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Evidence that the substance P-induced enhancement of pacemaking in lymphatics of the guinea-pig mesentery occurs through endothelial release of thromboxane A_2

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1 *In vitro* studies were performed to examine the mechanisms underlying substance P-induced enhancement of constriction rate in guinea-pig mesenteric lymphatic vessels.

2 Substance P caused an endothelium-dependent increase in lymphatic constriction frequency which was first significant at a concentration of 1 nM (115±3% of control, $n=11$) with 1 μM, the highest concentration tested, increasing the rate to 153±4% of control ($n=9$).

3 Repetitive 5 min applications of substance P (1 μM) caused tachyphylaxis with tissue responsiveness tending to decrease (by an average of 23%) and significantly decreasing (by 72%) for application at intervals of 30 and 10 min, respectively.

4 The competitive antagonist of tachykinin receptors, spantide (5 μM) and the specific NK_1 receptor antagonist, WIN51708 (10 μM) both prevented the enhancement of constriction rate induced by 1 μM substance P.

5 Endothelial cells loaded with the Ca^{2+} sensing fluophore, fluo 3/AM did not display a detectable change in $[Ca^{2+}]_i$ upon application of 1 μM substance P.

6 Inhibition of nitric oxide synthase by N^G nitro-L-arginine (L-NOARG; 100 μM) had no significant effect on the response induced by 1 μM substance P.

7 The enhancement of constriction rate induced by 1 μM substance P was prevented by the cyclo-oxygenase inhibitor, indomethacin (3 μM), the thromboxane A_2 synthase inhibitor, imidazole (50 μM), and the thromboxane A_2 receptor antagonist, SQ29548 (0.3 μM).

8 The stable analogue of thromboxane A_2, U46619 (0.1 μM) significantly increased the constriction rate of lymphangions with or without endothelium, an effect which was prevented by SQ29548 (0.3 μM).

9 Treatment with pertussis toxin (PTx; 100 ng ml^{-1}) completely abolished the response to 1 μM substance P without inhibiting either the perfusion-induced constriction or the U46619-induced enhancement of constriction rate.

10 Application of the phospholipase A_2 inhibitor, antiflammin-1 (1 nM) prevented the enhancement of lymphatic pumping induced by substance P (1 μM), without inhibiting the response to either U46619 (0.1 μM) or acetylcholine (10 μM).

11 The data support the hypothesis that the substance P-induced increase in pumping rate is mediated via the endothelium through NK_1 receptors coupled by a PTx sensitive G-protein to phospholipase A_2 and resulting in generation of the arachidonic acid metabolite, thromboxane A_2, this serving as the diffusible activator.

Keywords: Lymphatic vessels; endothelium; constriction rate; pacemaking; substance P; thromboxane A_2; arachidonic acid; calcium; pertussis toxin; NK_1 receptor

Introduction

The tachykinin, substance P (SP) has been shown to increase the frequency of constrictions in bovine lymphatic vessels (Foy *et al*., 1989). However, the pathways by which such modulation occurs are not known. This paper examines the second messenger pathways through studies on substance P-induced enhancement of the frequency of constriction of guinea-pig mesenteric lymphatics.

Many lymphatic collecting vessels actively propel lymph through phasic constrictions of the smooth muscle in the vessel wall. In this process, each chamber, formed by two adjacent unidirectional valves and termed a lymphangion (see Mislin, 1983), acts like a primitive heart to pump lymph rhythmically. An important determinant of the efficiency of these lymphangions is their constriction rate which is modulated by many factors including hormones or neurotransmitter substances (Florey, 1927; Mislin & Rathenow, 1961; Mawhinney & Roddie, 1973; McHale & Roddie, 1983). These modulators have traditionally been considered to act directly on the smooth muscle. However, recently it has been shown that some modulators act via the lymphatic endothelium. Thus, as for blood vessels, modulators such as acetylcholine cause release of endothelium-derived nitric oxide (EDNO), an action which reduces lymphatic smooth muscle tone (Ohhashi & Takahashi, 1991; Ferguson, 1992). Most importantly, EDNO also acts on the lymphatic pacemaker to slow the frequency of lymphatic constrictions (Yokoyama & Ohhashi, 1993; von der Weid *et al*., 1996).

Our interest in substance P arises from the likelihood of it having a physiological role in lymphatic pacemaking, in that it is present in or associated with lymphatic vessels (Foy *et al*., 1989). More specifically, it is present in the primary afferents which innervate lymphatic vessels (Guarna *et al*., 1991) and it may also be released from the endothelium, as has been shown in vascular tissues (Ralevic *et al*., 1990). It is also very likely to be released into the interstitium as a result of tissue injury/inflammation (Pernow, 1989) and consequently transported by

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lymphatic vessels. Indeed, the fact that substance P increases lymphatic pumping may be of considerable physiological advantage, particularly in the case of local inflammation.

Substance P receptors are generally considered to be coupled to a G protein positively coupled to phospholipase C (PLC) with activation causing an increase in $[Ca^{2+}]_i$ through PLC-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG), the former activating store Ca^{2+} release (Hanley *et al*., 1980; Hershey *et al*., 1991). Our findings indicate that substance P acts differently to this, increasing lymphatic pacemaking of the smooth muscle by acting on the lymphatic endothelium to liberate thromboxane A_2, a metabolite of arachidonic acid. Of particular interest is the finding that this action occurs independent of a measurable increase in $[Ca^{2+}]_i$.

