositol phosphates
Cerebral vessels from guinea-pigs, 200–250 g, were preincubated for 30 min at 37°C in buffer solution containing 1% bovine serum albumin (BSA; Sigma), 10^{-2} mol LiCl (BDH, Poole, UK) and 2×10^{-6} mol imipramine (Sigma), and aerated with a mixture of 5% CO_2 in O_2 to give a pH of 7·4. The blood vessels were divided into small groups of approximately 1 mg each, as even as possible in size, one group for each tube. One guinea-pig gave enough vessel material for 4 tubes. The vessels were then incubated for another 15 min at 37°C. [^{3}H] *Myo*-Inositol (0·5 μCi) (Amersham, Bucks, UK) was added to each tube and incubated for 2·5 h at 37°C. The appropriate concentration of drug (according to the protocol) was added to each tube and the tubes were incubated as above for 30 min. The incubation was terminated by the addition of 2 mL CH_3OH/$CHCl_3$/conc. HCl (40 : 20 : 1 v/v/v) and the samples were homogenized in a glass-glass tissue grinder for 20 s. After the addition of 600 μL $CHCl_3$ and 600 μL distilled water, the samples were centrifuged at 2500 *g* for 10 min to facilitate phase separation. The aqueous layer was removed from the tubes for assay of inositol phosphates. Each sample was run

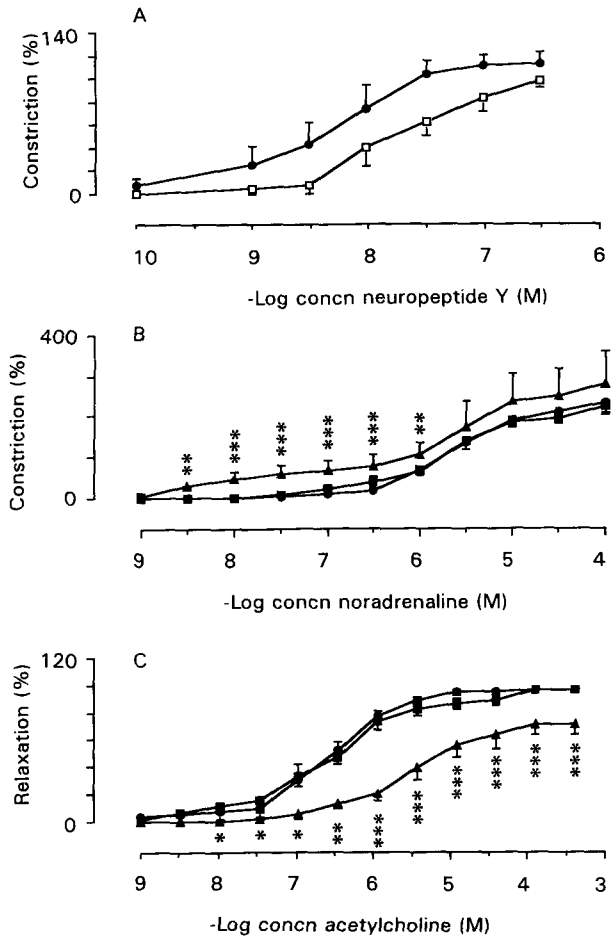

FIG. 2. A. Inhibitory effect of α-trinositol (□, 1 μM) on vasoconstriction evoked by neuropeptide Y in circular segments of human subcutaneous arteries. B. Inhibition by 10 nM α-trinositol (■) of the potentiating effect of neuropeptide Y (100 nM) on noradrenaline-evoked constriction of the guinea-pig uterine artery (▲). C. α-Trinositol (■, 10 nM) abolished neuropeptide Y (30 nM) elicited inhibition of relaxation of the guinea-pig basilar artery (▲); such a relaxation was induced by acetylcholine in vessels that had been preconstricted by 3 μmol prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Values are expressed as means + s.e.m. (n = 6–10). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ using paired Student's *t*-test, compared with control values (●).

