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European Journal of Pharmacology 484 (2004) 277–285
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Endothelium-dependent noradrenergic hyperresponsiveness induced by thapsigargin in human saphenous veins: role of thromboxane and calcium

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Received 24 July 2003; received in revised form 31 October 2003; accepted 7 November 2003

Abstract

To further investigate the mechanisms which regulate sympathetic vascular tone, we studied the effects of the sarcoplasmic reticulum Ca^{2+}-ATPase inhibitor, thapsigargin, on the vasoconstriction induced by transmural nerve stimulation and noradrenaline in superfused human saphenous vein rings. The contractions induced by both transmural nerve stimulation and noradrenaline were potentiated by thapsigargin in endothelium-intact, but not in endothelium-denuded vessels. This potentiation was unaffected by the non-selective endothelin $ET_{A/B}$ receptor antagonist, Ro 47-0203 (4-tert-Butyyl-*N*-[6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2,2′-bipyrimidin-4yl]benzene sulfonamide), or by the nitric oxide (NO) synthase inhibitor, L-NNA (N^{ω}-nitro-L-arginine), but was inhibited by the thromboxane A_2 receptor antagonist, Bay u3405 (3(**R**)-[[(4-flurophenyl) sulphonyl]amino-1,2,3,4-tetrahydro-9H-carbazole-9-propanoic acid]) or by the thromboxane A_2 synthase inhibitor, UK 38485 (3-(1H-imidazol-1-yl-methyl)-2-methyl-1H-indole-1-propanoic acid). Moreover, the thapsigargin-induced noradrenergic hyperresponsiveness, as well as that produced by subthreshold concentrations of the thromboxane A_2 mimetic, U 46619, were blocked by the Ca^{2+} channel antagonist, verapamil. In conclusion, our results indicate that thapsigargin enhances the contractions produced by sympathetic nerve stimulation in human saphenous vein rings through the endothelial release of thromboxane A_2 that potentiates the vasoconstriction induced by the noradrenergic mediator with a verapamil-sensitive mechanism.

Keywords: Thapsigargin; Saphenous vein, human; Sympathetic vascular tone; Thromboxane A_2; Endothelin; Verapamil

1. Introduction

It is well recognized that endothelial cells play a key role in regulating vascular tone, namely through the release of both endothelium-derived relaxing (EDRF) or contracting (EDCF) factors (Vanhoutte et al., 1986; Furchgott and Vanhoutte, 1989; Moncada et al., 1991; Lüscher et al., 1992). Depending on the stimulus or agonist utilized, numerous physiological actions of both EDRF or EDCF have been demonstrated in studies on various isolated blood vessels from experimental animals and humans (Furchgott and Vanhoutte, 1989; Moncada et al., 1991; Li et al., 1994).

As far as the sympathetic control of vascular tone by EDRF is concerned, nitric oxide (NO) has been shown to play an important modulatory (inhibitory) role in noradrenergic contractions in various arteries of different species (Liu et al., 1991; Vö et al., 1991; Thorin and Atkinson, 1994; Aldasoro et al., 1993). There is less information about the influence of NO on the noradrenergic tone of veins. Nevertheless, we previously described (Fabi et al., 1996) the inhibitory action induced by NO on the sympathetic contraction of a large human capacitance vessel, i.e. the human saphenous vein. Regarding the modulation of vascular tone by EDCF (Furchgott and Vanhoutte, 1989), endothelins have been shown to potentiate noradrenergic vasoconstriction in rat mesenteric arteries (Tabuchi et al., 1989). In addition, endothelial thromboxane A_2 has been shown to be involved in both the vasoconstriction elicited by various agonists in different animal arteries (Altiere et al., 1986; Descombes et al., 1993; Nishimura et al., 1995; Shirahase et

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doi:10.1016/j.ejphar.2003.11.017

al., 1995) and the noradrenergic hyperresponsiveness induced by neuropeptide Y in human saphenous vein (Fabi et al., 1998). Therefore, taking into account that the endothelium of human saphenous vein has been shown to produce both NO and thromboxane A_2, we deemed that a further study on these vessels might add to the insight into the mechanisms of endothelial regulation of vascular tone, in particular of sympathetic venous tone.

