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Inhibition of neuropeptide Y-induced potentiation of noradrenaline-induced vasoconstriction by PP56 (D-*myo*-inositol 1,2,6-tris-phosphate)

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1 Although neuropeptide Y (NPY) is a potent vasoconstrictor in many vascular beds, nanomolar concentrations of this peptide potentiate the noradrenaline-induced contractions in rabbit gastroepiploic and femoral arteries, and guinea-pig mesenteric and uterine arteries.

2 The potentiating effect of NPY on noradrenaline-induced contraction was present in endothelium-denuded femoral arteries.

3 The potentiating effect of NPY on noradrenaline-induced contraction was antagonized by PP56 (D-*myo*-inositol 1,2,6-trisphosphate) in low concentrations (down to 0.1 nM). This antagonistic effect was observed in all four types of vessels studied. Contractions induced by noradrenaline, histamine, endothelin-1 and potassium were not altered by PP56 in concentrations upto 1 μM in femoral artery of rabbit.

4 We provide evidence that a non-peptide (PP56) can selectively antagonize NPY-induced effects in rabbit and guinea-pig peripheral arteries without affecting the vasoconstrictor response to noradrenaline.

Keywords: Neuropeptide Y; vasoconstriction; D-*myo*-1,2,6-inositol trisphosphate; inhibition of potentiation

Introduction

Sympathetic neurotransmission is now known to be mediated not by a single neurotransmitter, but may rather involve the interaction of several substances released from the nerve terminal. Whereas noradrenaline (NA) is recognized to be the primary neurotransmitter for the sympathetic nervous system, recent attention has focused on the importance of neuropeptide Y (NPY) in influencing sympathetic nerve activity (Edvinsson *et al.*, 1987). NPY is co-localized with NA in perivascular sympathetic nerve fibres and is co-released with NA following sympathetic nerve activation, suggesting that NPY acts as a co-transmitter (for references see Edvinsson *et al.*, 1987).

The vascular effects of NPY vary with species and tissue. The effects of NPY on blood vessels include direct vascular smooth muscle contraction (Edvinsson *et al.*, 1983; 1984), potentiation of agonist-evoked responses (Ekblad *et al.*, 1984; Edvinsson *et al.*, 1984), inhibition of relaxation (Han & Abel, 1987; Fallgren *et al.*, 1989) and inhibition of the release of NA (Pernow *et al.*, 1986). NPY causes direct contraction in some tissues, but not all, and this effect occurs at relatively high concentrations of NPY. However, although a number of arteries fail to contract upon NPY administration, a potentiation of contractile responses to exogenous NA and transmural nerve stimulation occurs at nanomolar concentrations of NPY (Ekblad *et al.*, 1984; Edvinsson *et al.*, 1984). This response seems to prevail in the peripheral circulation, since coronary arteries and cerebral arteries preferably contract upon NPY administration (Edvinsson *et al.*, 1983; Clarke *et al.*, 1987).

The mechanism responsible for the potentiating effect of NPY is unclear. However, it seems to depend on calcium released from intracellular stores (Wahlestedt *et al.*, 1985). In parallel, NPY appears to facilitate the refill of the intracellular calcium stores from the extracellular medium, conceivably due to its ability to evoke membrane depolarization (Fallgren *et al.*, 1990). Other mechanisms proposed to explain the potentiating effect include inhibition of adenylate cyclase activity (Fredholm *et al.*, 1985) and enhanced accumulation of inositol trisphosphate (Häggblad & Fredholm, 1987).

To understand further the functional role of NPY in autonomic transmission there is a need of a selective NPY-antagonist. PP56 is a new chemical entity with NPY-blocking properties (Edvinsson *et al.*, 1990). It is at present being developed for therapeutic use in cardiovascular and inflammatory disorders by Perstorp Pharma, Sweden. The purpose of the present study was to characterize the effect of NPY on rabbit and guinea-pig arteries and to examine the NPY-antagonistic effects of PP56 (D-*myo*-inositol 1,2,6-trisphosphate; U.S. Pat. No. 4735963, EPO Pat. No. 179439, Dr M.J. Sirén, 1984). The initial observations indicated that NPY had no direct contractile effect but preferentially potentiated the responses to NA both in the presence and absence of endothelium. Furthermore, PP56 was found to be a very potent NPY blocker.