through a column containing 1 mL Dowex anion exchange resin (1 × 8, 100–200 mesh; Fluka, Switzerland) in the formate form. The columns were first washed with 3 mL distilled water before the addition of the aqueous layer, one tube to each column. The columns were then washed with 12 mL unlabelled myo-inositol (5 mmol). Glycerophosphoinositol (A), inositol monophosphate (B), inositol diphosphate (C) and inositol triphosphates (D) were eluted stepwise by the addition of 12 mL of each of the following solutions: A, 5 mmol disodium tetraborate and 60 mmol sodium formate; B, 200 mM ammonium formate and 100 mmol formate; C, 400 mM ammonium formate and 100 mmol formate; D, 1 mol ammonium formate and 100 mmol formate. Between each solution the columns were moved to a new set of scintillation tubes in which the eluents were collected and scintillant added (Hi Safe III, LKB, Sweden). Samples were assayed in a liquid scintillation counter.

Radioreceptor binding studies using [^{125}I] peptide YY

The cloned human neuropeptide Y/peptide YY Y1-receptor cDNA (hY1-5), inserted into the mammalian expression vector, pCDM8, was used for transfection of COS1 monkey kidney cells grown in 150 mm dishes. Plasmid DNA was transfected over 3·5 h using DEAE/dextran (Pharmacia, Uppsala, Sweden) in DMEM with 10% Nuserum (Collaborative Research) in 5% CO_2. Within 60 h, cells were harvested and suspended in 50 mmol ice-cold Tris-HCl buffer (pH 7·4) with 5 mmol EDTA and 1 mmol β-mercaptoethanol and then homogenized using a Polytron (Brinkman; setting 6) for 10 s. The homogenate was centrifuged at 1000 *g* for 10 min using a swinging bucket rotor. The supernatant was then subjected to ultracentrifugation at 100 000 *g* for 30 min. The resulting pellet was resuspended using a Polytron homogenizer in fresh binding buffer of the following composition (mM): (NaCl 137, KCl 5·4, KH_2PO_4 0·44, $CaCl_2$ 1·26, $MgSO_4$ 0·81, HEPES 20, bovine serum albumin 0·3% and bacitracin 0·01%; pH 7·4) and membranes from 2×10^6 cells were used per assay tube in a final volume of 0·4 mL. Samples were then incubated with [^{125}I] Peptide YY ([^{125}I] PYY; New England Nuclear, USA; 2200 Ci mmol^{-1}; 22°C; 100 or 150 min) as previously described. In displacement-type experiments, 0·1 nmol radioligand was used. In kinetic experiments, dissociation was induced by addition of excess unlabeled porcine peptide YY (300 nmol) after 150 min of incubation with radioligand. Association experiments employed use of a 10-fold diluted [^{125}I] PYY solution. The incubations were terminated by centrifugation (Eppendorf Microfuge) for 2 min, followed by washing of the pellets' surface by ice-cold buffer. Pellets were counted in a Packard gamma-counter. Binding data were analysed using LIGAND and KINETIC software (Biosoft).

Results

In-vitro studies

To examine the vasomotor effects of α-trinositol, circular ring segments of arteries with intact endothelium were studied using a sensitive in-vitro method. Neuropeptide Y caused a strong constriction of the human subcutaneous arteries (Fig. 2A). This effect was inhibited after preincubation for 20 min with 1 μmol α-trinositol (Fig. 2A).

Table 1. Lack of effect of α-trinositol (100 μM) on neuropeptide Y- and clonidine-induced suppression of electrically-evoked, sympathetic nerve-mediated, twitches of rat isolated vas deferens.

Agonist	pD_2 (control)	pD_2 (α-trinositol)
Neuropeptide Y	8·14 ± 0·19	8·04 ± 0·16
Clonidine	7·52 ± 0·14	7·61 ± 0·13

Values given are means ± s.e.m. (n = 6–8). Student's two-tailed t-test.