It has been clearly demonstrated that agonist-induced Ca^{2+} entry and changes in intracellular free Ca^{2+} concentration are the major determinants for the synthesis or release of endothelial factors (Sudjarwo et al., 1992; Moritoki et al., 1994; Fukao et al., 1999; Taniguchi et al., 1999; Huang et al., 2000; Tran et al., 2000). As a valuable tool in these studies, the naturally occurring sesquiterpene lactone, thapsigargin, the specific and potent inhibitor of the sarcoplasmic reticulum Ca^{2+}-ATPase (Thastrup et al., 1990; Tran et al., 2000), has been widely used. However, discrepant results have been obtained on the type of thapsigargin-released endothelial factor. Indeed, thapsigargin has been described to cause endothelium-dependent relaxation in guinea pig and rat aorta as well as in porcine coronary artery (Moritoki et al., 1994; Huang et al., 2000; Matsuyama et al., 1993; Kuroiwa-Matsumoto et al., 2000), whereas it inhibited the acetylcholine- and substance P-induced relaxations in rabbit aorta (Amerini et al., 1996) and caused triphasic responses in porcine renal artery which were ascribed to the simultaneous release of NO, EDHF and thromboxane A_2 (Ihara et al., 1999). In addition, concerning the Ca^{2+} entry pathway into vascular endothelium, although it has been generally found that the dominant Ca^{2+} influx into endothelial cells is the capacitative Ca^{2+} entry pathway (Putney and McKay, 1999), a certain reduction of the agonist-stimulated cytosolic Ca^{2+} entry has also been observed in the presence of voltage-dependent Ca^{2+} channels blockers (Bossu et al., 1992; Iouzalen et al., 1995).

Therefore, main purposes of our work on human saphenous vein were: (i) to investigate whether endothelial factor(s) produced by thapsigargin may affect the sympathetic tone of human saphenous vein; (ii) to characterize pharmacologically the type of endothelial factor(s) released by thapsigargin and (iii) to examine whether a voltage-sensitive Ca^{2+} channel blocker, like verapamil, can affect the thapsigargin-induced action.

A preliminary account of this work was presented at the XVIth World Congress of Pharmacology, at San Francisco, in 2002 (Del Basso Orsini et al., 2002).

2. Material and methods

2.1. Preparation of tissue

The experimental procedure and the techniques for measuring the contractile responses of the isolated human saphenous vein have been described in detail previously (Fabi et al., 1998) and will be given only briefly here. The investigation conforms to the principles outlined in the Declaration of Helsinki. Human saphenous vein segments were taken from patients undergoing surgery for aorta-coronary bypass grafting. During surgical preparation of the saphenous vein, the dilation procedure was avoided. Immediately after excision the tissue was placed in an oxygenated Krebs solution at 4 °C. 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 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 (pH 7.4). The tension of the circular muscle layer was recorded with a Grass FT 0.3 T isometric force transducer (Grass Instrument, Quincy, MA, USA) coupled to a polygraph (Grass model 7D).

Transmural nerve stimulation of the vessel rings was delivered for 1 min through platinum wire electrodes placed on both sides of the vessel. The rectangular pulses applied by a programmable electrical stimulator (type BM ST6; Biomedica Mangoni, Pisa, Italy) at 0.5–16 Hz were 0.3 ms in duration and had supramaximal voltage (14 V measured across the electrodes). As already described (Fabi et al., 1993, 1996), the contractions induced by transmural nerve stimulation, but not by exogenous noradrenaline, were blocked by superfusing the vessel rings with Krebs containing 2 μM tetrodotoxin or with 10 μM guanethidine, thus demonstrating their sympathetic origin.

2.2. Experimental protocols

After the preparations had been allowed to equilibrate and a stable tension was obtained, they were stimulated with 1-min transmural nerve stimulation at 16 Hz and with a 1-min infusion of Krebs containing noradrenaline in a concentration (10 μM) which had been shown to cause maximal contraction in pilot experiments. The preparations were then allowed to return to baseline tension. Submaximal tone was then elicited by exposing the rings to 1 μM noradrenaline. The tone was maintained for the period during which relaxant responses to 1-min infusion of Krebs containing 3–10 μM acetylcholine were tested. A control series of contractile responses to transmural nerve stimulation and noradrenaline 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 noradrenaline was administered by superfusing the vessel segment for 1 min with Krebs solution containing 0.1,

0.3, 1, 3 μM noradrenaline. After the control series of transmural nerve stimulation and noradrenaline responses was completed, thapsigargin or thapsigargin plus antagonists was added to the superfusing medium and allowed to bathe the blood vessels for 30 min. A second series of transmural nerve stimulation and noradrenaline addition was repeated in the presence of thapsigargin. Preliminary experiments confirmed that contractile responses to transmural nerve stimulation and to exogenous noradrenaline were reproducible in two experimental periods 30 min apart ($n=3$; data not shown).

In the endothelium denudation study, 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 denudation 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 transmural nerve stimulation and noradrenaline had given higher responses than the control series, we now compared only the second series of transmural nerve stimulation- and noradrenaline-induced contractions in two experimental groups of endothelium-denuded rings superfused with or without thapsigargin. This experimental procedure was also used for the studies with verapamil. Indeed, as already described (Fabi et al., 1993), in the presence of this Ca^{2+} antagonist, the contractions evoked by the second series of both transmural nerve stimulation and noradrenaline were lower than those of the first control series. Thus, after the first series was performed, the second series of two vessel rings from the same patient, which had been superfused with Krebs containing verapamil or verapamil plus thapsigargin, was compared. The same experimental procedure was utilized in the studies concerning the effect of verapamil on the noradrenergic hyperresponsiveness induced by the thromboxane A_2 mimetic, U 46619.