Methods

Tissue preparation

Guinea-pigs (aged 2–8 days) were killed by an overdose of the inhalation anaesthetic halothane (5–10% in air) followed by decapitation. Experiments were performed on mesenteric lymphatic vessels isolated from the ileal region of the intestine and pinned onto a sylgard-covered (Dow Corning) base of a small organ bath (volume 0.5 ml). The tissue was viewed with an inverted microscope (Nikon Diaphot) and superfused at a rapid rate (5 ml min^{-1}) with a physiological saline solution of the following composition (mM): NaCl 120, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 2, $NaHCO_3$ 25, NaH_2PO_4 1 and glucose 10, maintained at a pH of 7.2 by bubbling with a 95%:5% O_2:CO_2 gas mixture, with the solution maintained at a temperature of 33–35°C. Lymphatic pumping was activated by luminally perfusing the tissues, by means of a fine glass cannula, with the same physiological solution but with a lower calcium concentration ($CaCl_2$ 1.2 mM) to minimize any precipitation of Ca^{2+} and hence blockage of the cannula. The tissue was perfused with the cannula loosely inserted into the distal end of each isolated vessel. This procedure allowed some backflow thus damping out pressure changes associated with constrictions. The perfusion flow rate through the cannula was maintained at about 2.5 μl min^{-1}, a flow rate which was marginally suprathreshold to maintaining rhythmical lymphatic constrictions. Tissues were normally used within 1–4 h of isolation and were stored at 4°C in physiological saline until use.

Recording technique

The pumping activity of lymphangions was monitored by a video camera attached to an inverted microscope (Nikon Diaphot), with the output recorded on videotape and analysed visually or by a computer-based edge detection system (sampling frequency 25 Hz; see Beresford-Smith *et al*., 1993).

Intracellular calcium measurements

Experiments involving measurements of changes in the endothelial $[Ca^{2+}]_i$ were made with the calcium sensing dye (fluo 3/AM, Molecular Probes Pty Ltd) and a confocal laser system (BIORAD MRC 600) attached to an inverted microscope (Zeiss Axiovert 10) with a $\times 40$ oil immersion objective (NA 1.3). The endothelium was loaded with fluo 3/AM by luminally perfusing each vessel with 2 μM fluo 3/AM and pluronic acid (0.2%, w v^{-1}) for 30 min at 35°C. Movement artifacts were avoided by undertaking the experiments without luminal perfusion. The Ca^{2+} imaging experiments were made at a frame capture rate of 2 Hz.

Treatment with pertussis toxin (PTx)

Tissues were superfused with physiological saline solution containing PTx (100 ng ml^{-1}) for 4 h at 35°C (see Burch *et al*., 1986) with experiments done subsequent to this in the absence of PTx.

Experimental protocols

The experimental protocol used in the pharmacological studies normally involved a 15 min control period, a 5 min test period when an agonist was applied (substance P or U46619), followed by a 15–30 min washout period. Measurements were made by recording the lymphangion pumping frequency for 4 min periods at the end of the initial control period, 1 min after application of the agonist and at the end of the washout period. When pharmacological inhibitors were used, this protocol was in part repeated but now in the presence of the inhibitor which was applied for a total of 20 min (15 min before and during application of SP). There followed a 30 min washout period before substance P was again applied. Throughout the experiments, with the exception of the substance P-induced tachyphylaxis experiment, there was an interval of at least 30 min between applications of substance P. Except where noted, analysis has been based on comparisons of the test pumping rate of individual lymphangions to that of the relevant control immediately preceding the response, both averaged over a 4 min period.

Lysis of endothelium

Some experiments were made after lysis of the endothelium. Lysis was performed by use of a method developed by Juncos *et al*. (1994), by use of factor VIII immunoreactivity to destroy endothelial cells selectively whilst leaving the underlying smooth muscle intact. This method involved perfusion of the lymphatic vessel with low calcium physiological saline solution containing BSA (5%, w/v), antibodies against human von Willebrand factor (factor VIII R:Ag; 1/1000 dilution) and rabbit complement (2%, v/v) both purchased from Sigma Immunochemicals (Sydney). The vessel was perfused for 30 min at 35°C with an intraluminal flow rate of 5–8 μl min^{-1}. The vessel was then left for 2 h without intraluminal perfusion and maintained at 35°C to allow completion of endothelial lysis. The lumen was then washed out with low calcium physiological saline solution. This procedure is a slight modification of that used by von der Weid *et al*. (1996) where the endothelium from this same tissue was successfully destroyed, as tested both morphologically and pharmacologically. The effectiveness of the lysing procedure was tested based on the study by von der Weid *et al*. (1996), who demonstrated two populations of vessels. They found that while the majority of lymphatic vessels were inhibited by both ACh and sodium nitroprusside (SNP), there was a 'non responding' group which did not respond in any way to either ACh (0.01–0.1 mM) or SNP (0.1 mM). Therefore, the relative success of the lysing procedure was tested by firstly applying ACh (0.1 mM), followed by the application of SNP (0.1 mM). A negative response to ACh and a positive response (i.e. inhibition) to SNP indicated successful lysis of endothelial cells. Based on this testing procedure, it was found that approximately 50% of attempts to lyse endothelial cells proved successful.