Methods

Adult male rabbits (2–3 kg) were anaesthetized with pentobarbitone (30 mg kg^{-1}) and exsanguinated. The gastroepiploic and femoral arteries were rapidly dissected out and placed in an ice-cold oxygenated 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. Adult female guinea-pigs (300–400 g) were anaesthetized with pentobarbitone (80 mg kg^{-1}) and decapitated. The mesenteric and uterine arteries were rapidly removed and placed in the same buffer solution as above. Ring segments of the arteries, 2–3 mm long, were immersed in temperature controlled tissue baths (2.5 ml in volume) containing the buffer solution aerated with 5% CO_2 in O_2, giving a pH of 7.4. The specimens were mounted on two L-shaped metal holders (0.1 or 0.2 mm in diameter depending on vessel diameter). The position of one of the holders could be changed by means of a movable unit allowing fine adjustments of the vascular tension. The other holder was connected to a force displacement transducer (Grass FT03C). The transducer signals were amplified by a Transbridge TBM4 amplifier, digitalized by Maclab TM analog-digital converter and recorded by a MacIntosh Plus computer. Increase in isometric tension was expressed in mN. The arteries were given an initial tension of 2–4 mN depending on vessel type and allowed to maintain this level of tension for a resting period of 90 min. After the resting period

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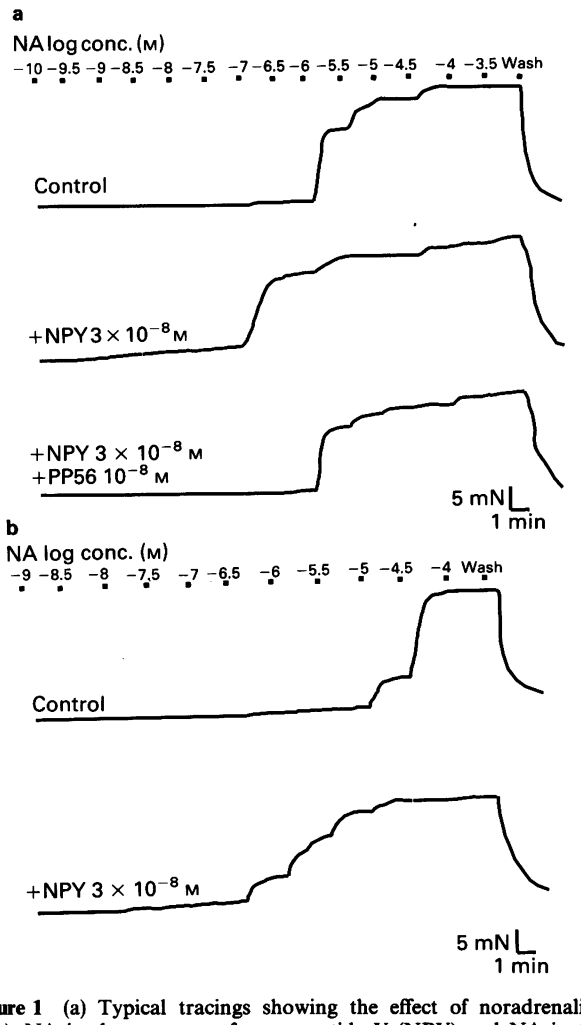

Figure 1 (a) Typical tracings showing the effect of noradrenaline (NA), NA in the presence of neuropeptide Y (NPY) and NA in the presence of both NPY and D-*myo*-inositol 1,2,6-trisphosphate (PP56) in rabbit femoral artery. Calibration bars are inserted. (b) NPY-induced potentiation occurs in the absence of endothelium in the rabbit femoral artery. The endothelium was removed by gentle rubbing of the vessel with a wooden stick and verified by lack of dilator response to acetylcholine of arteries precontracted with NA. Calibration bars are inserted.