To normalize the data, the contractile responses of each preparation were expressed as a percentage of the maximum force generated in response to 16 Hz and to 10 μM noradrenaline at the beginning of the experiments. Mean frequency- and concentration–response curves were obtained with rings from different patients. Each ring was exposed to only one antagonist, but various antagonists were tested at the same time using separate venous rings from the same patient.

2.3. Drugs

All chemicals used were of analytical grade. Thapsigargin was obtained from Biomol, Plymouth Meeting, PA, USA; (−)-noradrenaline bitartrate, acetylcholine chloride, N^{ω}-nitro-L-arginine (L-NNA), verapamil hydrochloride, endothelin-1, U 46619 (9,11-dideoxy-9α,11α-epoxymethanoprostaglandin-$F_{2\alpha}$) were all obtained from Sigma, St. Louis, MO, USA. Ro 47-0203 (4-tert-Butyyl-*N*-[6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2,2′-bipyrimidin-4yl]benzene sulfonamide) was a generous gift from Hoffmann-La Roche, Milano, Italy. Bay u3405 (3(**R**)-[[(4-flurophenyl) sulphonyl]amino-1,2,3,4-tetrahydro-9H-carbazole-9-propanoic acid]) was a generous gift from Bayer Research, Milano, Italy. UK 38485 (3-(1H-imidazol-1-yl-methyl)-2-methyl-1H-indole-1-propanoic acid) was kindly supplied from Pfizer Italiana, Roma, Italy.

Thapsigargin was dissolved to a concentration of 5 mg/ml in absolute ethanol and stored frozen (−20 °C). Working solutions were prepared from the stock solution, and diluted on a daily basis with Krebs solution. Noradrenaline was dissolved in 0.9% saline containing 0.1% ascorbic acid and kept at +4 °C. Bay u3405 was diluted in NaOH 1N and the pH was 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.

2.4. Statistical methods

The data are expressed as means ± S.E.M. and n indicates the number of experiments in each group. The comparison of the contractile responses in the absence and presence of thapsigargin or thapsigargin plus antagonists on the same experimental vessel or in two different experimental groups was made by repeated measures analysis of variance (ANOVA) with Bonferroni-Dunn's procedure for multiple comparisons when appropriate, calculated with a Macintosh MacOS 9 computer using the data analysis package Stat View (Abacus Concepts, Berkeley, CA, USA, 1992). A P value <0.05 was considered to be significant, unless differently required for the statistical analysis.

3. Results

3.1. Effect of thapsigargin on the vasoconstrictor responses to transmural nerve stimulation and exogenous noradrenaline in human saphenous veins with or without endothelium

Transmural nerve stimulation of the superfused venous rings with intact endothelium produced a frequency-dependent vasoconstriction which reached its maximum at 16 Hz (6.8 ± 1.5 g, $n=9$). In the same preparations, 1-min infusion of noradrenaline produced a concentration-dependent vasoconstriction that mimicked the response to transmural nerve stimulation (maximum contraction at 10 μM 7.8 ± 2.0 g; $n=9$).

In superfused venous rings with intact endothelium ($n=9$), the frequency- and concentration-dependent vasoconstrictions produced by both transmural nerve stimulation and noradrenaline were significantly enhanced ($P<0.05$ for both transmural nerve stimulation and noradrenaline) by the presence of thapsigargin in a concen-

tration (50 nM) that did not itself cause any contractile effect on the vein (Fig. 1A,B).

To examine the possible involvement of endothelium in the thapsigargin-induced noradrenergic hyperresponsiveness, frequency– and concentration–response curves for transmural nerve stimulation and noradrenaline were obtained in two experimental groups of human saphenous vein rings denuded of endothelium in the presence or absence of thapsigargin ($n=9$, each). Under these conditions (Fig. 1C,D), the responses induced by both transmural nerve stimulation and noradrenaline in the control group, i.e. perfused with Krebs in the absence of thapsigargin, overlapped the responses in the experiments performed on vein rings superfused with Krebs added with thapsigargin ($P>0.05$ for both transmural nerve stimulation and noradrenaline).

