British Journal of Pharmacology (1998) 124, 101–110

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Neuropeptide Y-induced potentiation of noradrenergic vasoconstriction in the human saphenous vein: involvement of endothelium generated thromboxane

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1 We investigated the potentiating effect of low concentrations of neuropeptide Y (NPY) on the vasoconstriction induced by transmural nerve stimulation (TNS) and noradrenaline (NA) in human saphenous veins. The effects of (i) endothelium removal; (ii) the addition of the NO pathway precursor L-arginine; (iii) the ET_A/ET_B endothelin receptor antagonist Ro 47-0203; (iv) the cyclo-oxygenase inhibitor, indomethacin; (v) the selective thromboxane A_2 (TxA_2) receptor antagonists Bay u3405 and ifetroban, and (vi) the TxA_2 synthase inhibitor, UK 38485, were studied in order to gain information about the mechanisms of NPY-induced potentiation.

2 Contractile response curves for TNS (0.5–8 Hz) and for exogenously administered NA (0.1–3 μM) were obtained in superfused saphenous vein rings. The contractions induced by both TNS and NA at all tested frequencies and concentrations, respectively, were significantly potentiated by 50 nM NPY in endothelium intact veins. Conversely, in endothelium-denuded vessel rings the contractile-response curves to TNS and NA overlapped both in the absence and presence of NPY, thus suggesting that a release of vasoactive substances from endothelial cells could account for the noradrenergic NPY-induced potentiation.

3 In vessels with intact endothelium, the potentiating action of NPY on TNS and NA was unaffected by the presence of high concentrations of the NO precursor L-arginine (3–10 mM) or the non-selective ET_A/ET_B endothelin receptor antagonist, Ro 47-0203 (10 μM). These data indicate that the NPY-induced effect does not involve either the endothelium-derived vasodilator nitric oxide or the vasoconstrictor endothelin. Conversely, in the presence of the cyclo-oxygenase inhibitor, indomethacin (30 μM), NPY failed to potentiate the vasoconstrictions produced by either nerve stimulation or by exogenous NA, thus providing evidence that arachidonic acid metabolites through the cyclo-oxygenase pathway are mainly responsible for the potentiation evoked by NPY.

4 When the TxA_2 receptor antagonists, Bay u 3405 (1 μM) and ifetroban (1 μM) were added to the superfusing medium, NPY did not alter either the frequency- or the concentration-response curves for either TNS or NA. Accordingly, both TNS- and NA-induced contractions were not potentiated by NPY in the presence of the TxA_2 synthase inhibitor, UK 38485 (10 μM). This clearly demonstrates the pivotal role of TxA_2 in NPY-induced potentiation.

5 In superfused vein rings with endothelium, a subthreshold concentration (0.2 nM) of the TxA_2 mimetic U 46619 potentiated both TNS- and NA-induced vasoconstrictions. This potentiation was higher at low stimulation frequencies and low NA concentrations, and resembled that produced by NPY.

6 Our results indicate that in the human saphenous vein NPY potentiates the contractions produced by sympathetic nerve stimulation acting at the postjunctional level, primarily on endothelial cells. In particular, the NPY-induced release of a cyclo-oxygenase metabolite, namely TxA_2, may have a synergistic effect on the vasoconstriction induced by the noradrenergic mediator. Thus, such a mechanism may play a key role in the maintenance of the sympathetic tone of large human capacitance vessels.

Keywords: Neuropeptide Y; human saphenous veins; sympathetic neurotransmission; thromboxane; endothelium; cyclo-oxygenase

Introduction

Neuropeptide Y (NPY) is now well known to be co-localized in perivascular sympathetic nerve fibres and co-released with noradrenaline (NA) from sympathetic nerve endings. At the prejunctional level, it reduces the release of NA (for reviews, Edvinsson *et al*., 1987; Potter, 1988), whereas at the postjunctional level, at relatively high concentrations, probably *via* activation of NPY_1 receptors (Oellerich & Malik, 1993; Westfall *et al*., 1995) it is a vasoconstrictor in most but not all vessels (Edvinsson *et al*., 1984; Wahlenstedt *et al*., 1985). At lower concentrations, NPY can increase the vasoconstriction induced by various contractile agents (Edvinsson *et al*., 1984; Andriantsitohaina & Stoclet, 1988; Macho *et al*., 1989; Westfall *et al*., 1995) or by perivascular nerve stimulation (Wong-Dusting & Rand, 1988; Vu *et al*., 1989; Saville *et al*., 1990).

However, NPY-induced vasoconstriction potentiation has been observed to be a tissue- and species-dependent effect, and discrepant results have also been obtained with regard to its mechanism of action. The responses to NA were enhanced in rabbit gastroepiploic and femoral arteries, but not in veins (Edvinsson *et al*., 1984), and in rat mesenteric arteries, but not

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in femoral veins (Pernow *et al*., 1986). However, NPY potentiating effects on both transmural nerve stimulation (TNS) and exogenous NA were observed in canine saphenous veins (Hieble *et al*., 1989). Concerning the mode of action of NPY, differences have been found with respect, for example, to the role of endothelium. In rat isolated tail and mesenteric arteries, NA-induced contractile responses were potentiated by NPY in the absence of endothelium (Gustafsson & Nilson, 1990; Small *et al*., 1992). Conversely, in the rabbit isolated perfused ear artery and in canine saphenous vein, an intact endothelium was required for the potentiation of NA- and TNS-mediated contractions by NPY (Daly & Hieble, 1987; Hieble *et al*., 1989). In addition, in bovine isolated retinal arteries, the release of an endothelium-derived contracting factor has been recently described to account for the potentiating effect of NPY in the proximal, but not the distal, part of the vessel (Prieto *et al*., 1995).

Therefore, we deemed it interesting to verify whether NPY was able to enhance the vascular autonomic tone of large human venous capacitance vessels, and to study the role of the endothelium in the phenomenon. Thus with regard to the mechanisms of this NPY-induced effect, we hypothesized that either (i) the inhibition of the release of a relaxing factor, or (ii) the stimulation of the release of a contractile factor by the endothelium and/or the muscular layer might be responsible for the NPY-potentiating effect.

The possibility that NPY could inhibit the release of an endothelium-derived relaxing factor (particularly NO) seemed reasonable in view of the observations that in human omental arteries (Aldasoro *et al*., 1993) and saphenous veins (Fabi *et al*., 1996) the contractile responses to both TNS and NA were enhanced by endothelium removal or by the presence of the NO synthase inhibitors. On the other hand, we could not exclude the speculation that the ability of NPY to potentiate the responses to nerve stimulation and exogenous NA might be explained through the release of vasoconstrictor substance(s), which can synergistically act with the noradrenergic mediator at the postjunctional level. Indeed, it is well recognized that different stimuli induced endothelial and/or smooth muscle cells to release different contractile factors. Endothelin (Furchgott & Vanhoutte, 1989; Yang *et al*., 1990) and/or prostanoids, particularly thromboxane A_2 (TxA_2; Chester *et al*., 1993; Cocks *et al*., 1993), are the endogenously released substances most likely to act as secondary mediators in vasoconstriction.