A second type of postjunctional effect, i.e. the potentiation by neuropeptide Y of noradrenaline-evoked vasoconstriction, was studied in the guinea-pig isolated uterine arteries. As shown in Fig. 2B, such potentiation was abolished by pretreatment with α-trinositol (as low as 10^{-8} mol).

Neuropeptide Y may also act as a potent inhibitor of vasodilator responses, a phenomenon that appears synergistic with enhancement of constriction, and results in the production of higher vascular tone. In the present study we found the peptide to inhibit acetylcholine-induced relaxation of segments of guinea-pig basilar artery that had been preconstricted using prostaglandin $F_{2\alpha}$ at low nanomolar concentrations (Fig. 2C).

In contrast, Ins[1,2,6]P_3 did not affect the prejunctional inhibitory effect of neuropeptide Y or of clonidine (an α_2-adrenoceptor agonist) on electrically induced twitches of the rat isolated vas deferens (Table 1).

In-vivo studies

The systemic (in-situ) action of α-trinositol was examined in pithed rats. In doses that did not affect basal blood pressure, α-trinositol potently inhibited neuropeptide Y-induced elevations of blood pressure. Only at markedly higher doses did the phosphoinositide inhibit pressor responses to phenylephrine and vasopressin (Fig. 3).

When preganglianic nerves were stimulated using low

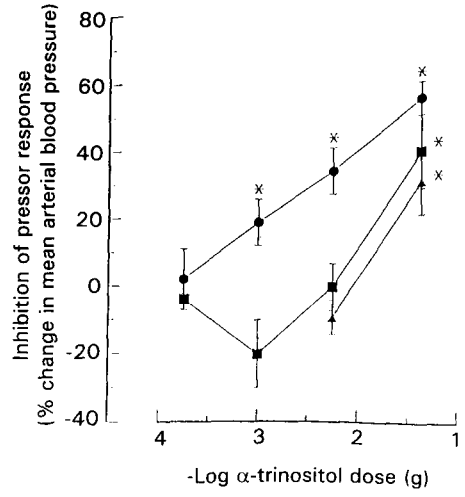

FIG. 3. Dose-dependent inhibitory effect of α-trinositol on the increase in mean arterial blood pressure in response to neuropeptide Y infusion (●, 2 μg kg^{-1} min^{-1}, 10 min) phenylephrine (■, 25 mmol kg^{-1} in bolus) or vasopressin (▲, 0·75 μg kg^{-1} i.v. bolus). Values given represent mean ± s.e.m.; *P < 0·05 compared with control.

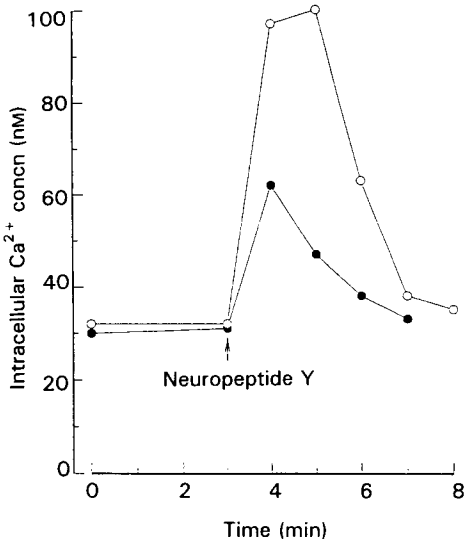

FIG. 4. Inhibitory effect of α-trinositol, 10 μM (●) on intracellular Ca^{2+} concentrations elevated by neuropeptide Y, 0·1 μM (○) as studied in a single fura-2 containing mesangial cell from rat. Values given are means ± s.e.m.