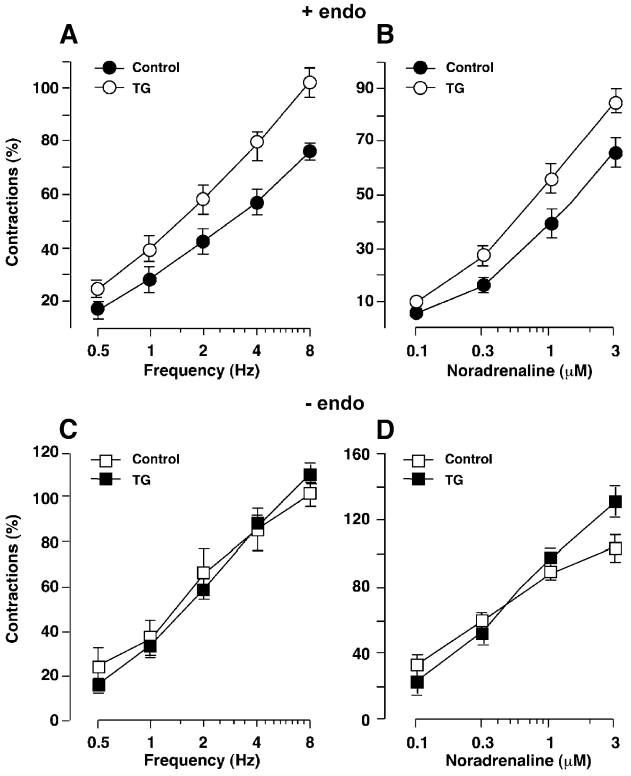

Fig. 1. (A,B) Comparison of the first series (Control) of vasoconstrictor responses to 1-min transmural nerve stimulation (A, Frequency) and 1-min noradrenaline infusion (B) with the second series of vasoconstrictor responses to the same stimuli obtained 30 min after the addition of 50 nM thapsigargin (TG) in superfused human saphenous vein rings with intact endothelium ($n=9$). The contractile effects of the first series of transmural nerve stimulation and noradrenaline are significantly less than those of the second series carried out in the presence of thapsigargin. $P<0.05$ for both transmural nerve stimulation and noradrenaline vs. control. (C,D) Frequency– and concentration–response curves for transmural nerve stimulation (C) and noradrenaline (D) in two separate groups of human saphenous vein rings without endothelium. The contractions induced by the second series of transmural nerve stimulation and noradrenaline in endothelium-denuded vein rings superfused with Krebs solution (Control; $n=9$) do not differ ($P>0.05$ for both) from those of the group superfused with the medium containing 50 nM thapsigargin (TG; $n=9$). Contractions are expressed as percentages of the maximal contraction in response to transmural nerve stimulation (16 Hz) and noradrenaline (10 μM) at the beginning of the experiments. Data points represent means, and vertical lines show S.E.M.

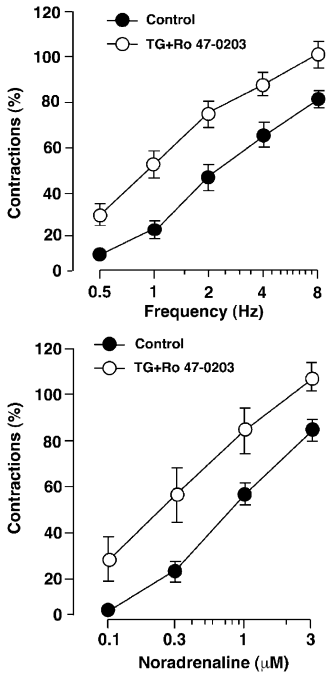

Fig. 2. Frequency– and concentration–response curves for transmural nerve stimulation and noradrenaline in human saphenous vein rings with intact endothelium. The contractile curves obtained in the absence (Control) are significant lower than those obtained in the combined presence of thapsigargin (50 nM; TG) and the endothelin $ET_{A/B}$ receptor antagonist Ro 47-0203 (10 μM). $P<0.005$ for transmural nerve stimulation and $P<0.05$ for noradrenaline, respectively vs. control. Values are means, and vertical lines show S.E.M. of nine experiments.

3.2. Lack of effects of the endothelin $ET_{A/B}$ receptor antagonist, Ro 47-0203, on the thapsigargin-induced noradrenergic potentiation

The possible involvement of thapsigargin-induced endothelial release of endothelins in the hyperresponsiveness to noradrenergic contraction was tested by using the non-specific endothelin $ET_{A/B}$ receptor antagonist, Ro 47-0203. In the presence of this drug, at a concentration (10 μM) that completely blocked the almost maximal contraction induced by a bolus injection of endothelin-1 (0.5–1 μg; $n=3$; data not shown), the addition of thapsigargin in the perfusing medium was still able to potentiate the contractions induced by both transmural nerve stimulation and noradrenaline (Fig. 2). Indeed, the frequency and the concentration–response curves for transmural nerve stimulation and for noradrenaline in the simultaneous presence of the endothelin antagonist and thapsigargin were significantly shifted to the left compared to those of the control series ($n=9$; $P<0.005$ and $P<0.05$ for transmural nerve stimulation and noradrenaline, respectively).