Drugs

Special chemicals included acetylcholine chloride (ACh), the thromboxane synthase inhibitor imidazole, the cyclo-oxygenase inhibitor indomethacin, N^G nitro-L-arginine (L-NOARG), substance P (acetate salt), the nonspecific tachykinin receptor antagonist spantide ([D-Arg^1,D-$Trp^{7,9}$,Leu^{11}]-substance P) (all from Sigma-Aldrich Pty Ltd, Sydney), the non-peptide NK_1 tachykinin receptor antagonist WIN51708 (17-β-hydroxy-17-α-ethynyl-5-α-androstano[3,2-*b*]pyrimido[1,2-*a*]benzimidazole), the thromboxane A_2 mimetic U46619 (9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$), the thromboxane A_2 receptor antagonist SQ29548 ([1S-[1α,2α(Z),3α,4α]]-7-[3-[[2-

[(phenylamino)carbonyl] hydrazino] methyl]-7-oxabicyclo [2.2.1]hept-2-yl]-5-heptanoic acid) and the phospholipase A_2 inhibitor antiflammin-1 (Met-Gln-Met-Lys-Lys-Val-Leu-Asp-Ser) all from RBI (Australian Laboratory Services, Sydney). Stock solutions were made up in either distilled water or dimethylsulphoxide (DMSO) at concentrations of between 0.1–100 mM and stored at −20°C. These were thawed and diluted to appropriate concentrations in physiological saline solution when required. Test solutions were always applied as superfusate. The concentration of vehicle (DMSO) was always considerably less than 0.1%, a concentration which had no significant effect on lymphatic pumping.

The choice of inhibitor concentrations arose from preliminary experiments with initial concentrations based on published data obtained in other tissues as referenced (below). All drugs were effective without obvious non-specific effects at or near the published concentrations except for antiflammin-1, which we used at a substantially lower concentration (1 nM) as it had non-specific effects on lymphatic pumping rate at higher concentrations (>10 nM).

Data analysis

Data are presented as the mean ±s.e.mean with statistical comparisons made by two tailed paired Student's *t*-test, with $P<0.05$ considered significant.

Results

Lymphatic vessels were cannulated and perfused at the minimum rate necessary to induce spontaneous contractile activity of individual lymphangions which once activated, normally exhibited regular constrictions at frequencies of between 6 and 25 min^{-1}. In all, about 10% of tissues were rejected including some 5% of tissues which could not be induced to constrict irrespective of perfusion rate and some 5% of tissues which exhibited irregular pumping activity under control conditions. A total of 127 lymphangions from 59 guinea-pigs were studied. These chambers had diameters of between 90–286 μm and chamber lengths of between 135–720 μm.

Responses to substance P

As in bovine mesenteric lymphatics (Foy *et al.*, 1989), application of substance P caused an increase in the pumping rate of guinea-pig mesenteric lymphatics, with 1 μM substance P resulting in a significant (15–80%) increase in pumping rate (see Figure 1a).

The effects of repetitive application of substance P (1 μM) were examined. There was a trend for the substance P-induced enhancement of lymphatic pumping to decrease upon repetitive application. This is shown in Figure 1b, which demonstrates that while larger intervals (30 min) between removal and 5 min re-application of substance P led to a small (but not significant) decline in the substance P-induced enhancement of constriction rate, a small interval (10 min) between removal and 5 min re-application of substance P caused a marked and highly significant ($P<0.01$) decline in the substance P-induced enhancement of constriction rate (from 122±4% to 106±2% of control; $n=6$). The control pumping rate did not change significantly over this same period (data not shown).

Concentration-response curve for substance P and the significance of an intact endothelium

The response to increasing concentrations of substance P is presented in Figure 2. Here the different concentrations of substance P were applied randomly for each tissue. The resultant log concentration-response curve (Figure 2) demonstrates that substance P significantly increased the pumping rate at concentrations of 1 nM with the magnitude of the effect