Therefore, the main aims of our work were (1) to investigate whether NPY was able to enhance the frequency- and concentration-response curves to TNS and to NA, respectively, in human saphenous veins, (2) to verify the role of the endothelium in such potentiation, and (3) to characterize the endothelium-derived factor, if any, involved in the phenomenon.

Methods

Preparation of tissue

Vessels were prepared as described elsewhere (Fabi *et al*., 1993). Briefly, human saphenous vein segments were taken from patients undergoing surgery for aorta-coronary bypass grafting. We selected veins from patients who were not receiving adrenoceptor agonists or antagonists, or drugs that influence the storage or release of NA, but who were on Ca^{2+} channel antagonists, angiotensin converting enzyme inhibitors and/or nitrovasodilators. We used the terminal segment of the vessel, 2 to 3 cm in length, before it penetrates the fascia lata (less desirable for bypass purposes). Immediately after excision the tissue was placed in an oxygenated Krebs solution at 4°C and transported to the laboratory within 10 min.Most vessels were used on the day of surgery, and all tissues were used within 18 h. The vessels were cleaned of the adherent connective tissue and cut into 4 to 5 mm wide rings. The vein segments were mounted in an organ chamber on L-shaped stainless steel rods, to record the smooth muscle force. The preparations were superfused with Krebs oxygenated solution at 37°C by a constant perfusion pump (Gilson Minipuls II, Villiers Le Bel, France) at a flow rate of 5 ml min^{-1} under a resting tension of 2 g and allowed to equilibrate for 90–120 min. The composition of the Krebs solution was (mM): $NaHCO_3$ 25, NaCl 118, KCl 4.7, $CaCl_2$ $2H_2O$ 2.5, KH_2PO_4 1.2, $MgSO_4$ $7H_2O$ 1.17 and glucose 5.6; the solution was aerated with a mixture of 95% O_2 and 5% CO_2. The tension of the circular muscle layer was recorded with a Grass FT 0.3 T isometric force transducer (Grass Instrument Company, Quincy, MA, U.S.A.) coupled to a polygraph (Grass model 7D).

TNS of the vessel rings

TNS was delivered for 1 min through platinum wire electrodes placed on both sides of the vessel. The rectangular pulses applied by an electronic stimulator (Grass model S 11) at 0.5–16 Hz were 0.3 ms in duration and had supramaximal voltage (14 V measured across the electrodes). Contractile response curves for TNS and for exogenously administered NA were obtained. To verify whether the contractions evoked by TNS were mediated by endogenous NA released from adrenergic nerves, the vessel rings were superfused with Krebs containing 2 μM tetrodotoxin or with 10 μM guanethidine for 30 min. The contractions induced by TNS, but not by exogenously administered NA, were inhibited by the two antagonists, confirming that TNS-induced contractions were neurogenic and mediated by endogenous NA released from adrenergic nerves. Following the addition of phentolamine (1 μM) to the perfusing medium, the contractile responses produced by both TNS and NA were substantially blocked, indicating the involvement of α-adrenoceptors (data not shown).

Experimental protocols

After the preparations had been allowed to equilibrate and a stable tension obtained, they were stimulated with 1 min TNS at 16 Hz and with a 1 min infusion of Krebs containing NA (10 μM), which caused near maximal contraction in pilot experiments. The preparations were then allowed to return to base-line tension. Submaximal tone was then elicited by exposing rings to 1 μM NA. The tone was maintained for the period of time during which relaxant responses to a 1 min infusion of Krebs containing 3–10 μM acetylcholine were tested. A control series of contractile responses to TNS and NA was then performed. Stimulations lasting 1 min were applied at 0.5, 1, 2, 4 and 8 Hz, with a period of at least 10 min between each stimulation. Exogenous NA was administered by superfusing the vessel segment for 1 min with Krebs solution containing 0.1, 0.3, 1 and 3 μM NA. After the control series of TNS and NA responses was completed, NPY or NPY plus antagonists were added to the superfusing medium and allowed to bathe the blood vessels for 30 min. A second series of TNS and NA addition was repeated in the presence of the neuropeptide. Preliminary experiments demonstrated that

contractile responses to TNS and to exogenous NA were reproducible in two experimental periods 30 min apart ($n=3$). In the study involving endothelium denuded vessels, paired vessel rings from the same patients were prepared. The endothelium was removed mechanically by inserting a roughened stainless steel wire into the lumen and gently rolling the rings on wet filter paper. Endothelium removal or integrity was confirmed in each experiment by the loss or the presence of vasorelaxant responses to exogenous acetylcholine, respectively. Since in our previous experiments on endothelium-denuded saphenous vein rings (Fabi *et al*., 1996), the second series with TNS and NA had given higher responses than the control series, we compared only the second series of TNS- and NA-induced contractions in two experimental groups of endothelium-denuded rings with and without NPY.

To normalize the data, the contractile responses of each preparation were expressed as the percentage of the maximum force generated in response to 16 Hz and to 10 μM NA in the control series. The mean frequency- and concentration-response curves were obtained in rings from different patients. Each ring was exposed to only one antagonist, but various antagonists were tested at the same time by use of separate venous rings from the same patient.

Drugs

The following drugs were used: neuropeptide Y, human, (−)-NA bitartrate, acetylcholine chloride, guanethidine sulphate, tetrodotoxin, phentolamine hydrochloride, indomethacin, L-arginine, endothelin-1, U46619, (obtained from Sigma Chemical Co, St Louis, MO, U.S.A.), Bay u3405 (3(**R**)-[[(4-flurophenyl) sulphonyl] amino-1,2,3,4-tetrahydro-9 H-carbazole-9-propanoic acid]) was a generous gift from Bayer Research (Milano, Italy). Ifetroban ([1**S**-(1a,2a,4a)]-2-[[3-[4-[(pentylamino) carbonyl]-2-oxazolyl]-7-oxabicyclo [2.2.1] hept-2-yl] methyl] benzene propanoic acid; formerly BMS-180291) was kindly provided by Bristol-Myers Squibb Pharmaceutical Research Institute (Princeton, NJ, U.S.A.), Ro 47-0203 (4-tert-butyyl-N-[6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2,2′-bipyrimidin-4yl]benzene sulphonamide) was a generous gift from Hoffmann-La Roche (Milano, Italy) and U.K. 38485 (3-(1H-imidazol-1-yl-methyl)-2-methyl-1H-indole-1-propanoic acid) was from Pfizer Italiana (Roma, Italy).