3.3. Inhibition of the thapsigargin-induced noradrenergic hyperresponsiveness by a thromboxane A_2 receptor antagonist and a thromboxane A_2 synthase inhibitor

In the attempt to determine whether endothelial production of thromboxane A_2 could be involved in the thapsigargin-induced potentiation of the noradrenergic vasoconstrictions, we tested the effect of the presence in the perfusing medium of the thromboxane A_2 receptor antagonist, Bay u3405. In the presence of the antagonist, at a concentration (1 μM) able to completely block the contractions elicited by 0.5–1 μg bolus injections of the thromboxane A_2 mimetic U 46619 ($n=3$; data not shown), thapsigargin failed to potentiate the transmural nerve stimulation- and noradrenaline-induced contractions. As shown in Fig. 3A,B, no significant differences were found between the contractile curves for transmural nerve stimulation and noradrenaline in the absence or in the presence of the thromboxane A_2 antagonist plus thapsigargin ($n=6$; $P>0.05$ for both transmural nerve stimulation and noradrenaline).

According to the results obtained with the thromboxane A_2 receptor antagonist, in the presence of the thromboxane

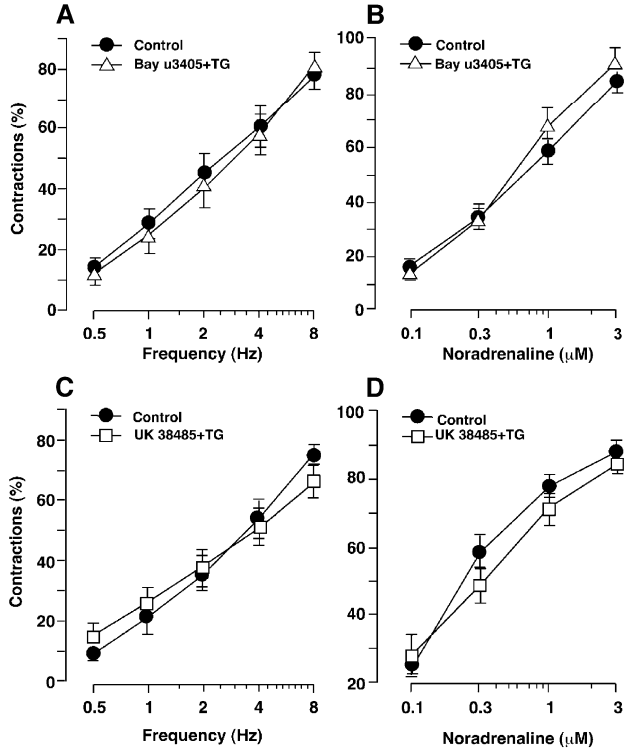

Fig. 3. Frequency– and concentration–response curves for transmural nerve stimulation (A,C,) and noradrenaline (B,D,) in human saphenous vein rings with intact endothelium. The contractile curves obtained in the absence (Control) and in the combined presence of 50 nM thapsigargin (TG) with either the thromboxane A_2 receptor antagonist Bay u3405 (1 μM; A,B, $n=6$) or the thromboxane A_2 synthase inhibitor, UK 38485 (10 μM; C,D; $n=6$) overlap. $P>0.05$ for all vs. own controls. Values are means, and vertical lines show S.E.M.

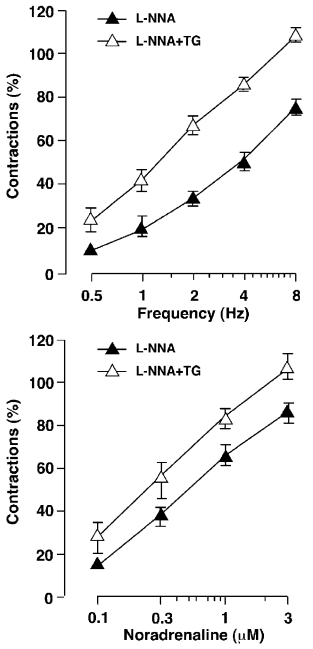

Fig. 4. Frequency– and concentration–response curves for transmural nerve stimulation and noradrenaline in human saphenous vein rings with intact endothelium. The contractile curves obtained in the presence of the nitric oxide synthase inhibitor, L-NNA (3 μM), alone are significantly lower than those carried out in the combined presence of L-NNA and 50 nM thapsigargin (TG). $P<0.001$ for transmural nerve stimulation and $P<0.05$ for noradrenaline, respectively, vs. control. Values are means and vertical lines show S.E.M. of six experiments.

A_2 synthase inhibitor, UK 38485 (10 μM; $n=6$; Fig. 3C,D), further addition of thapsigargin did not alter the noradrenergic contractions and the frequency– and concentration–response curves for transmural nerve stimulation and noradrenaline overlapped those of the control series ($P>0.05$ for both transmural nerve stimulation and noradrenaline).