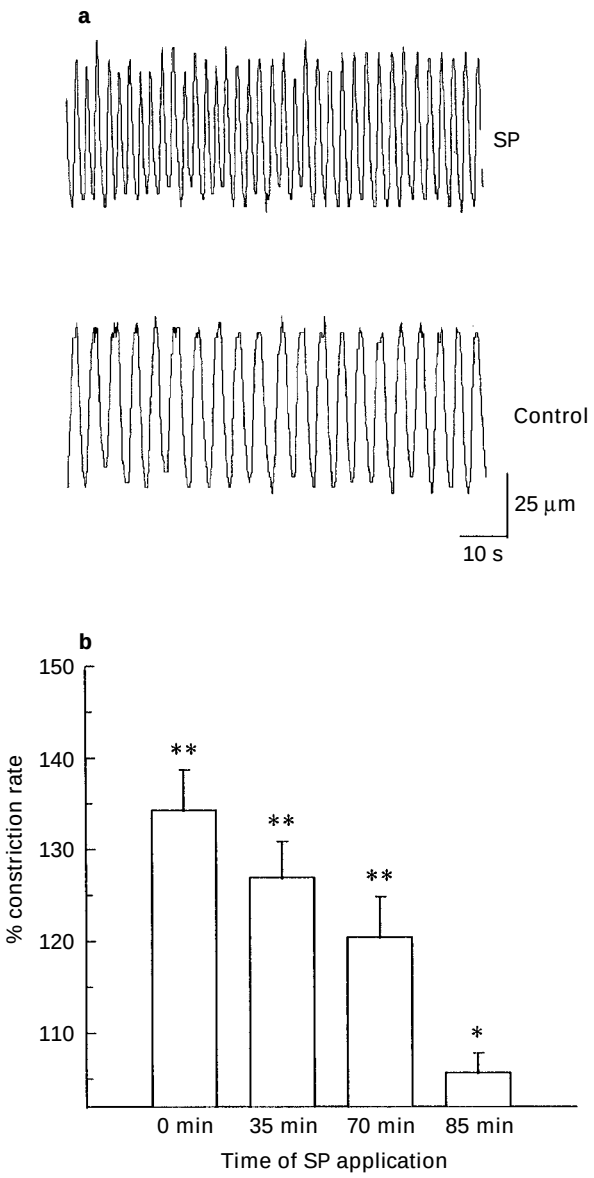

Figure 1 The effects of substance P (SP) on lymphangion constriction rate. (a) Sample edge traces of a constricting lymphangion in the presence of substance P (1 μM) and under control conditions. (b) The effects of repetitive application of substance P (1 μM) applied for 5 min at two repeated intervals (between removal and re-application of substance P) of 30 min and a final interval of 10 min. Consecutive measurements were made in each of 6 lymphangions with values normalized with respect to the initial constriction rate. Vertical lines denote s.e.mean. $*P<0.05$, $**P<0.01$.

increasing with the higher substance P concentrations tested. This effect was most likely due to an action of substance P on the endothelium as removal of the endothelium entirely inhibited the substance P-induced enhancement of lymphatic pacemaking over the same concentration range (Figure 2).

Effects of spantide and WIN51708 on the effects of substance P

A role for the NK_1 receptor was examined. We first examined the effect of spantide, a peptide analogue known to be a competitive antagonist of substance P (Folkers *et al.*, 1984; Appell *et al.*, 1992). The antagonist was applied 15 min before and during a 5 min application of substance P, with measurements taken over a 4 min period just before and 1 min after application of substance P. Spantide (5 μM) prevented the

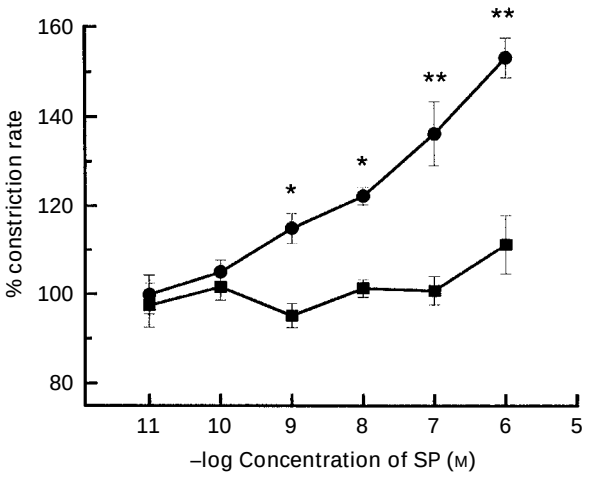

Figure 2 The effects of substance P (SP) on lymphangion constriction rate with (●) or without (■) endothelium present. All concentrations of substance P were applied in a random order. All data were normalized with respect to the corresponding control lymphangion constriction rate with $n=8-11$ lymphangions for all points. Vertical lines denote s.e.mean. $*P<0.05$, $**P<0.01$.

enhancement of constriction rate induced by 1 μM substance P (Figure 3a). This effect of spantide was not readily reversed as the response to substance P after 30 min wash was not significantly different from the control rate in the absence of substance P ($n=7$).

The inhibition by spantide was likely to be an action on NK_1 tachykinin receptors as a similar application of WIN51708 (10 μM), an antagonist selective for the NK_1 receptor (Snider *et al*., 1991), completely inhibited the enhancement of lymphatic pacemaking induced by 1 μM substance P (Figure 3b). This effect was at least partly reversible after a 30 min wash, with substance P causing a significant ($P<0.01$) enhancement in pumping rate to $123\pm3\%$ ($n=6$) back in control solution.

Substance P does not increase endothelial $[Ca^{2+}]_i$

The second messenger cascade was examined by application of substance P (1 μM) to fluo 3 loaded lymphatic vessels viewed by confocal microscopy. Importantly, no measurable increase in fluorescence occurred (fluorescence intensity $100\pm1\%$ of control, $n=7$), indicating that activation of substance P receptors does not increase endothelial $[Ca^{2+}]_i$. By comparison, ACh which is known to increase $[Ca^{2+}]_i$ in the lymphatic en-