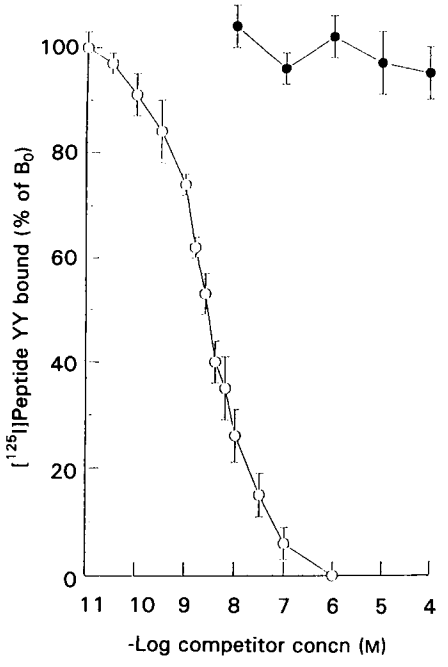

FIG. 5. Lack of inhibitory effect of α-trinositol on the binding of [^{125}I] PYY to membranes of human vascular-type neuropeptide Y/peptide YY Y1-receptor (pCDM8/hY1-5) transfected COS1 monkey kidney cells. This binding was concentration-dependently inhibited by unlabelled neuropeptide Y (○) while α-trinositol did not displace [^{125}I] PYY binding even at 100 μmol (●) α-trinositol did not exert any effect on the observed association rate (k_{obs}) control 0·8 ± 0·2, treated 0·75 = 0·15 min^{-1} or dissociation rate (k_{-1}) of [^{125}I] PYY control 0·12 ± 0·05; treated 0·1 ± 0·05 min^{-1} as determined in kinetic experiments, indicating lack of allosteric action of the inositol phosphate on Y1-receptors. Values given are means ± s.e.m. of triplicate experiments repeated at least twice.

frequency stimulation (0·8 Hz), a condition that may favour the release of ATP relative to noradrenaline, we found that the α_1-adrenoceptor antagonist, prazosin, was unable to completely block the pressor response to its stimulation (control + 70 ± 5·46 mm Hg; with prazosin +33·4 ± 3·08 mm Hg; $P < 0·01$ compared with control). This pressor response was also significantly attenuated by α-trinositol at a dose (10 mg kg^{-1}) that did not affect exogenous phenylephrine (Fig. 3a). The effects of prazosin and α-trinositol were additive (control + 99.4 ± 4.30 mm Hg; with prazosin + α-trinositol +18 ± 2·61 mm Hg; $P < 0·001$ compared with control), indicating that the two drugs affect different components of the stimulated pressor response.

Mode of action

Inhibition of Ca^{2+} elevations. We examined whether α-trinositol was capable of preventing Ca^{2+} elevations evoked by neuropeptide Y in rat mesangial cells by studying changes in the Ca^{2+}-sensitive fluorescent dye, fura-2, neuropeptide Y was found to increase intracellular Ca^{2+} concentrations concentration-dependently. Furthermore, the results showed that α-trinositol in micromolar concentrations inhibited

Table 2. Inhibitory effect of α-trinositol on the formation of inositol phosphates in guinea-pig brain vessels.

	Inositol phosphates (counts min^{-1} mg^{-1} guinea-pig brain)		Inositol phosphates (counts min^{-1} mg^{-1} guinea-pig brain)
Control	49 ± 16	Control	52 ± 17
ATP (10^{-6} M)	129 ± 30*	Endothelin-1 (10^{-8} M)	187 ± 40*
+α-Trinositol (10^{-8} M)	149 ± 34	+α-Trinositol (10^{-8} M)	158 ± 35
+α-Trinositol (10^{-7} M)	188 ± 45	+α-Trinositol (10^{-7} M)	218 ± 45
+α-Trinositol (10^{-6} M)	152 ± 40	+α-Trinositol (10^{-6} M)	147 ± 34
Control	83 ± 29	Control	54 ± 18
Noradrenaline (10^{-8} M)	223 ± 47*	Histamine (10^{-6}M)	80 ± 25
+α-Trinositol (10^{-8} M)	165 ± 53	+α-Trinositol (10^{-8} M)	83 ± 22
+α-Trinositol (10^{-7} M)	213 ± 46	+α-Trinositol (10^{-7} M)	74 ± 24
+α-Trinositol (10^{-6} M)	236 ± 51	+α-Trinositol (10^{-6} M)	104 ± 28
Control	60 ± 18	Control	62 ± 18
Neuropeptide Y (10^{-8} M)	181 ± 35*	5-HT (10^{-6}M)	177 ± 35*
+α-Trinositol (10^{-8} M)	62 ± 20#	+α-Trinositol (10^{-8} M)	130 ± 38
+α-Trinositol (10^{-7} M)	70 ± 19#	+α-Trinositol (10^{-7} M)	181 ± 46
+α-Trinositol (10^{-6} M)	63 ± 21#	+α-Trinositol (10^{-6} M)	179 ± 40

