Br. J. Pharmacol. (1994), **113**, 522–526

Inhibition by SR140333 of NK_1 tachykinin receptor-evoked, nitric oxide-dependent vasodilatation in the hamster cheek pouch microvasculature *in vivo*

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1 This study investigated tachykinin-evoked vasodilatation in the microvasculature of the hamster cheek pouch *in vivo*. Arterioles and venules were observed by intravital microscopy with video recording, and vasodilatation and constriction, defined as changes in blood vessel diameter, measured by image analysis. All agents were applied topically by superfusion. None of the agents tested had a significant effect on venule diameter.

2 When arterioles were preconstricted (by ca. 50%) with endothelin-1 present in the superfusing medium, substance P (0.3–30 nM) was a potent vasodilator, being 10 fold more active than both neurokinin A and the NK_1 receptor-selective agonist, substance P methyl ester. The NK_2 receptor-selective agonist, [β-Ala^8]-NKA(4-10)(0.1–10 μM) was active only at high concentrations, and the NK_3 receptor-selective agonist senktide (0.1–10 μM) was virtually inactive ($n = 8$ hamsters). Dilatation evoked by tachykinins and analogues was rapid in onset (<0.5 min) and readily reversible.

3 At low concentrations (1–10 nM), the non-peptide tachykinin NK_1 receptor antagonist SR140333 ((S)1-{2-[3(3,4-dichlorophenyl)-1-(3-iso-propoxyphenylacetyl)piperidin-3-yl]ethyl}-4-phenyl-1-azoniabicyclo[2.2.2]octone, chloride) had no effect on the diameter of preconstricted arterioles *per se*, but potently inhibited dilator responses to substance P methyl ester (apparent pK_B 9.9 ± 0.2; $n = 5$ hamsters, $n = 10$ estimates). SR140333 (10 nM) did not inhibit submaximal dilator responses evoked by human alpha calcitonin gene-related peptide (αCGRPh; 1.0 nM; $P > 0.05$; $n = 5$).

4 The nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; 10 μM) caused a 51.3 ± 5.4% arteriolar constriction. In the presence of L-NAME, submaximal vasodilator responses to substance P (10–100 nM) and carbachol (0.1–1.0 μM) were significantly attenuated ($n = 5$ hamsters; $P < 0.05$) as compared to responses obtained in preparations that were preconstricted to a similar extent by endothelin-1 (48.0 ± 5.6%). L-NAME (10 μM) was without effect on submaximal vasodilator responses to αCGRPh (0.1 nM) or sodium nitroprusside (10 nM) ($n = 5$ hamsters; $P > 0.05$).

5 We conclude that tachykinin-evoked arteriolar vasodilatation in the hamster cheek pouch is mediated via NK_1 receptor activation and depends, at least in part, on the release of nitric oxide. The NK_1 receptors mediating vasodilatation can be blocked by topical application of SR140333; which may therefore be useful in the investigation of the role of NK_1 receptors in neurogenic inflammation in the microvasculature.

Keywords: Cheek pouch (hamster); substance P; neurokinin; tachykinin; NK_1-receptor; nitric oxide; microvasculature; SR140333; L-NAME; vasodilatation

Introduction

Peripherally released sensory neuropeptides, including the tachykinin, substance P and calcitonin gene-related peptide (CGRP), have been shown to have potent effects in the vasculature in a number of species including man (see Holzer, 1992). In the microvasculature, CGRP has been clearly demonstrated to cause long-lasting vasodilatation in many preparations, such as the hamster cheek pouch (Brain *et al.*, 1985; Raud *et al.*, 1991; Hall & Brain, 1993); and has been shown to mediate the increased blood flow resulting from sensory nerve stimulation by capsaicin in rabbit skin (Hughes & Brain, 1991), and by capsaicin and saphenous nerve stimulation in rat skin (Escott & Brain, 1993). Studies with the non-peptide tachykinin NK_1 receptor antagonists, CP-96,345 and RP67580, have established that tachykinins act via NK_1 receptors on post-capillary venules to increase microvascular permeability leading to oedema formation (e.g. Garret *et al.*, 1992; Lembeck *et al.*, 1992; see Holzer, 1992; Hall, 1994). In the hamster cheek pouch, Gao and coworkers (1993) have recently demonstrated inhibition by CP-96,345 of capsaicin and substance P-evoked plasma extravasation. These studies provide further evidence for a role for NK_1 receptors in the microvasculature.