NPY was dissolved to a concentration of 0.5 mg ml^{-1} in 0.9% saline and stored frozen. Working solutions were prepared from the stock solution, and diluted on a daily basis with Krebs solution. NA was dissolved in 0.9% saline containing 0.1% ascorbic acid and kept at +4°C. Indomethacin was dissolved in a small amount of absolute ethanol and sodium bicarbonate (150 mM) solution and then in Krebs solution readjusted to pH 7.4 with HCl before use. Bay u3405 was diluted in NaOH 1 N and the pH readjusted with HCl to about 7.4 before use. UK 38485 was dissolved in 0.1 N NaOH and the pH was adjusted to 8.5 with 0.1 N HCl. All the other drugs were dissolved in distilled water and freshly prepared upon use.

Statistical analysis of results

Values are presented as means ± s.e.mean and n indicates the number of experiments in each group. Frequency-response curves for TNS and concentration-response curves for NA in the groups (1) without endothelium and (2) in the presence of NPY and U 46619 were compared by analysis of variance with repeated measures according to Winer (1971). The comparison of the contractile responses in the absence and presence of NPY or NPY plus antagonists on the same experimental vessel was made by analysis of variance and covariance with repeated measures (BMDP Statistical Software, Inc., Los Angeles, Ca). P value <0.05 was considered to be significant.

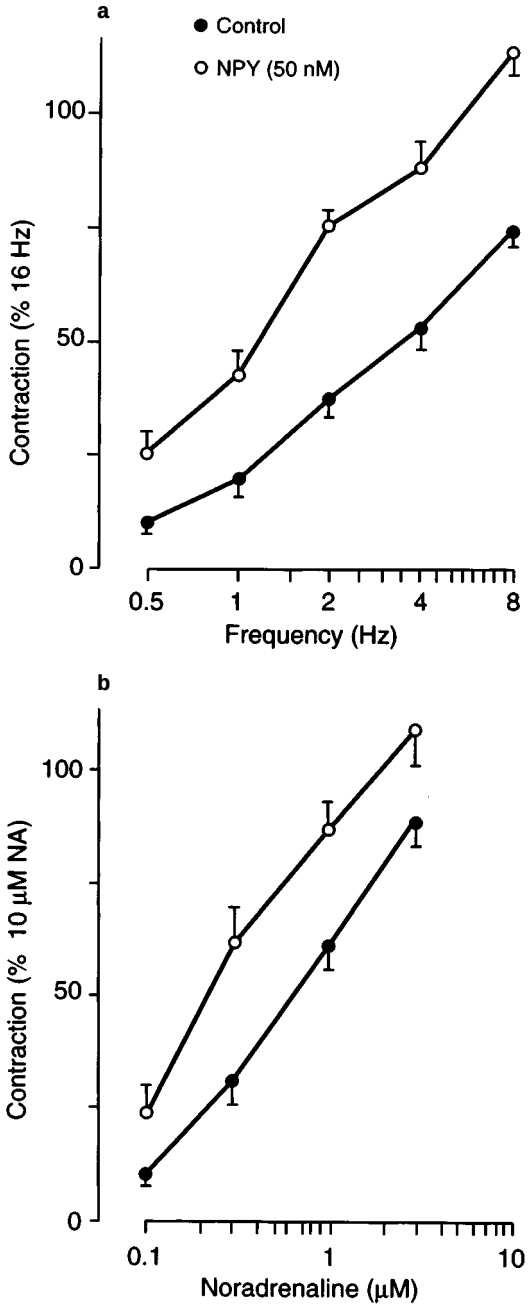

Figure 1 Comparison of the first series (Control) of vasoconstrictor responses to 1 min transmural nerve stimulation (a) and 1 min noradrenaline infusion (b) with the second series of vasoconstrictor responses to the same stimuli obtained 30 min after the addition of 50 nM NPY in superfused human saphenous vein rings with intact endothelium ($n=10$). Contractions are expressed as the percentage of the maximal contraction to transmural nerve stimulation (16 Hz) and noradrenaline (NA, 10 μM) at the beginning of the experiments. The contractile effects of this first series of transmural nerve stimulation and noradrenaline are significantly different from those of the second series in the presence of NPY; $P<0.001$ and $P<0.005$ *vs* control period for transmural nerve stimulation and noradrenaline, respectively. Data points represent means and vertical lines show s.e.mean.

Results

Potentiation by NPY of the vasoconstrictor responses to transmural nerve stimulation and exogenous noradrenaline: influence of endothelium removal

In superfused venous rings with intact endothelium ($n=10$), the frequency- and concentration-dependent vasoconstrictions produced by both TNS and NA infusions were significantly ($F=64.9$, $P<0.001$ and $F=17.5$, $P<0.005$, respectively) enhanced by the presence of NPY (Figure 1), at a concentration (50 nM) that did not by itself cause any contractile effect on the vein rings.

As shown in Figure 2, frequency- and concentration-dependent vasoconstrictions in response to TNS and NA were also obtained in two experimental groups of human saphenous vein rings denuded of endothelium. By comparison between the second series of contractions, according to the protocol described in Methods, the responses induced by both TNS and NA in the control group, i.e. perfused with Krebs in the