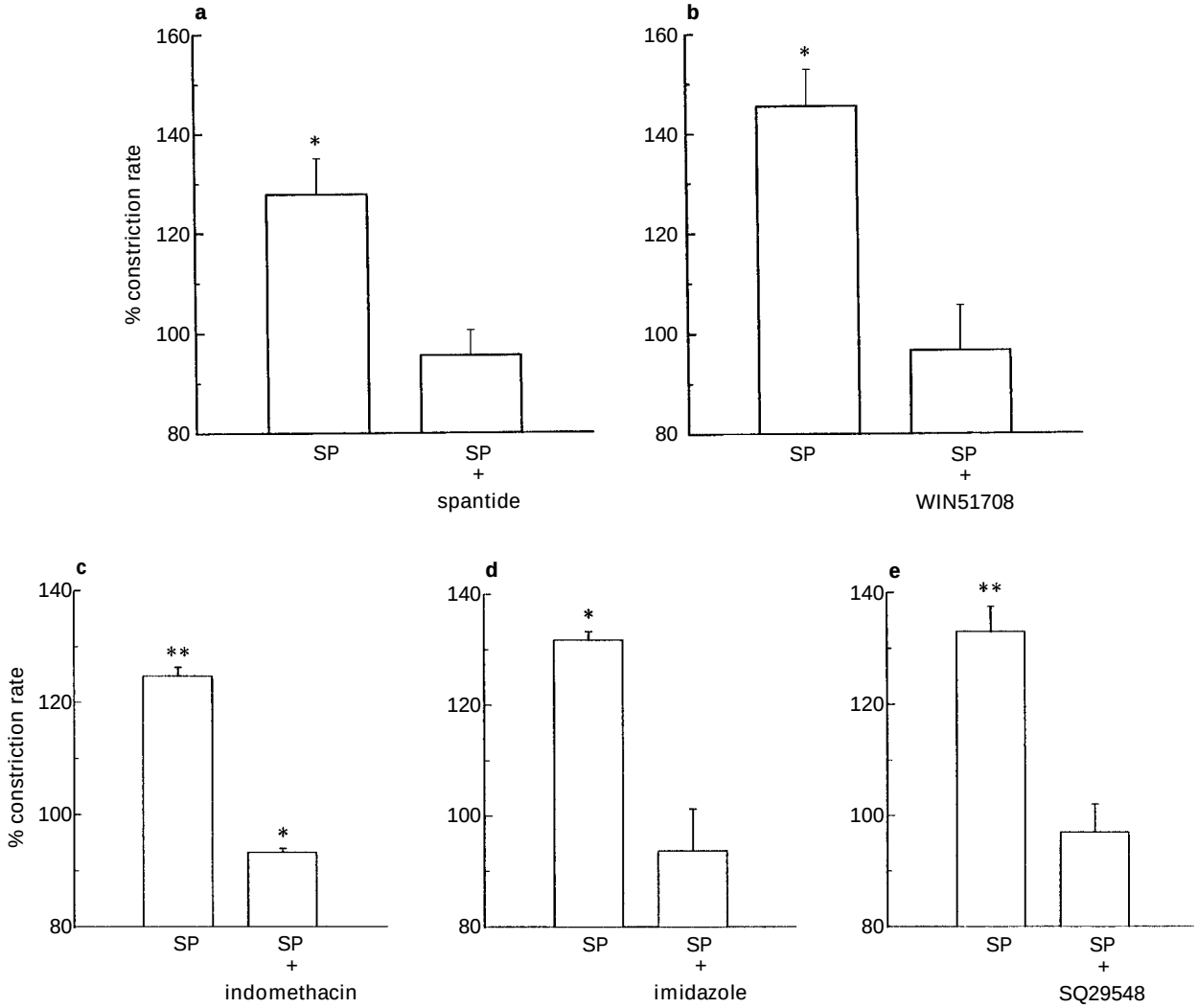

Figure 3 The effects of antagonists of tachykinin receptors, a cyclo-oxygenase inhibitor, a thromboxane A_2-synthase inhibitor and a thromboxane A_2-receptor antagonist on the increase in lymphangion constriction rate induced by substance P (SP). Shown are the effects of (a) the competitive antagonist of tachykinin receptors, spantide (5 μM; $n=7$), (b) an antagonist selective for the NK_1 tachykinin receptor, WIN51708 (10 μM; $n=6$), (c) the cyclo-oxygenase inhibitor, indomethacin (3 μM; $n=6$), (d) the thromboxane A_2 synthase inhibitor, imidazole (50 μM; $n=6$) and (e) the thromboxane A_2-receptor antagonist, SQ29548 (0.3 μM; $n=11$) on the substance P-induced response. Data for each lymphangion have been normalized with respect to the control constriction rate just before application of either substance P or substance P plus inhibitor. Vertical lines denote s.e.mean. $*P<0.05$, $**P<0.01$.

dothelium (von der Weid *et al*., 1996), applied to these same tissues caused a significant ($P<0.05$) increase in the relative levels of endothelial $[Ca^{2+}]_i$ (fluorescence intensity $115\pm7\%$ of control; $n=7$).

Substance P action does not occur through inhibition of endogenous release of EDNO

It has been shown previously that perfused lymphatic vessels constrict at reduced frequency because of an NO synthase induced release of EDNO (von der Weid *et al*., 1996). Therefore, the possibility that substance P was acting by inhibiting release of EDNO was examined by use of the nitric oxide synthase inhibitor L-NOARG (100 μM), applied 20 min before and during application of 1 μM substance P. In paired studies on 6 lymphangions the substance P-induced enhancement of pumping was $135\pm7\%$ of control and the substance P-induced enhancement in the presence of L-NOARG (100 μM) was $131\pm7\%$ of control. These data indicate that the substance P-induced action occurs independently of the generation of EDNO.