3.4. Effect of nitric oxide synthase inhibition on the thapsigargin-induced noradrenergic potentiation

To examine whether thapsigargin-induced release of NO by endothelium could also be involved in the action of thapsigargin on noradrenergic contractions, experiments were performed on vein rings with endothelium superfused with Krebs solution plus the NO synthase inhibitor, L-NNA (3 μM). As shown in Fig. 4, addition of thapsigargin to the superfusing medium containing L-NNA, enhanced the contractions induced by transmural nerve stimulation and noradrenaline and the frequency– and concentration–response curves for both transmural nerve stimulation and noradrenaline were shifted to the left as compared to those of the control series ($P<0.001$ for transmural nerve stimulation and $P<0.05$ for noradrenaline, respectively). Moreover, the presence of L-NNA did not appear to affect the potentiating action of thapsigargin, which was similar

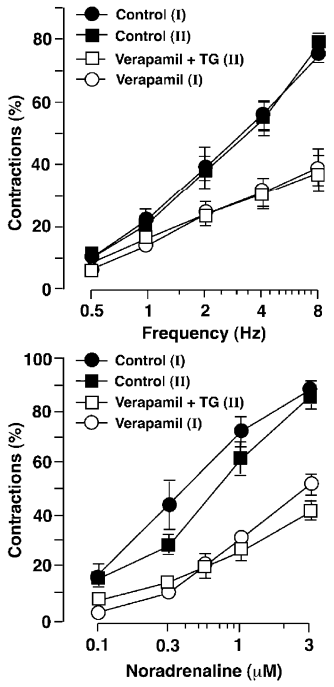

Fig. 5. Frequency– and concentration–response curves for transmural nerve stimulation and noradrenaline in two separate groups (I, $n=8$; II, $n=7$) of human saphenous vein rings with endothelium. The contractions induced by the first series of transmural nerve stimulation and noradrenaline in the two groups (Control I and Control II) overlap ($P>0.05$ for both frequency– and noradrenaline–contraction responses). Similarly, no significant differences were found between the second series of responses to transmural nerve stimulation and noradrenaline ($P>0.05$ for both) superfused with a medium containing the Ca^{2+} antagonist, verapamil, alone (10 μM; verapamil I) or verapamil plus 50 nM thapsigargin (verapamil + TG; II). Values are means, and vertical lines show the S.E.M.

to the potentiation observed in the control and in the Ro 47-0203-treated groups (Figs. 1 and 2).

3.5. Inhibition by verapamil of the noradrenergic hyperresponsiveness induced by thapsigargin

As already observed with superfused human saphenous vein (Fabi et al., 1993), addition of verapamil to the medium caused a time-dependent reduction of the transmural nerve stimulation- and noradrenaline-induced contractile responses, so that two reproducible experimental periods for transmural nerve stimulation and noradrenaline could not be obtained with the same vessel ring. Therefore, to test the effect of verapamil on the noradrenergic hyperresponsiveness produced by thapsigargin, after the first series of transmural nerve stimulation- and noradrenaline-induced contractions had been performed with two vessel rings from the same patient (control series), in the second series, the vessels were superfused with medium containing 10 μM verapamil alone ($n=8$) or verapamil with thapsigargin added ($n=7$).

As shown in Fig. 5, the control series of responses produced by both transmural nerve stimulation and exogenous noradrenaline in the two experimental groups overlapped ($P>0.05$ for both transmural nerve stimulation and noradrenaline). In the same manner, by comparing the second series of transmural nerve stimulation- and noradrenaline-induced contractions, according to the protocol described above, the noradrenergic responses in the group perfused with Krebs containing verapamil overlapped the responses of vein rings superfused with Krebs containing verapamil plus thapsigargin ($P>0.05$ for both transmural nerve stimulation and noradrenaline).

3.6. Inhibition by verapamil of the noradrenergic hyperresponsiveness induced by the thromboxane A_2-mimetic, U 46619

As we previously reported (Fabi et al., 1998), in superfused human saphenous veins without endothelium, the presence of U 46619 at a concentration (0.2 nM) that did not itself induce any contractile effect potentiated the contractions induced by both transmural nerve stimulation and noradrenaline in comparison to the controls. Therefore, to verify whether the inhibition by verapamil of the thapsigargin-induced noradrenergic potentiation could be ascribed either to the inhibition of the synthesis/release of thrombox-