Values given are mean ± s.e.m. *$P < 0·05$ compared with control values, #$P < 0·05$ compared with neuropeptide Y alone.

elevation of intracellular Ca^{2+} evoked by neuropeptide Y (Fig. 4). Interestingly, α-trinositol did not by itself alter the resting Ca^{2+} levels.

Effect on binding of neuropeptide Y. We examined the possibility that α-trinositol might interfere with binding of [^{125}I] PYY to membranes of human Y1-receptor transfected COS1 cells, i.e. the neuropeptide Y/peptide YY-receptor that is thought to be expressed in vasculature. As can be seen in Fig. 5, neuropeptide Y concentration-dependently inhibited binding of the radioligand. In contrast, α-trinositol, even at 100 μmol was unable to displace [^{125}I] PYY binding to this receptor (Fig. 5A). The possibility however remained that α-trinositol might exert a more subtle effect on neuropeptide Y/peptide YY binding, perhaps as an allosteric modulator of peptide binding acting at a site other than the peptide recognition site. We, therefore, performed kinetic experiments, i.e. studying peptide association and dissociation. These latter experiments however, did not reveal any such allosteric properties of α-trinositol (see legend to Fig. 5).

Activation of the phosphoinositol production. We investigated whether α-trinositol could interfere with neuropeptide Y mediated increases in intracellular inositol phosphate accumulation. These experiments (Table 2) showed that α-trinositol (10^{-6}–10^{-8} mol) significantly inhibited the formation of inositol phosphates (total as well as IP_3 and IP_2) in guinea-pig basilar arteries. Notably, no significant effects by α-trinositol were observed on ATP-, noradrenaline-, endothelin-1-, histamine- and 5-HT-induced increases of inositol phosphate accumulation in the same type of vessel preparations (Table 2).

Discussion

In-vitro contraction studies

The present study has demonstrated that Ins[1,2,6]P_3 possesses vasomotor effects in circular ring segments of arteries in-vitro as well as in-vivo. Clearly, α-trinositol inhibits direct vasoconstriction elicited by neuropeptide Y, potentiation by neuropeptide Y of noradrenaline-evoked constriction and inhibition of the neuropeptide Y-mediated blockade of acetylcholine-induced vasodilatation, without influencing the presynaptic inhibition by neuropeptide Y of sympathetic neurotransmitter release in rat vas deferens. These observations are in accordance with our previous studies of α-trinositol in the guinea-pig basilar artery and in the pithed and conscious rat, in which α-trinositol caused concentration- and dose-dependent inhibition of neuropeptide Y-induced vascular constriction without affecting the responses evoked by noradrenaline or a range of other vasoconstrictors (Edvinsson et al 1990). The functional effects of α-trinositol on neuropeptide Y induced vascular contraction may differ between tissues (Edvinsson et al 1994). In addition, recent work indicates that α-trinositol interacts with a single population of binding sites, found in varying densities in different tissues and cultured cells; bovine aortic endothelial cells > rat vena cava vascular smooth muscle cells > rat heart > rat lung > rat liver (Yoo et al 1994).

It is unlikely that α-trinositol acts as a non-selective inhibitor of Ca^{2+} influx to selectively block vasoconstriction elicited by neuropeptide Y, since many of these vasoconstrictors, including neuropeptide Y, were less efficacious in the absence of extracellular Ca^{2+} or in the presence of the dihydropyridine Ca^{2+} channel antagonist, nifedipine (Edvinsson et al 1990), in vessels from laboratory animal and human subcutaneous arteries.