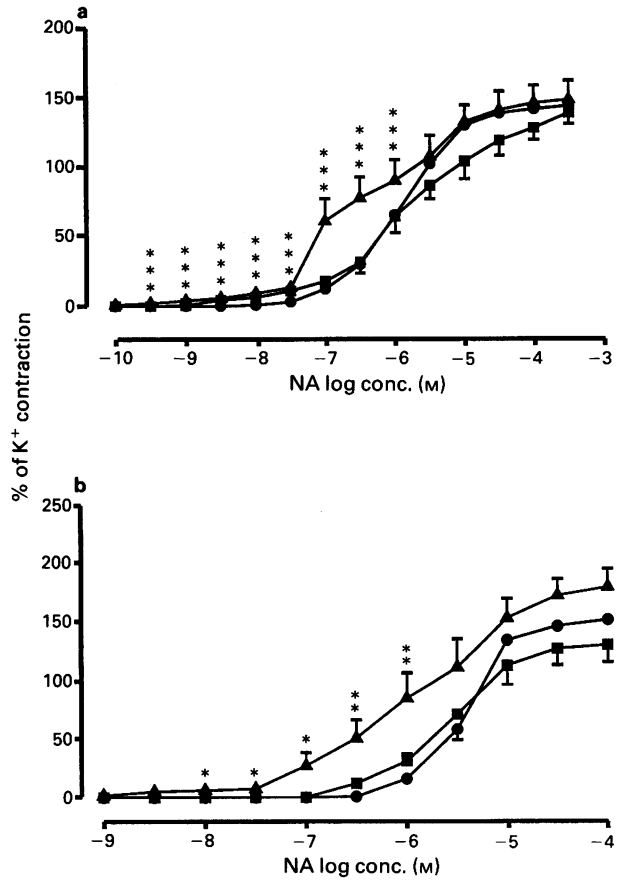

Figure 2 Effect of neuropeptide Y (NPY) on contraction elicited by noradrenaline in the absence and presence of D-*myo*-inositol 1,2,6-trisphosphate (PP56) in the rabbit (a) femoral and (b) gastroepiploic arteries. Control experiments [●]; tests in the presence of 30 nM NPY [▲]; and tests in the presence of 30 nM NPY and 10 nM PP56 [■]. Values given as the mean with the error bars representing s.e.mean. Number of experiments, $n = 6$–8. Unpaired Student's t test for control versus NPY treatment; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$.

the contractile capacity of each specimen was examined by exposure to a potassium-rich buffer solution (60 mM) obtained by equimolar substitution of NaCl by KCl in the above buffer. After two reproducible tests (variation less than 10%), concentration-response curves for NA (1 nM–0.1 mM) were obtained. The experiments were performed in 4 tissue baths, with 4 neighbouring arterial segments. One preparation served as control and received NA only. The enhancing effect of NPY was tested in the other three preparations by addition of NPY (30–100 nM) 2–3 min before the first concentration of NA. In two of the preparations receiving NPY, the NPY-antagonistic property of D-*myo*-inositol 1,2,6-trisphosphate (10 pM–10 μM) was examined by incubation of the vessels with PP56 for 20 min before NPY was added. In this manner each arterial segments were used for experimentation only once, i.e. the vessels were never exposed to NPY and PP56 on more than one occasion and, therefore, the risk of desensitization of the preparations was unlikely. This schedule was repeated from the different arteries of several animals in order to obtain reliable and statistically significant results.

In separate experiments on rabbit femoral arteries the endothelium was removed mechanically by gently rubbing the internal surface of the vascular segment with a wooden stick. The absence of endothelium was verified by loss of the dilator response to acetylcholine (Furchgott & Zawadski, 1980). In ring segments of femoral arteries a modulatory effect of PP56 was examined on contraction induced by cumulatively applied histamine, endothelin-1 and potassium.

Data analysis

Statistical analysis was performed by comparing the concentration-response curves to NA obtained alone, in the presence of NPY, or in the presence of NPY and PP56. Results are expressed as the means ± s.e.mean. Student's unpaired t test was used to determine statistical differences between means.

Drugs

Noradrenaline, acetylcholine and histamine (Sigma, U.S.A.), endothelin-1 (Peninsula, U.S.A.), neuropeptide Y (Porcine, Sigma, U.S.A.) and cocaine (ACO, Sweden) were dissolved and diluted in saline immediately before the *in vitro* tests in order to avoid possible breakdown or adsorption to walls of containers. D-*myo*-Inositol 1,2,6-trisphosphate (PP56, Perstorp Pharma, Sweden) was dissolved in 0.9% saline with 1% bovine serum albumin. A fresh solution of PP56 was made every day to avoid breakdown. All concentrations given below are the final molar concentration in the tissue bath during the experiments.

Results

Noradrenaline (NA) evoked a concentration-dependent contraction of rabbit and guinea-pig arteries. This response was sensitive to prazosin but not rauwolscine (Wahlestedt *et al.*,