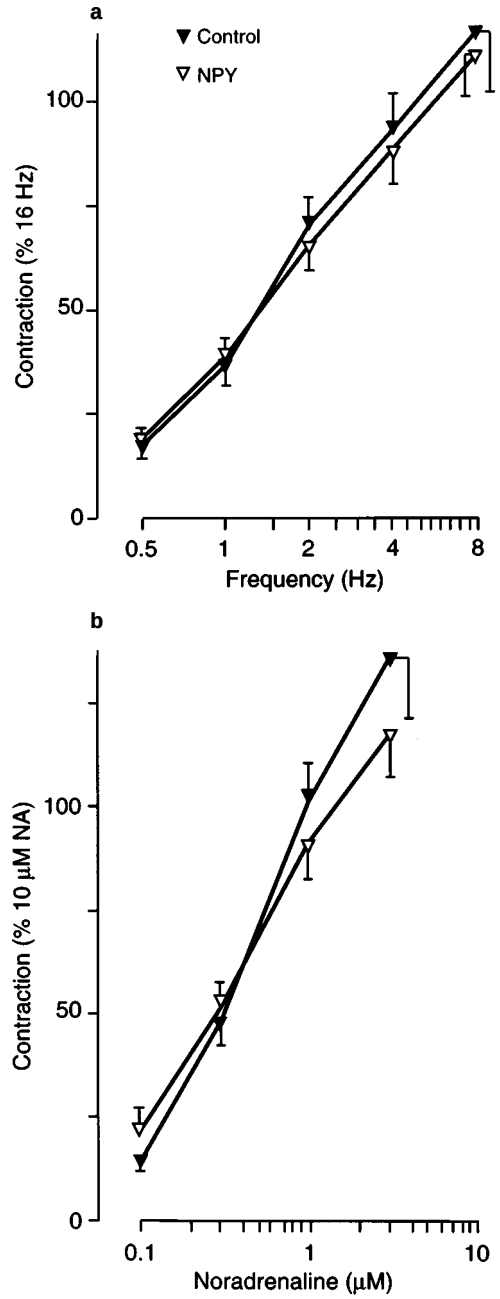

Figure 2 Frequency- and concentration-response curves to transmural nerve stimulation (a) and noradrenaline (b) in two separate groups of human saphenous vein rings without endothelium. The contractions induced by transmural nerve stimulation and noradrenaline in endothelium-denuded vein rings superfused with Krebs solution (Control; $n=10$) did not differ ($P>0.05$ for both) from those of the group superfused with a medium containing 50 nM NPY ($n=10$). Values are means and vertical lines show s.e.mean. For experimental details, see Methods.

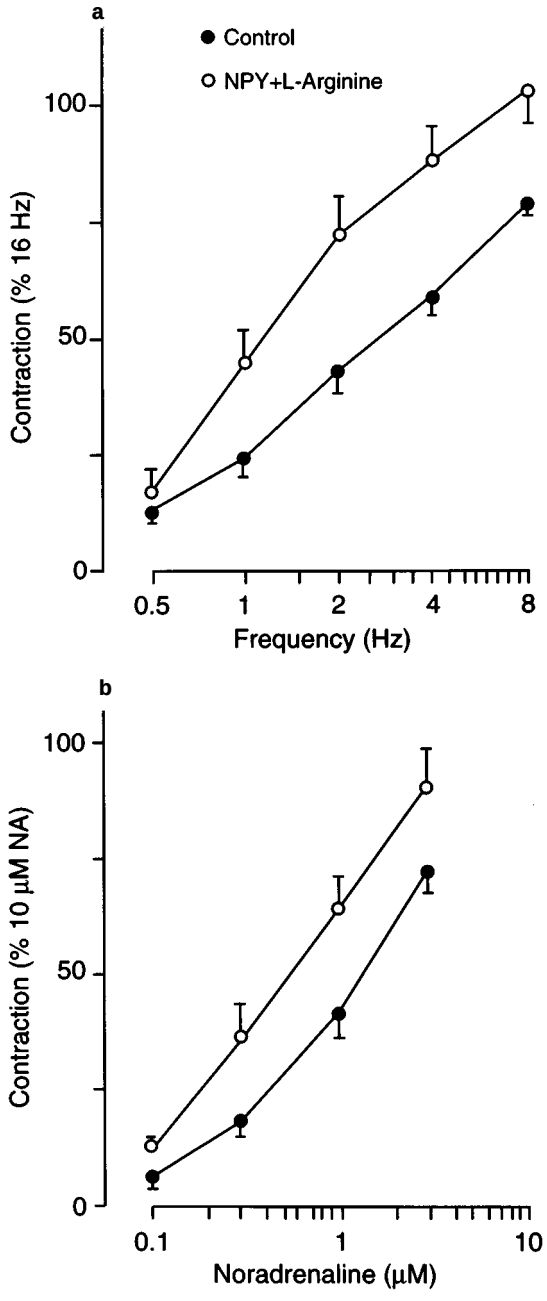

Figure 3 Frequency- and concentration-response curves to transmural nerve stimulation (a) and noradrenaline (b) in the absence (Control) and in the combined presence of 50 nM NPY and 3–10 mM L-arginine in human saphenous vein rings with intact endothelium. The NO precursor L-arginine was not able to counteract the potentiating effects of NPY and the second series of contractile responses induced by either transmural nerve stimulation or noradrenaline were significantly higher than those of the control series ($P<0.025$ and $P<0.01$, respectively). Values are means and vertical lines show s.e.mean of 8 experiments.

absence of NPY, overlapped those of the vein rings superfused with Krebs added with 50 nM NPY ($F=0.2$ and $F=2.7$ for TNS and NA, respectively; $P>0.05$ for both).

Lack of involvement of the nitric oxide pathway or of endothelins in the potentiating action of NPY

In order to verify whether the endothelium-dependent potentiating effect of NPY on noradrenergic contractions may be ascribed to inhibition of NO synthase, as we recently observed for N^{ω}-nitro-L-arginine (L-NOARG) in the same experimental model (Fabi *et al*., 1996), high concentrations of the NO precursor L-arginine (3–10 mM; $n=8$) were added to the perfusing medium together with NPY. However, as shown in Figure 3, after the first series of TNS- and NA-induced contractions had been elicited in venous rings with endothelium, NPY in the combined presence of L-arginine was still able to potentiate both contractions. Indeed, the frequency- and concentration-response curves to TNS and NA were higher than those of the control series ($F=9.9$, $P<0.025$ and $F=12.5$, $P<0.01$, respectively).