Effects of a cyclo-oxygenase inhibitor on the substance P-induced response

The actions of substance P appeared to involve one or more metabolites of arachidonic acid (AA) as inhibition of cyclo-oxygenase by indomethacin (3 μM) markedly and significantly ($P<0.05$) decreased the enhancement in constriction rate induced by 1 μM substance P (Figure 3c). The action of indomethacin did not significantly reverse upon 30 min wash. Indomethacin (3 μM) itself did not significantly alter the constriction rate of the perfused lymphangions (rate $103\pm3\%$ of control, $n=5$), indicating that perfusion-induced constrictions primarily by pathways independent of cyclo-oxygenase induced metabolites of arachidonic acid.

Role of thromboxane A_2

Evidence that the substance P-induced enhancement of constriction rate resulted from the arachidonic acid metabolite thromboxane A_2 was provided by the finding that imidazole (50 μM), an inhibitor of thromboxane A_2 synthase (see Needleman *et al*., 1977; 1986; Moncada & Vane, 1979) abolished the action of 1 μM substance P (Figure 3d). This effect was reversible, with substance P inducing a mean relative constriction rate of $123\pm9\%$ ($n=6$) after 30 min wash. Imidazole (50 μM) itself, did not significantly affect lymphatic pumping rate with a mean relative rate of $114\pm8\%$ of control ($n=6$).

Further evidence for a primary involvement of thromboxane A_2 was provided by application of the thromboxane A_2 receptor antagonist SQ29548 (0.3 μM; see Monshizadegan *et al*., 1992; Hunt *et al*., 1992), which completely inhibited the substance P-induced action (Figure 3e). SQ29548 (0.3 μM) alone did not significantly alter the frequency of lymphatic pumping with a mean relative rate of $105\pm6\%$ of control ($n=11$).

The stable analogue of thromboxane A_2, U46619 (0.1 μM; see Coleman *et al*., 1981), which has been shown to enhance the frequency of lymphatic pumping in both sheep and bovine mesenteric lymphatics (Johnston & Gordon, 1981), significantly increased the pumping rate of lymphangions in a manner analogous to that of substance P (Figure 4). This enhancement of pumping activity persisted following lysis of the endothelium (Figure 4). Importantly, as for the substance P response, SQ29548 (0.3 μM) completely abolished the mean increase in constriction rate to U46619 (0.1 μM; rate $97\pm2\%$ of control, $n=5$).

Effects of pertussis toxin (PTx) on the substance P-induced response

The action of substance P is likely to occur through activation of a receptor coupled to a G protein. That this is the case was supported by the finding that treatment with PTx completely abolished the response to substance P. The finding that the response to U46619 persisted in the same PTx-treated lymphangions indicated that this was a specific action on the substance P receptor-associated G protein (Figure 5). Furthermore, the fact that perfusion still activated constrictions in the PTx-treated tissues (allowing these measurements to be made) indicates that perfusion-induced lymphatic pumping was itself PTx-insensitive.