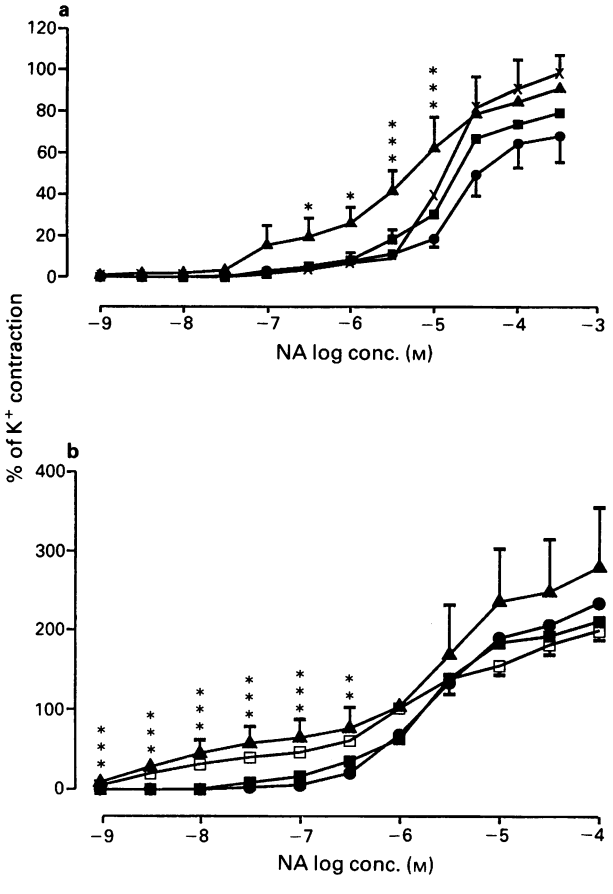

Figure 3 Effect of neuropeptide Y (NPY) on contraction elicited by noradrenaline in the absence and presence of D-*myo*-inositol 1,2,6-trisphosphate (PP56) in the guinea-pig (a) mesenteric and (b) uterine arteries. Control experiments [●]; tests in the presence of 0.1 μM NPY [▲] and tests in the presence of 0.1 μM NPY and 1 μM [×], 10 nM [■] or 1 nM [□] PP56. Values given as the mean with error bars representing s.e.mean. Number of experiments, $n = 6$–8. Unpaired Student's t test for control versus NPY: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$.

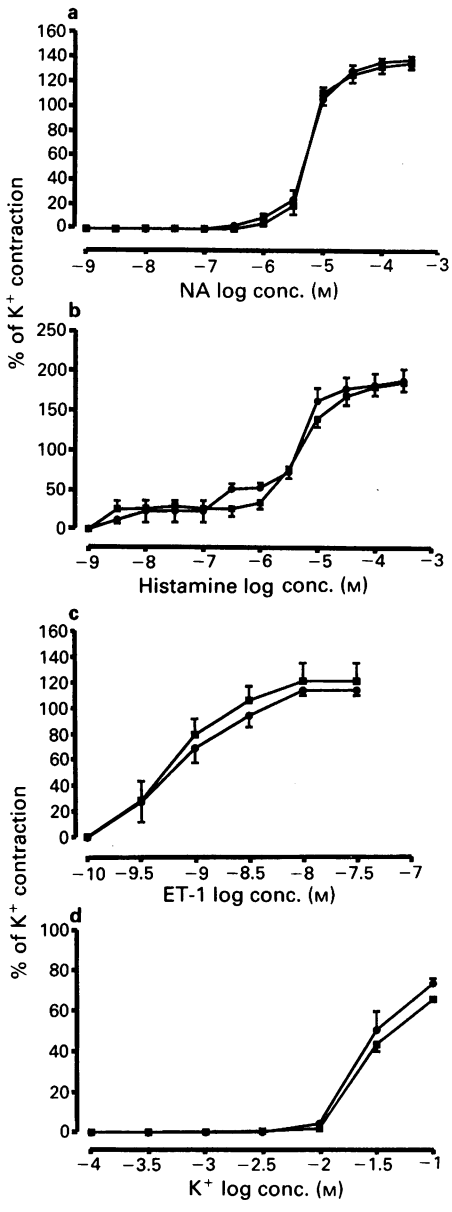

Figure 4 Effect of D-*myo*-inositol 1,2,6-trisphosphate (PP56) on contractions induced by (a) noradrenaline (NA), (b) histamine, (c) endothelin-1 (ET-1) and (d) potassium (K^+) in the rabbit femoral artery. Control experiments [●] and tests in the presence of 1 μM PP56 [■]. Values given as the mean with error bars representing s.e.mean. Number of experiments, $n = 6$.

1985; Fallgren & Edvinsson, 1986), demonstrating an action predominantly mediated via α_1-adrenoceptors. The experiments were performed in the presence of 1 μM cocaine to inhibit prejunctional uptake of NA. In the previous studies propranolol was found not to modify the contractile response to NA. NPY *per se* did not induce any constriction in concentrations up to 0.1 μM.