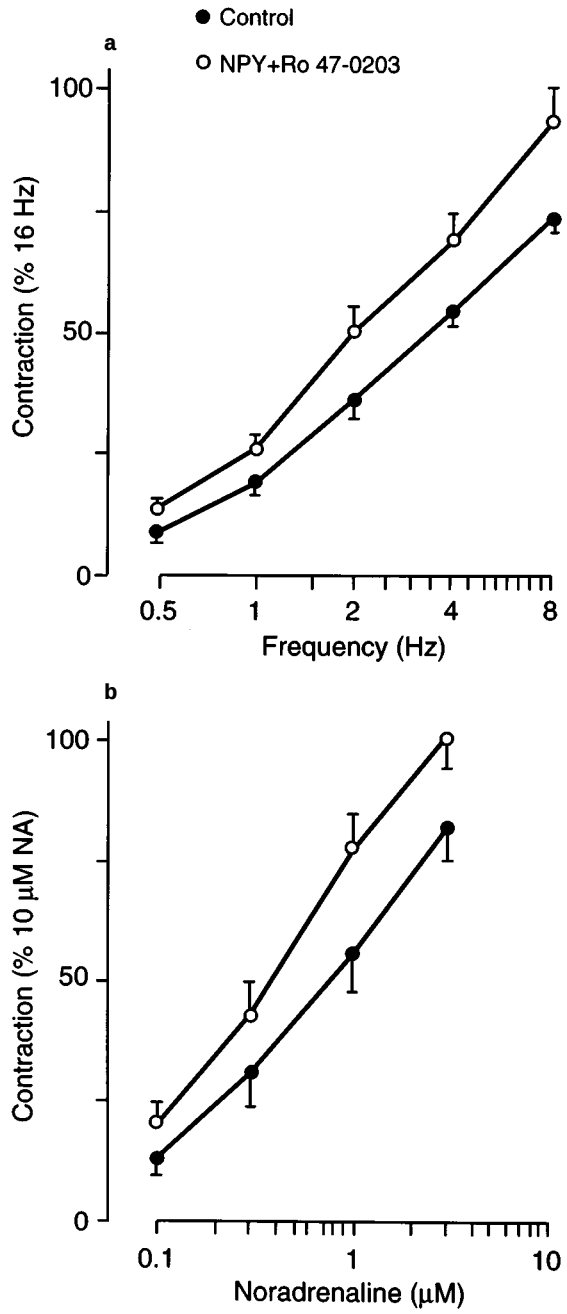

Figure 4 Frequency- and concentration-response curves to transmural nerve stimulation (a) and noradrenaline (b) in human saphenous vein rings with intact endothelium. The contractile curves obtained in the absence (Control) and in the combined presence of 50 nM NPY and the $ET_{A/B}$ receptor antagonist Ro 47-0203 (10 μM) were significantly different ($P<0.005$ for both transmural nerve stimulation and noradrenaline *vs* control). Values are means and vertical lines show s.e.mean of 8 experiments.

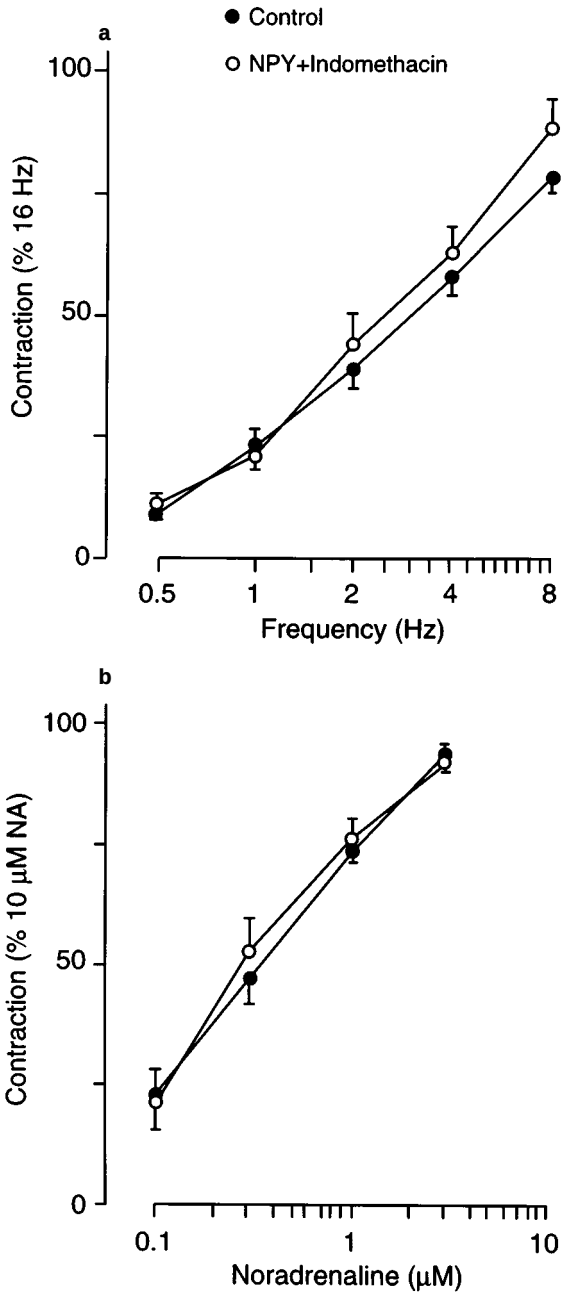

Figure 5 Effects of the combined presence of 50 nM NPY and 30 μM indomethacin ($n=8$) on the frequency- and concentration-contractile curves to transmural nerve stimulation (a) and noradrenaline (b) in human superfused saphenous vein rings with intact endothelium. The contractions evoked by transmural nerve stimulation and noradrenaline in the presence of NPY plus the cyclo-oxygenase inhibitor indomethacin did not differ significantly ($P>0.05$ for both) from those of the control series. Values are means and vertical lines show s.e.mean.

In another experimental group ($n=8$; Figure 4) the possible role of endothelins was verified by using the non specific ET_A/ET_B endothelin receptor antagonist Ro 47-0203. Even in this case, NPY in the presence of the endothelin receptor antagonist in the superfusing medium, significantly potentiated the contractile response curves to both TNS and NA ($F=17.9$, $P<0.005$ and $F=27.8$, $P<0.005$, respectively, as compared to the control series). In preliminary experiments ($n=6$), Ro 47-0203 at the concentration used (10 μM), did not alter the contractions elicited by either TNS or NA, but was able to block completely the contractions evoked by bolus injections of 0.5–1 μg endothelin-1.

Cyclo-oxygenase dependence of the potentiating effect of NPY

As shown in Figure 5, when the cyclo-oxygenase inhibitor indomethacin, in a concentration (30 μM) that had no effect by itself on TNS and NA-induced contractions ($n=8$; data not shown), was added to the medium together with 50 nM NPY, the frequency- and concentration-dependent contractile responses of the venous rings to TNS and exogenous NA overlapped those of the control series ($F=0.56$ and $F=0.08$, respectively; $P>0.05$ for both responses).

Involvement of endogenous production of TxA_2 in the NPY-induced effect

In an attempt to determine whether TxA_2 was the main prostanoid produced by the endothelial cells involved in the potentiating effect of NPY, the effects of the presence of two different TxA_2 receptor antagonists, Bay u3405 (1 μM; $n=9$) and ifetroban (1 μM; $n=8$), in the perfusing medium were examined. At the concentrations used, both TxA_2 antagonists failed to affect the contractions elicited by TNS and NA ($n=8$ and $n=7$ for Bay u3405 and ifetroban, respectively; $P>0.05$ for both) but completely blocked the contractions elicited by 0.5–1 μg bolus injections of the TxA_2 mimetic U 46619 (data not shown). In the presence of both these antagonists, NPY failed to potentiate the TNS- and NA-induced contractions and no significant differences were found between the control contractile curves to TNS and NA in the presence of Bay u 3405 ($n=9$; $F=0.27$ and $F=0.87$ for TNS and NA, respectively; $P>0.05$ for both; Figure 6a,b) or of ifetroban ($n=8$; $F=0.003$ and $F=0.01$ for TNS and NA, respectively; $P>0.05$ for both; Figure 6c,d).

As found with the TxA_2 receptor antagonists, the sympathetic- and the exogenous NA-induced contractile curves in the combined presence of NPY and the TxA_2 synthase inhibitor UK 38485 (10 μM; $n=7$) overlapped ($F=1.84$ and $F=0.12$ for TNS and NA, respectively, $P>0.05$ for both) the control frequency- and concentration-response curves (Figure 6 e,f).