In addition to the direct vasoconstriction, neuropeptide Y may also potentiate the effects of various vasoconstrictors, such as noradrenaline. In the guinea-pig isolated uterine arteries α-trinositol attenuated the neuropeptide Y-evoked potentiation of noradrenaline-induced vasoconstriction. These data are in accordance with in-vivo results obtained in the pithed as well as conscious normotensive and hypertensive rats (Sun et al 1991, 1992, 1993).

In the present study we also investigated the effects of α-trinositol on neuropeptide Y-mediated inhibition of acetylcholine-induced relaxation. Even at low nanomolar concentrations, α-trinositol abolished the inhibitory action of neuropeptide Y on acetylcholine-induced relaxation of segments of guinea-pig basilar artery, thereby restoring the relaxant responses to acetylcholine. Taken together, these three types of inhibitory effects of α-trinositol in-vitro may act synergistically to reduce the sympathetic vascular tone, locally and possibly systemically.

In contrast, α-trinositol did not affect the prejunctional inhibitory effect of neuropeptide Y (Y_2-mediated) or of clonidine (an α_2-adrenoceptor agonist) on electrically-induced twitches of the rat isolated vas deferens which is a preparation frequently used for studying release of sympathetic transmitters (Stjärne et al 1986; Wahlstedt et al 1986).

In-vivo studies

In the pithed rats, the effects of α-trinositol potently inhibited neuropeptide Y-induced elevations of blood pressure in a dose range that did not affect basal blood pressure. In this in-vivo model, α-trinositol did not inhibit pressor responses to phenylephrine and vasopressin. The preganglionic nerve-stimulation pressor response was also significantly attenuated by α-trinositol. Moreover, when stimulation was by using low frequency stimulation (0·8 Hz), a condition that may favour the release of ATP relative to noradrenaline (Stjärne et al 1986), we found that the effects of prazosin and α-trinositol were additive, indicating that the two drugs affect different components of the stimulated pressor response.

Exogenous neuropeptide Y caused enhancement of the pressor response to preganglionic nerve-stimulation as well as the pressor response to phenylephrine, which is in agreement with earlier studies (Dahlöf et al 1985; Zukowska-Grojec et al 1986); both these enhancing effects of neuropeptide Y have been demonstrated to be abolished by α-trinositol at 2 mg kg^{-1} (Sun et al 1992). Thus, the inositol phosphate appears to be effective in-vivo as well as in-vitro in inhibiting neuropeptide Y-induced potentiation of vasoconstriction evoked by noradrenaline, in addition to inhibiting the pressor response to sympathetic nerve stimulation.

Mode of action

Firstly, we attempted to investigate if α-trinositol might

interfere with the binding of neuropeptide Y, or its radioactive analogue, [^{125}I] PYY, to the recently cloned (Larhammar et al 1992) vascular Y1-type of neuropeptide Y/peptide YY-receptor (Wahlestedt et al 1986, 1990a,b). The receptor binding studies in membranes of human Y1-receptor transfected COS1 cells, i.e. the neuropeptide Y/peptide YY-receptor that is thought to be expressed in vasculature (Wahlestedt et al 1986, 1990a,b) demonstrated that α-trinositol did not interfere with binding of [^{125}I] peptide YY even at 100 μM. This receptor was recently cloned from a human brain cDNA library and its corresponding mRNA was found to be present in human cultured vascular smooth muscle cells (Larhammar et al 1992). The possibility however remained that α-trinositol might exert a more subtle effect on neuropeptide Y/YY binding, perhaps as an allosteric modulator of peptide binding acting at a site other than the peptide recognition site. We therefore performed kinetic experiments, i.e. studying peptide association and dissociation which did not reveal any such allosteric properties of α-trinositol. We therefore conclude that the novel inositol phosphate, despite being able to inhibit many vascular actions of neuropeptide Y, does not affect binding of the peptide to its vascular receptor. Instead, α-trinositol should be viewed as a functional, i.e. non-receptor, neuropeptide Y antagonist. If binding data are taken together with the above-mentioned Ca^{2+} data, it appears that α-trinositol interferes with Ca^{2+} signalling elicited by neuropeptide Y through a mechanism that comes into play only after the peptide is recognized by its receptor.