The effect of NPY on NA-evoked constriction was manifested in a left-ward shift of the lower half of the concentration-response curve for NA without any change in maximum response (Figures 1, 2 and 3). Removal of the endothelium in the rabbit femoral artery abolished acetylcholine-induced relaxation in arteries precontracted with NA (not shown). NA caused a concentration-dependent contraction which was potentiated in the same manner by NPY in endothelium-denuded vessels (Figure 1b) as were seen in intact vessels (Figure 1a).

PP56 selectively blocked the NPY-evoked potentiation of the NA-induced constriction without further affecting the concentration-response curve of NA (Figures 1a, 2 and 3). As can be seen in Figure 3 the NPY-blocking effect of PP56 was concentration-dependent. Inhibition started to appear in concentrations of around 0.1 nM (not shown). Total inhibition was obtained at 10 nM PP56 (Figures 2 and 3), and higher concentrations of PP56 (up to 1 μM) equally inhibited the NPY effect without altering the shape of the NA-evoked concentration-response curve (Figure 3).

As shown by the lack of effect on vascular tension during the 20 min incubation, PP56 did not show any effect *per se* on resting artery segments in concentrations up to 1 μM and it did not significantly change the NA-induced contraction in any of the vessels studied. In addition, PP56 was without effect on contractions induced by NA, histamine, endothelin-1 or potassium, respectively (Figure 4 and data not shown).

Discussion

The present results are in favour of the view that the NPY-induced potentiation involves a direct stimulation of smooth muscle cell-surface receptors (Gustafsson & Nilsson, 1990), rather than activation of an endothelium-dependent mechanism (Daly & Hieble, 1987). Receptor stimulation in general may initiate hydrolysis of a membrane-bound inositol lipid with subsequent formation of two second messengers, one of which is inositol 1,4,5-trisphosphate (1,4,5-IP_3) and the other diacylglycerol (DAG) (Berridge & Irvine, 1989). This may be particularly relevant for the NPY-induced potentiation of contraction in smooth muscle since Häggblad & Fredholm

(1987) found an enhanced accumulation of inositol trisphosphate after NPY administration in the vas deferens. 1,4,5-IP_3 regulates intracellular calcium both by mobilizing calcium from internal stores and, perhaps indirectly, by stimulating calcium entry. It has been postulated that NPY causes potentiation by facilitating the mobilization of intracellularly stored calcium ions (Wahlestedt *et al.*, 1985). Indeed, the potentiation was most prominent in low NA concentrations which putatively are associated with second messenger-mediated increase of intracellular calcium levels (for reference see Fallgren, 1988).

Recently we found evidence for two calcium-dependent mechanisms involved in the NPY-induced potentiation (Fallgren *et al.*, 1990 and unpublished): it was hypothesized that NPY first evoked intracellular release of calcium which may act as a second (or third) messenger that signals an increased sensitivity to the membrane-bound adrenoceptors. Accordingly, chelation of intracellular calcium pools with quin-2 blunted the potentiation without depressing the NA-induced concentration-response curve (Fallgren *et al.*, 1990), an effect similar to that of PP56 in the present study. Second, extracellular calcium influx seems to be a prerequisite for the observed effect of NPY, as evidenced by an inhibitory effect of different blockers of calcium entry (Fallgren *et al.*, 1990). PP56 does not inhibit extracellular calcium influx, as shown in the guinea-pig basilar artery (Edvinsson *et al.*, 1990, and unpublished). Hence, from a functional point of view, PP56 mimics the effect of quin-2, obviously by obstruction of the subtle intracellular calcium fluxes mediating the enhanced contractile response to NA in the presence of NPY.

The exact mechanism of action of D-*myo*-inositol 1,2,6-trisphosphate (PP56) is not known and further studies are required to elucidate its NPY-antagonistic property. However, due to the molecular structure of PP56 an interaction with 1,4,5-IP_3 is possible, although the weak lipid solubility of PP56 would contradict a direct effect on endogenous 1,4,5-IP_3. If so, the demonstration that PP56 inhibited the NPY effect would be in agreement with a suggestion that its NPY-blocking effect occurs via activation of the recently identified cell surface receptors for inositol phosphates (Vallejo *et al.*, 1987). Such inositol phosphate receptors may functionally be linked to the NPY-receptors due to the rather selective inhibition of NPY evoked by PP56 (Edvinsson *et al.*, 1990).

In conclusion, the present results support the view that PP56 antagonizes NPY by interference with an inositol phosphate-sensitive mechanism, conceivably associated with cell surface receptors coupled to intracellular calcium fluxes.

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