Augmentation of the responses to TNS and NA by U 46619

As shown in Figure 7, in human saphenous venous rings with intact endothelium ($n=10$) the addition to the superfusing medium of U 46619 at a concentration (0.2 nM) that did not

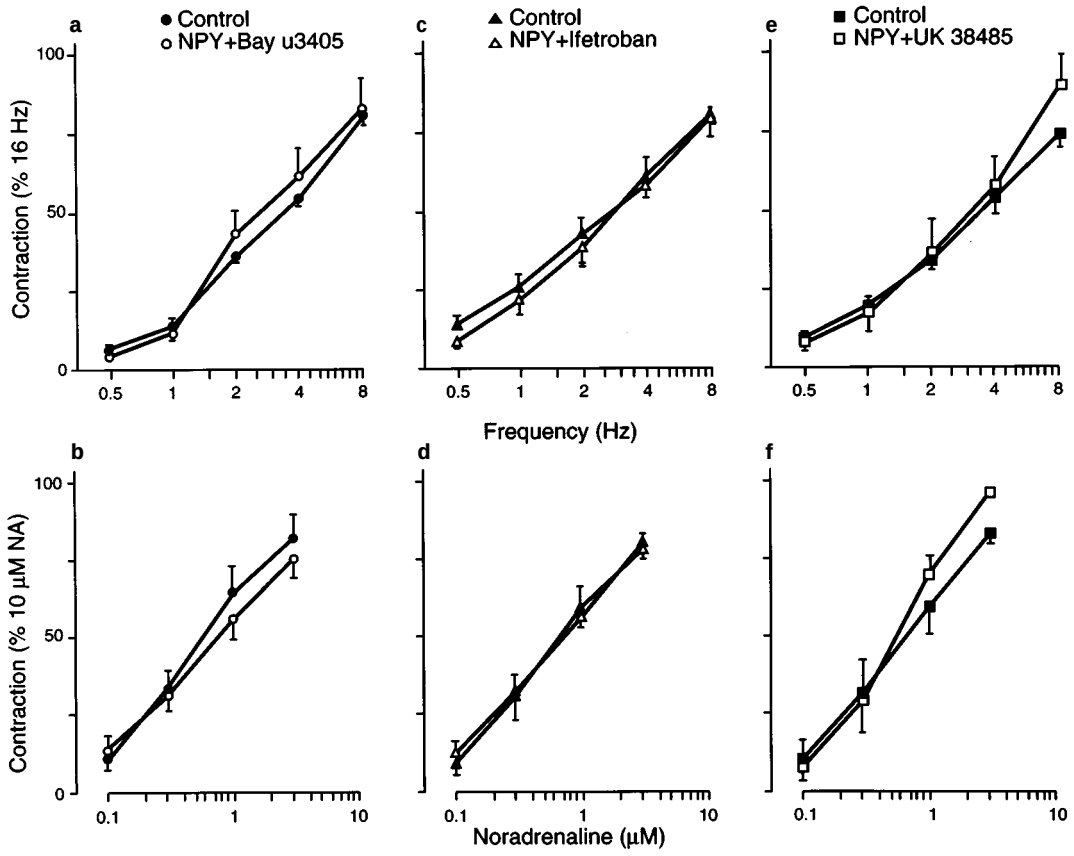

Figure 6 Frequency- and concentration-response curves for transmural nerve stimulation (a, c, e) and noradrenaline (b, d, f) in human saphenous vein rings with intact endothelium. The contractile curves, obtained in the absence (Control) and in the combined presence of 50 nM NPY and either the thromboxane A_2 receptor antagonists Bay u3405 (1 μM; a, b; $n=9$) and ifetroban (1 μM; c, d; $n=8$), or the thromboxane synthase inhibitor UK 38485 (10 μM; e, f; $n=7$) overlapped ($P>0.05$ for all *vs* own controls). Values are means and vertical lines show s.e.mean.

induce any contractile effect by itself, significantly enhanced the contractions induced by both TNS and NA in comparison to the corresponding control contractile curves ($F=27.8$ and $F=46.7$ for TNS and NA, respectively; $P<0.001$ for both).

Discussion

The present study demonstrated firstly that NPY potentiates the contractions elicited by the transmural stimulation of sympathetic nerves in human saphenous veins *in vitro*. This effect is most likely due to an action of the neuropeptide at the postjunctional sites to increase the vascular reactivity to the neurotransmitter released from the sympathetic nerve fibres since, in the same experimental vessels, NPY also potentiated the contractions induced by exogenously administered noradrenaline (NA). This confirms the data previously obtained in several isolated blood vessels from various experimental

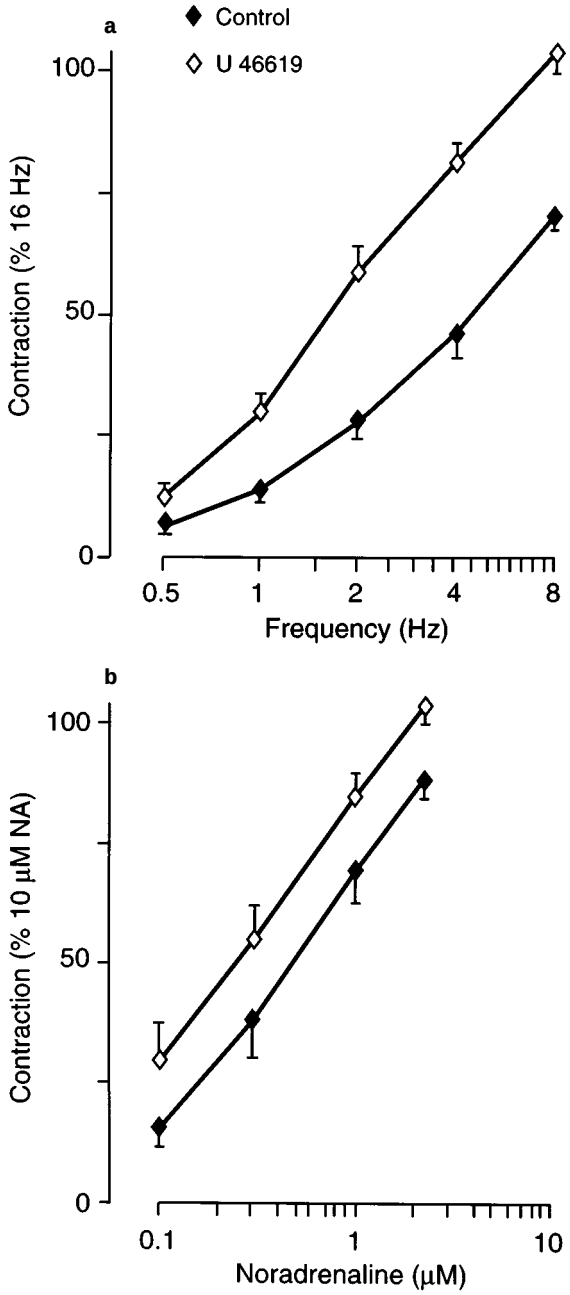

Figure 7 Comparison of the first series (Control) of vasoconstrictor responses to transmural nerve stimulation (a) and noradrenaline (b) with the second series of vasoconstrictor responses to the same stimuli obtained in the presence of U 46619 (0.2 nM) in human superfused saphenous vein rings with intact endothelium. The contractions evoked by transmural nerve stimulation and noradrenaline in the presence of the thromboxane mimetic were significantly different from those of the control series ($P<0.001$ for both). Values are means and vertical lines show s.e.mean of 10 experiments.