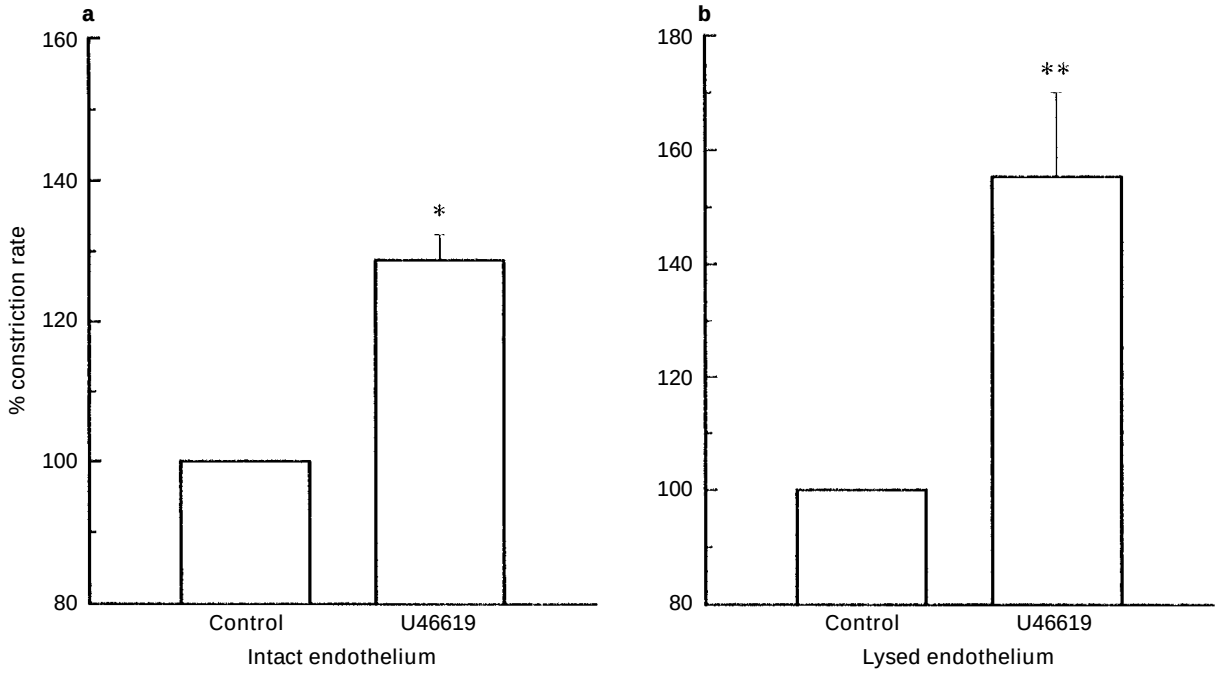

Figure 4 The effect of a thromboxane A_2 agonist on lymphangion constriction rate (a) with and (b) without endothelium. The stable thromboxane A_2 analogue, U46619 (0.1 μM) enhanced the constriction rate in a manner analogous to that of substance P in (a) normal ($n=6$) and (b) endothelium lysed ($n=6$) tissues. Data for each lymphangion have been normalized with respect to the preceding control constriction rate. Vertical lines denote s.e.mean. $^*P<0.05$, $^{**}P<0.01$.

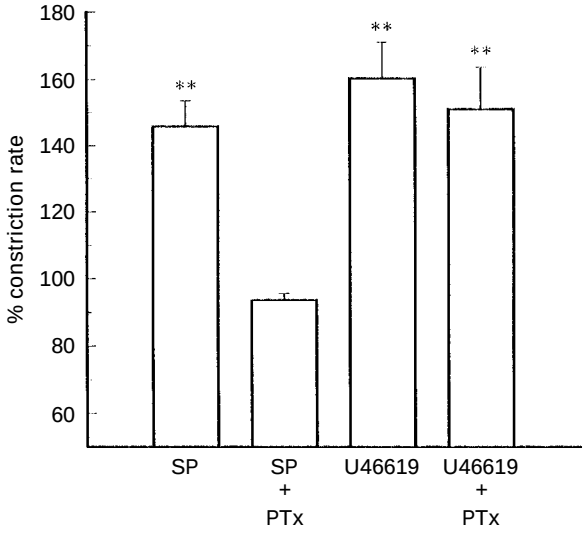

Figure 5 The effect of pertussis toxin (PTx) treatment on substance P(SP)- and a thromboxane A_2-receptor agonist-induced enhancement of lymphatic pumping. The effects of pertussis toxin (100 ng ml^{-1}) treatment on the response to (a) substance P (1 μM) and (b) U46619 (0.1 μM) were compared in the same lymphangions ($n=5$). Values obtained from individual lymphangions were normalized with respect to the control obtained just preceding application of the agonist before or after pertussis toxin treatment. Vertical lines denote s.e.mean. Statistical comparisons were made between the test values and the relative control constriction rate. $^{**}P<0.01$.

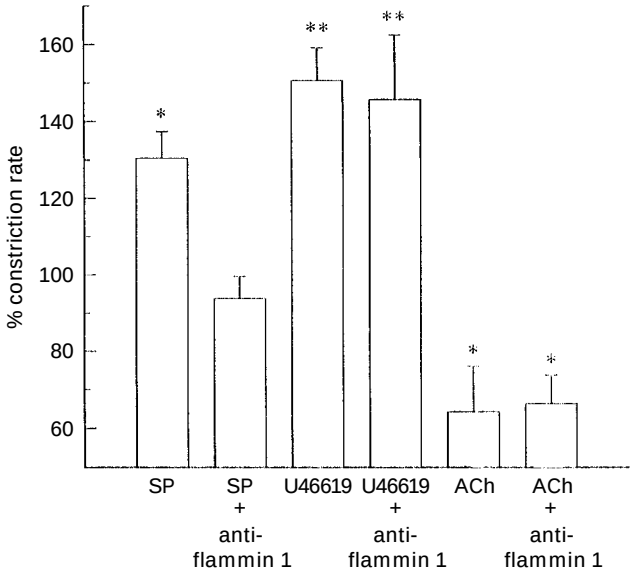

Figure 6 The effects of a phospholipase A_2 inhibitor on the substance P (SP)-induced enhancement of lymphangion constriction rate. The phospholipase A_2 inhibitor antiflammin-1 (1 nM) inhibited the enhancement of constriction rate induced by substance P (1 μM) while having no effect on the enhancement caused by the thromboxane A_2 receptor agonist U46619 (0.1 μM) or the inhibition caused by acetylcholine (ACh, 10 μM) in the same 6 lymphangions. Values were obtained from individual lymphangions and normalized with respect to the control immediately preceding each test measurement. Vertical lines denote s.e.mean. $^{*}P<0.05$, $^{**}P<0.01$.

Effects of inhibiting PLA_2 on the substance P-induced response

The possibility that substance P mobilizes arachidonic acid through activation of phospholipase A_2 (PLA_2; see Hershey *et al.*, 1991) was investigated by use of the inhibitor of PLA_2, antiflammin-1 (Lloret & Moreno, 1992). Application of antiflammin-1 (1 nM) prevented substance P acting to modulate lymphatic pumping, an effect which appeared specific in that there was no inhibition of either the response to U46619 (0.1 μM) or to ACh (0.1 mM) (Figure 6). Antiflammin-1 (1 nM) alone significantly inhibited pumping activity ($P<0.05$), slowing lymphatic pumping to $79\pm7\%$ of control, indicating that AA metabolites other than cyclo-oxygenase metabolites (eg. thromboxane A_2) may be involved in the perfusion-induced pumping.