Secondly, there is much evidence that neuropeptide Y elevates intracellular Ca^{2+} in various cell types, including vascular smooth muscle cells (Mihara et al 1989; Lobaugh & Blackshear 1990) and that such elevations are intimately coupled to myosin light chain phosphorylation and contraction (Kamm & Stull 1985).

In our study, we found that α-trinositol in micromolar concentrations inhibited elevations of intracellular Ca^{2+} evoked by neuropeptide Y in agreement with other independent experiments which have indicated that α-trinositol also inhibits neuropeptide Y-evoked $^{45}Ca^{2+}$-influx in rat cultured vena cava smooth muscle cells (Wahlstedt et al 1991). It is thus possible that both Ca^{2+} mobilization and influx can be inhibited by the inositol phosphate, concurring with the capacitative model of intracellular Ca^{2+} elevation (Putney et al 1978), but the level at which α-trinositol exerts its inhibitory effect yet remains unknown. However, since the compound, as noted above, acts only to inhibit selected vasoconstrictors, it distinguishes itself from dihydropyridine-type Ca^{2+} channel antagonists, suggesting that Ca^{2+} elevation elicited by neuropeptide Y and ATP in vascular smooth muscle is triggered either at an intracellular site(s) or at other Ca^{2+} entry channels.

Thirdly, we found that the mechanism of action of α-trinositol requires the involvement of changes in inositol phosphate turnover. Thus, α-trinositol significantly inhibited the formation of neuropeptide Y induced activation of inositol phosphates (total as well as IP_3 and IP_2) in guinea-pig basilar arteries while there were no effects on the activation of the phosphoinositide pathway mediated by ATP, noradrenaline, 5-HT, endothelin-1 or histamine.

The kinetics and metabolism of α-trinositol is not yet clarified in detail. However, preliminary data from man (Hedner et al unpublished), indicate that there is a conversion to di- and monophosphate compounds. Interestingly, recent work (Sun et al 1995) have demonstrated that α-trinositol remains the most potent functional neuropeptide Y inhibitor of a series of inositol derivatives.

Potential clinical implications

Neuropeptide Y has been implicated in the pathophysiology of cardiovascular disorders such as congestive heart failure and hypertension (Edvinsson et al 1991; Zukowska-Grojec & Wahlestedt 1993). Since these suggestions have been based primarily on increased levels of circulating neuropeptide Y, the availability of a non-peptide antagonist, such as α-trinositol would make such hypotheses testable. Indeed, in a preliminary controlled clinical trial, the inositol phosphate was found to reduce blood pressure in hypertensive patients but not in normotensive control subjects (Lind et al 1994). This difference appeared more pronounced when these two groups, hypertensives and normotensives, had been test-bicycling, in order to increase sympathetic outflow. Interestingly, Pernow et al (1986, 1989) found that sympathetic activation in man occurs concomitantly with elevated levels of circulating neuropeptide Y, and that high sympathetic nerve activity is associated with preferential release of neuropeptide Y (as compared with noradrenaline). Moreover, elevated sympathetic nerve activity is likely to be implicated in the pathophysiology of mild to severe hypertension (Anderson et al 1989). Because vasoconstriction has been found to persist after α_1-adrenoceptor blockade in hypertensive patients (Taddei et al 1989), it is conceivable that sympathetic co-transmitters (Edvinsson et al 1991; Zukowska-Grojec et al 1986) are involved. This is also supported by recent clinical studies showing that α-trinositol possesses blood pressure lowering properties and that the inositol phosphate may modulate the blood pressure response after dynamic exercise in hypertensive patients (Lind et al 1994).

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