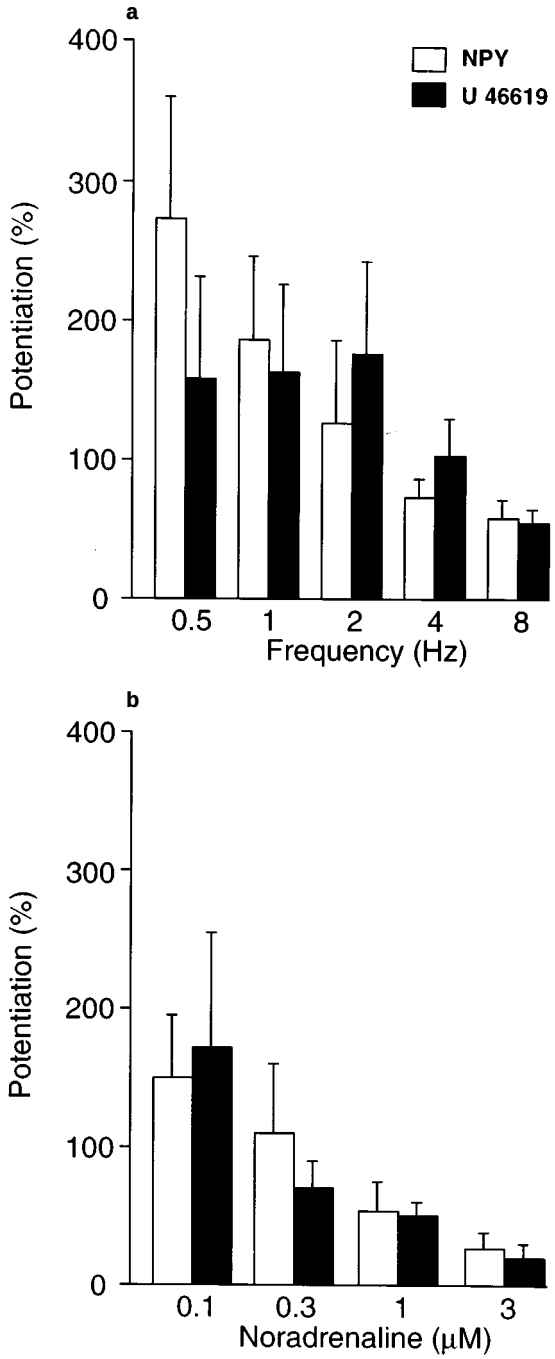

Figure 8 Comparison of the potentiating effects of 50 nM NPY (data from Figure 1) with those induced by 0.2 nM U 46619 (data from Figure 7) on the frequency- and concentration-response curves to transmural nerve stimulation (a) and noradrenaline (b) in superfused human saphenous vein rings with intact endothelium. The augmentations induced by NPY overlapped those induced by the thromboxane agonist ($P>0.05$ for both transmural nerve stimulation and noradrenaline). Potentiation was calculated as the percentage increase of the second series of contractions *vs* the corresponding contractile responses of the first series. Values are means and vertical lines show s.e.mean.

animals (Pernow *et al*., 1986; Wong-Dusting & Rand, 1988; Hieble *et al*., 1989; Saville *et al*., 1990). In addition, with regard to the mode of action of NPY in this phenomenon, our results are primarily in favour of the view that NPY-induced potentiation in human saphenous veins involves the activation of an endothelium-dependent mechanism.

As mentioned in the Introduction, the role of the endothelium on NPY-induced potentiation of noradrenergic vasoconstriction in isolated blood vessels is controversial. In the majority of the studies, carried out on several isolated mammalian vessels, the potentiating effects of NPY on TNS- and/or NA-induced contractions appear to be independent of the presence of an intact endothelium (Gustafsson & Nilsson, 1990; Adamsson *et al*., 1992; Small *et al*., 1992), but few studies have described an endothelium-dependent mechanism as well (Daly & Hieble, 1987; Hieble *et al*., 1989). Some doubt could arise about the role of endothelial cells in the phenomenon in view of the contradictory observations of an endothelium-dependent (Daly & Hieble, 1987) and -independent (Budai *et al*., 1989; Gustafsson & Nilson, 1990) NPY potentiation obtained by different authors in the same type of blood vessel, i.e. rabbit ear artery. Nevertheless, the recent results by Prieto *et al*. (1995) on the bovine retinal artery – which suggest that NPY triggers the release of an endothelium-derived contractile factor which facilitates the NA-induced contraction only in the proximal, but not in the distal, part of the artery – provide a reason for the contrasting results obtained in the rabbit ear artery, as well as confirming the role of the endothelium in NPY potentiation. In addition, an endothelium-dependent potentiation by NPY has also been described in a canine large capacitance vessel, i.e. the saphenous vein (Hieble *et al*., 1989). Thus, in agreement with these latter data, our results provide evidence that in human saphenous veins, the presence of an intact endothelium is a requirement for the NPY-induced potentiation of noradrenergic contractions.

As far as the mechanisms underlying the potentiating effect of NPY are concerned, most of the studies on the action of NPY have been performed on smooth muscle cells, supporting the theories that NPY may potentiate vasoconstriction by inhibiting adenylyl cyclase activity (Lundberg *et al*., 1988) or by promoting a G-protein-mediated calcium entry with increased accumulation of inositol triphosphate (Andriantsitohaina *et al*., 1990; 1993; Duckles & Buxton, 1994). Conversely, with regard to the mode of action of the neuropeptide in the endothelium-dependent potentiation, as far as we know, few data on the role of cyclo-oxygenase products and, by the way, with opposite results in different vessels, have been obtained (Hieble *et al*., 1989; Prieto *et al*., 1995). Therefore, as it is now well recognized that endothelial cells play a primary role in the regulation of the vascular function, mainly due to their capacity to produce strong vasodilator or contracting substances in response to a variety of stimuli (for review, see Li *et al*., 1994), we felt it reasonable to hypothesize that NPY potentiates the noradrenergic contractions of human saphenous veins either (i) by blocking the release of vasodilator substances which could modulate the contractions elicited by the sympathetic mediator or (ii) by producing contractile factors which act synergistically with the neuromediator.