Discussion

In this study the mechanisms by which substance P enhances the pumping rate of lymphangions of guinea-pig mesenteric lymphatics were investigated. The important finding was that substance P appears to act by a G protein-mediated generation of the arachidonic acid metabolite thromboxane A_2 in the endothelium, which acts on smooth muscle thromboxane A_2 receptors to produce an increase in pumping rate.

The substance P-induced response in lymphatic vessels appears to share many of the characteristics of substance P-mediated responses in other tissues, in that it is activated by an NK_1 type tachykinin receptor (see Hershey *et al.*, 1991), coupled to a pertussis toxin-sensitive G protein (Nakamura & Ui, 1985) and readily undergoes desensitization (see Hershey *et al.*, 1991). The major difference is that in most tissues investigated, substance P has been shown to cause an increase in $[Ca^{2+}]_i$ due to either inositol 1,4,5-trisphosphate ($InsP_3$)-mediated release of Ca^{2+} from intracellular stores (see Hershey *et al.*, 1991) and/or through an enhancement of Ca^{2+} entry (Gallacher *et al.*, 1990). Such mechanisms are believed to occur through positive coupling to phospholipase C(PLC), the activation of which catalyses the hydrolysis of inositol phospholipids into diacylglycerol and inositol polyphosphates (Hanley *et al.*, 1980; Weiss *et al.*, 1982; Abdel-Latiff, 1986). However, while such a second messenger cascade could lead to the generation of arachidonic acid (eg. from diacylglycerol; see Wolfe, 1982), it is not readily reconciled with the finding that substance P does not cause an increase in endothelial $[Ca^{2+}]_i$. Another possibility is that the substance P receptor and its associated G protein is coupled to PLA_2 (Irvine, 1982; Axelrod *et al.*, 1988). That PLA_2 was integral to the response was indicated by the finding that an inhibitor to PLA_2, antiflammin-1 at low concentrations (1 nM) completely and selectively inhibited the action of substance P.

The finding that substance P does not increase lymphatic endothelial $[Ca^{2+}]_i$ makes the underlying mechanisms distinct from those leading to the production of EDNO, which are associated with an increase in lymphatic endothelial $[Ca^{2+}]_i$ (von der Weid *et al.*, 1996). Indeed it is the enhancement of $[Ca^{2+}]_i$ which is likely to activate nitric oxide synthase to release EDNO (Palmer *et al.*, 1988; Busse & Mulsch, 1990). Stimulation of endothelial muscarinic receptors has been shown to activate PLC-mediated mobilization of $InsP_3$ (see Berridge, 1993) which initiates the generation of EDNO and consequent inhibition of lymphatic pumping (Yokoyama & Ohhashi, 1993). EDNO also appears to be released endogenously, reducing the pumping rate of perfused lymphatics (von der Weid *et al.*, 1996). However, the possibility that substance P exerts its effect by interfering with an EDNO-induced inhibition of pacemaking was not supported, as the effect of substance P was not altered in the presence of an inhibitor of NO synthase.

Studies on the vasculature have shown that substance P can cause an endothelium-dependent relaxation of pre-contracted arteries of the rabbit, dog and cat (Zawadski *et al.*, 1981; Furchgott *et al.*, 1984). Furthermore, as shown for porcine endothelial cells, substance P receptors coupled to a G protein are present in the endothelial cell membrane (Saito *et al.*, 1990). While, substance P normally causes endothelium-de-

pendent relaxation in blood vessels, it has also been found to cause vasoconstriction (Bayorh & Feuerstein, 1985; Shirahase *et al*., 1995). Importantly, a study of intrapulmonary arteries has demonstrated an endothelium-derived constriction caused by endothelial substance P-receptor mediated activation of thromboxane A_2 (Shirahase *et al*., 1995). However, whether substance P also acts in this tissue by a mechanism which does not increase $[Ca^{2+}]_i$ is not known.

The fact that substance P increases lymphatic pumping may be of considerable importance in dealing with tissue injury/inflammation. In these circumstances increased firing of the sensory nerves could release substance P known to be present in these nerves (Guarna *et al*., 1991) which may act directly on the endothelium (Pernow, 1989; Li & Duckles, 1992) and in so doing cause the release of thromboxane A_2 and enhance pumping. Alternatively substance P could be present in the interstitium arising from tissue inflammation (Pernow, 1989) and by increasing lymphatic pumping, speed up resolution of any injury-associated oedema.

In conclusion, we have shown that the known action of substance P to enhance lymphatic pumping occurs through stimulation of the endothelium to release thromboxane A_2. Our data support the hypothesis that substance P binds to an NK_1 tachykinin receptor coupled to a pertussis toxin-sensitive G protein. This leads to PLA_2-mediated activation of the arachidonic acid cascade which, without increasing $[Ca^{2+}]_i$, proceeds by the cyclo-oxygenase pathway to generate thromboxane A_2 and by releasing this activator increases the constriction frequency of the smooth muscle.

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