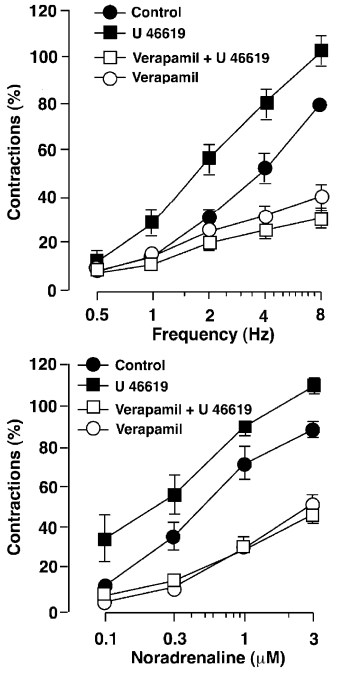

Fig. 6. Frequency– and concentration–response curves for transmural nerve stimulation and noradrenaline in four separate groups of human saphenous vein rings. In vessels superfused with Krebs solution (Control, $n=5$), the contractions evoked by transmural nerve stimulation and noradrenaline are less ($P<0.05$ for both) than those of vessels superfused with Krebs combined with the thromboxane A_2 mimetic U 46619 (0.2 nM; $n=6$). On the contrary, no significant differences were found between the responses to transmural nerve stimulation and noradrenaline ($P>0.05$ for both) in the presence of 10 μM verapamil alone ($n=8$; verapamil) or in the simultaneous presence of verapamil with U 46619 ($n=7$; verapamil + U 46619). Values are means, and vertical lines show the S.E.M.

ane A_2 by the endothelial cells or to the next step, that leads to the thromboxane A_2-induced noradrenergic potentiation, we examined the effect of the Ca^{2+} antagonist on the U 46619-induced noradrenergic hyperresponsiveness.

Fig. 6 shows that the contractions induced by both transmural nerve stimulation and noradrenaline in the venous rings superfused with the medium containing U 46619 ($n=6$) were stronger than those of the control vessels ($n=5$; $P<0.05$ for both transmural nerve stimulation and noradrenaline). On the contrary, U-46619 did not cause any noradrenergic potentiation in the presence of verapamil. Indeed, the contractions elicited by the second series of both transmural nerve stimulation and noradrenaline in two different groups superfused with verapamil alone ($n=8$) or with verapamil plus U 46619 ($n=7$) overlapped ($P>0.05$ for both transmural nerve stimulation and noradrenaline).

4. Discussion

The present study provided evidence that thapsigargin induces potentiation of the contractions elicited by transmural stimulation of sympathetic nerves in human saphenous veins in vitro. Because, in the same experimental vessels, thapsigargin also potentiates the contractions induced by exogenous administered noradrenaline, the effect is most likely due to an action of thapsigargin at postjunctional sites to increase vascular reactivity to the neurotransmitter released from the sympathetic nerve fibers. In addition, our results clearly indicate that the thapsigargin-induced noradrenergic hyperresponsiveness in human saphenous veins is dependent on the integrity of vascular endothelium.

As far as the mechanisms of the thapsigargin-induced action are concerned, it is now well established that a low concentration of this sesquiterpene lactone is able to specifically block the sarco/-endoplasmic Ca^{2+} pump (Thastrup et al., 1990) in non-excitable cells such as endothelium, resulting in a gradual emptying of Ca^{2+} stores, that leads to an increase of Ca^{2+} entry, mainly through capacitative channels (Gericke et al., 1993; Gibson et al., 1998; Treiman et al., 1998). Therefore, considering that Ca^{2+} ion-dependence of endothelial factor production has been demonstrated in isolated endothelial cells (Suttorp et al., 1985; Busse and Mülsch, 1990; Newby and Henderson, 1990), as well as in vascular endothelium in situ (Sato et al., 1990), we felt we could reasonably hypothesize that the release of contractile factors, namely endothelins and/or thromboxane A_2, might be responsible for the thapsigargin-induced noradrenergic hyperresponsiveness in human saphenous veins.

Regarding the hypothesis of a thapsigargin-induced release of endothelins, to the best of our knowledge a possible relationship between thapsigargin and endothelial release of endothelins has not been described. However, as endothelins, at subthreshold concentrations, have been reported to potentiate the contractions elicited by noradrenaline in human arteries (Yang et al., 1990) and also because both endothelin ET_A/ET_B receptors have been shown to be expressed in human saphenous vein (Maguire and Davenport, 1999), the activation of which exerts a strong contractile effect (Pate et al., 1999), we decided to test the possible involvement of these contractile peptides in the thapsigargin-induced noradrenergic hyperresponsiveness. However, results of our experiments in the presence of the endothelin $ET_{A/B}$ receptor antagonist, Ro 47-0203 (Clozel et al., 1994), did not favour a major role of endothelins in the phenomenon.