The hypothesis that an NPY-induced inhibition of the nitric oxide (NO) pathway in the endothelial cells may be responsible for its potentiating effect on the noradrenergic contractions come from our previous observations on human saphenous veins, in which the same protocols were used (Fabi *et al*., 1996). In those experiments, the NO synthase inhibitor N^{ω}-nitro-L-arginine (L-NOARG), potentiated the sympathetic contractions of vessels through an endothelium-dependent mechanism, probably blocking the noradrenaline-induced release of NO from the endothelial cells. This hypothesis was confirmed by the fact that the combined presence of L-NOARG and the NO precursor, L-arginine, did not cause any potentiation of the sympathetic contractions. Thus, our present finding that L-arginine at a very high concentration was not able to counteract the NPY-induced potentiation, reasonably suggests that an interference of NPY with the NO pathway or, more precisely, an L-NOARG-like action of NPY, is not the major mechanism of action of the neuropeptide.

A great deal of evidence is now available which demonstrates that in addition to mediating relaxation, the endothelium can also release contracting factors. In rat and canine basilar arteries, endothelium-dependent contracting factors have been suggested to facilitate the contractions elicited by various agonists (Katusic *et al*, 1988; Descombes *et al*., 1993). Moreover, it has been found that in certain large cerebral vessels and peripheral veins the normal endothelium has the propensity to release vasoconstrictor substances (Li *et al*., 1994). Among such endothelium-derived contracting factors, endothelins and cyclo-oxygenase metabolites appeared to us the most probable candidates to be involved in NPY-induced sympathetic potentiation. Indeed, endothelins, the family of peptides with 21 amino acids, which have potent vasoconstrictor properties (Yanagisawa *et al*., 1988; Inoue *et al*., 1989), have also been shown at subthreshold concentrations to potentiate the contractions elicited by NA in human arteries (Yang *et al*., 1990). However, our experiments in the presence of the ET_A/ET_B receptor antagonist Ro 47-0203, (Clozel *et al*., 1994) did not favour a major role of endothelins in the phenomenon. Conversely, the inability of NPY to potentiate noradrenergic contractions in the presence of indomethacin clearly indicates the involvement of cyclo-oxygenase metabolites in NPY potentiation.

The capacity of smooth muscle cells to produce contractile metabolites from arachidonic acid through the cyclo-oxygenase pathway, TxA_2 in particular, is associated in canine and rat veins with the contractions produced by various peptide autacoids, like bradykinin (Aksoy *et al*., 1990; Campos & Calixto, 1994; Marsault *et al*., 1997), but controversial data exist on the role and the production of TxA_2 from endothelial cells. Indeed, this potent vasoconstrictor substance is usually not considered the predominant endothelium-derived contractile factor (Furchgott & Vanhoutte, 1989), probably because, in certain blood vessels, the endothelium-dependent contractions evoked by various agonists are sensitive to cyclo-oxygenase inhibitors, but not abolished by TxA_2 synthase blockers (Furchgott & Vanhoutte, 1989; Vanhoute *et al*., 1991). However, some data exist on the production of TxA_2 from endothelial cells. The endothelium of rabbit pulmonary artery produced TxA_2 both spontaneously and after arachidonic acid stimulation (Buzzard *et al*., 1993). In addition, contractions elicited by certain agonists in the canine and rat basilar artery (Katusic *et al*., 1988; Descombe *et al*., 1993) and in the rabbit intrapulmonary artery (Altiere *et al*., 1986) have been shown to cause endothelial TxA_2 production, that contributes to the contractile response. Finally, as Lin *et al*. (1993) described the formation of TxA_2 by the endothelium in the human internal mammary artery and, Yang *et al*. (1991) suggested that the production of TxA_2 modulates the endothelium-dependent relaxation by acetylcholine in human saphenous veins, we deemed it reasonable to verify whether endogenously produced TxA_2 may be involved in the potentiation by NPY.

This hypothesis was investigated in two different ways. Firstly, we tested the effects of two chemically different receptor antagonists, namely Bay u3405 (McKenniff *et al*., 1991; Norel *et al*., 1991) and ifetroban (Ogletree *et al*., 1993). Next, we inhibited the formation of endogenous TxA_2 by perfusing the tissues with the TxA_2 synthase inhibitor, U.K. 47-0203 (Parry *et al*., 1982). The finding that NPY-induced potentiation was inhibited by TxA_2 receptor antagonists and the TxA_2 synthase inhibitor in the same way as it was by indomethacin strongly supports the notion that, among the endogenously produced cyclo-oxygenase metabolites, TxA_2 plays a substantial role in the NPY-induced potentiation of the noradrenergic contractions.

To give further support to this speculation, of particular relevance are the results of our experiments with U 46619, showing that threshold concentrations of the TxA_2 stable agonist that did not elicit significant contractions by themselves, were capable of potentiating the contractions induced by both sympathetic stimulation and exogenous NA. In addition, this augmentation mimicked in extent and shape the potentiation produced by NPY in these vessels when the same experimental procedure and protocol was utilized. Indeed, as shown in Figure 8, (a) the percentage potentiating effect of both U 46619 and NPY on TNS and NA was higher at lower stimulation frequencies and lower NA concentrations and (b) the augmentation induced by U 46619 at all frequencies and concentrations tested completely overlapped the potentiation produced by NPY (percentage potentiation calculated on the data of Figure 7 and Figure 1; $F=0.08$ and $F=0.10$ for TNS and NA, respectively; $P>0.05$ for both). All in all, our results suggest that endogenously produced TxA_2, acting synergistically with the noradrenergic mediator, enhances the responses to sympathetic stimulation in human saphenous veins. This action of TxA_2 may give some further insight into the mechanisms of vasospastic diseases, suggesting that not only the well-known platelet-derived, but also the vascular, release of TxA_2 can contribute to the increase in the contractile responses to noradrenergic mediators in a human large capacitance vessel.

Taking into account all our findings, we hypothesize that, in human saphenous veins, NPY potentiates the sympathetic vasoconstriction through a postjunctional action, by stimulating the release of TxA_2 from endothelial cells. Thus, given that it has been demonstrated that NPY, at a higher concentration than we used, also induces contractions in human saphenous veins without endothelium *in vitro* (Luu *et al*., 1992), our data further illustrates the complex interactions NPY has with perivascular neuroeffector mechanisms and show the modes by which NPY may also contribute to the sympathetic enhancement of the tone of venous capacitance vessels.

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(Received November 11, 1997
Revised February 2, 1998
Accepted February 3, 1998)