We then tested whether the endothelial release of thromboxane A_2 could be responsible for the noradrenergic hyperresponsiveness induced by thapsigargin as well, as we had found for that induced by neuropeptide Y (Fabi et al., 1998). The finding that the thapsigargin-induced potentiation was inhibited by both the thromboxane A_2 receptor antagonist, Bay u3405 (MCKenniff et al., 1991; Norel et al., 1991), and by the thromboxane A_2 synthase inhibitor, UK 38485 (Parry et al., 1982), strongly supported this suggestion. The endothelial release of subthreshold concentrations of a contractile factor, namely thromboxane A_2, which acted synergistically with the noradrenergic mediator, appeared to us the most likely mechanism responsible for the phenomenon. This finding is somehow different from the results of most studies carried out with isolated blood vessels. Indeed, although thapsigargin has been shown to increase the release of arachidonic acid in human umbilical endothelial cells (Millanvoye-Van Brussel et al., 1999) and to induce a thromboxane A_2-mediated contraction in the porcine renal artery (Ihara et al., 1999), thapsigargin-induced endothelium-dependent relaxation, ascribable to the production of NO, and in some case also to EDHF, has been mainly observed (Moritoki et al., 1994; Taniguchi et al., 1999; Huang et al., 2000; Kuroiwa-Matsumoto et al., 2000; Ihara et al., 1999). Therefore, we tested whether simultaneous synthesis of the vasodilator NO induced by thapsigargin could partially mask the potentiating effect of thromboxane A_2 on noradrenergic contractions. The results obtained did not support this hypothesis, as the potentiation by thapsigargin in the presence of the NO synthase inhibitor, L-NNA, was similar to that in its absence.

Finally, although it is generally accepted that endothelium is devoid of voltage-sensitive Ca^{2+} channels, we decided to investigate the possible effect of verapamil on the thapsigargin-induced noradrenergic potentiation, as this drug has been described to block the increase in $[Ca^{2+}]_i$ induced by carbachol in the endothelium of rat aorta (Sato et al., 1990) and as the presence of L-type Ca^{2+} channels in the endothelium of veins may be still an open question because isradipine had been shown to decrease the Ca^{2+} influx induced by thapsigargin in cultured human umbilical veins (Iouzalen et al., 1995). Moreover, dihydropyridine-sensitive Ca^{2+} channels were also observed in cultured endothelial cells from porcine valve and rat aorta (Huang et al., 2000; Kawasaki et al., 1999) and in bovine capillary endothelial cells (Bossu et al., 1989, 1992). The results

clearly demonstrated that, in the presence of verapamil, thapsigargin no longer exerted any potentiating effect on the noradrenergic contractions.

However these findings did not indicate whether verapamil inhibited Ca^{2+} entry into endothelial cells—thus blocking the Ca^{2+}-dependent synthesis/release of thromboxane A_2- or acted at the "post-endothelial" level, i.e. on the potentiating action induced by the released thromboxane A_2 on the noradrenaline-induced contraction at the smooth muscle level. Our previous results with human saphenous vein, indicating that subthreshold concentrations of the thromboxane A_2 mimetic, U 46619, enhanced the noradrenergic contractions (Fabi et al., 1998), prompted us to test the latter hypothesis first. Therefore, we tested the effects of the Ca^{2+} antagonist on the noradrenergic hyperresponsiveness induced by the thromboxane A_2 mimetic, U 46619. The results indicated that the potentiation by U 46619 of noradrenergic contraction, like that induced by thapsigargin, was blocked by verapamil. A recent report (Vila et al., 2001), substantiated our previous results on the ability of U-46619 to induce the potentiation of the noradrenergic responses in human saphenous veins, but did not agree with our present results as far as the effect of the Ca^{2+} channel antagonists on the phenomenon is concerned. Indeed, these authors observed that nifedipine did not affect the potentiation induced by U 46619 on the noradrenergic contractions in human saphenous veins. The likely explanation for these discrepant results may be either an "unspecific" effect of verapamil—i. e. independent from its Ca^{2+} channel blocking action—or the different extent of the Ca^{2+} channels blocking action under the two experimental conditions. Vila et al. (2001) used nifedipine at a concentration (1 μM) that did not completely block the KCl-induced contractions and did not alter the EC_{50} values for noradrenaline. In contrast, verapamil 10 μM completely blocked the KCl-induced contractions and significantly increased the EC_{50} values for noradrenaline (0.85 ± 0.10 and 0.41 ± 0.08 μM in the presence or absence of verapamil, data from Figs. 1 and 6, respectively) in our experiments. In any event, under our experimental conditions, the inhibition by verapamil of the U 46619-induced noradrenergic potentiation mimicked the effect observed on the thapsigargin-induced noradrenergic hyperresponsiveness, allowing us to suggest that a verapamil-sensitive mechanism, operating after the endothelial release of thromboxane A_2, is involved in the phenomenon.

In conclusion, our results with human saphenous veins indicate that endothelial production of thromboxane A_2 induced by thapsigargin enhances the responses to sympathetic stimulation. This phenomenon may be ascribed to the potentiation induced by the released thromboxane A_2 of the contraction elicited by the noradrenergic mediator through a verapamil-sensitive mechanism. Our data thus substantiate the notion that the endothelium of peripheral veins can release vasoconstrictor substances (Li et al., 1994), which play a substantial role in the regulation of vascular tone. In addition, the mechanism described for thapsigargin may also be relevant as, in vascular endothelial cells, an agonist-activated Ca^{2+} influx pathway and the pathway activated by the depletion of internal Ca^{2+} stores are indistinguishable (Schilling et al., 1992).

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