A role for tachykinins as arteriolar dilators in the microvasculature, however, is not well documented. Substance P has been found by intravital microscopy (Persson *et al.*, 1991; Raud *et al.*, 1991) and laser-Doppler flowmetry (Andrews & Helme, 1989; Kerezoudis *et al.*, 1993; Lam & Ferrell, 1993) to cause transient arteriolar dilatation, and the higher activity of substance P as compared to neurokinins A and B has led to the suggestion that arteriolar dilatation results, at least in part, from NK_1 receptor stimulation (see Lam & Ferrell, 1993). Confirmation of NK_1-receptor evoked arteriolar dilatation, however, requires evidence from studies with receptor-selective antagonists. There are few published studies involving the use of antagonists in the microvasculature. Limited examples include inhibition of the substance P-evoked increase in blood flow in the rat hind knee joint by the NK_1 receptor antagonist, CP-96,345 (Lam & Ferrell, 1993), and inhibition of substance P methyl ester-evoked dilatation in guinea-pig pial arterioles by the peptide NK_1 receptor antagonist, GR82334 (Beattie *et al.*, 1993).

The aim of the present study was to use intravital microscopy to carry out a quantitative investigation of the tachykinin receptors mediating vasodilatation in the hamster cheek pouch *in vivo*. We described here the effects of tachykinin receptor-selective agonists and the recently des-

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cribed non-peptide NK_1 receptor antagonist, SR140333 (Emonds-Alt *et al.*, 1993) on NK_1 receptor-mediated vasodilatation in the microvasculature. We confirm that NK_1 receptors mediate tachykinin-evoked arteriolar vasodilatation in the microvasculature, and demonstrate a role for nitric oxide in basal and NK_1 receptor-mediated arteriolar dilatation. We suggest that the novel non-peptide NK_1 receptor antagonist, SR140333, may be a valuable tool in investigating the role of NK_1 receptors in neurogenic inflammation since it is active following topical application, and unlike several other non-peptide NK_1 receptor antagonists, does not appear to have effects, such as ion channel blockade, unrelated to tachykinin receptor antagonism (Emonds-Alt *et al.*, 1993).

A preliminary account of this work has been communicated at the meeting of the European Neuropeptide Club, Strasbourg, France, April 1994 (Hall *et al.*, 1994).

Methods

Tissue preparation

Male golden (Syrian) hamsters (90–160 g) were anaesthetized with sodium pentobarbitone (Sagatal 50 mg kg^{-1}, i.p.) and anaesthesia was maintained with 15 mg kg^{-1} pentobarbitone as required. A single layer of vascular membrane was prepared as described by Duling (1973) with some modifications (Brain, 1989). Briefly, the hamster was placed on a specially designed stage with a central depressed well, the right cheek pouch was gently everted and placed in the well and pinned to a silicon rubber ring encircling a window. A single vascular layer was dissected out keeping an intact blood supply, and all connective tissue was removed. The tissue was superfused with Krebs-bicarbonate solution (composition mM: NaCl 120, KCl 47, $MgCl_2$ 0.12, $CaCl_2$ 0.18, glucose 10, $NaHCO_3$ 23) at a rate of 4 ml min^{-1} and the Krebs solution, warmed to 35°C, was gassed with 5% CO_2 in air. An area of microvasculature was selected which allowed an arteriole and adjacent venule (each between 20 and 40 μm in diameter) to be viewed concomitantly. The microvessels were observed with a Leiz dialux microscope having a 27 × salt-water dipping objective and 10 × eye pieces. Diameters of microvessels were measured using a computerised imaging and data analysis system (Kompira Ltd, Strathclyde, U.K.). In all experiments, unless stated otherwise, arterioles were preconstricted (by ca. 50%) with endothelin-1 (usually 30–300 pM) superfused 20 min before, and throughout, the experiment. All agents were applied to the cheek pouch topically by superfusion. Following application of dilator agents, vessel diameters were measured at 30 s, 1 min and every min thereafter until a maximal dilatation was obtained. In all studies, both arteriole and venule diameters were monitored concomitantly.

Tachykinin receptor studies

Up to five tachykinins and analogues (substance P, neurokinin A, substance P methyl ester, [β-Ala^8]-NKA(4-10) and Succ-[Asp^6,Me-Phe^8]-SP(6-11) (senktide)) were applied to each cheek pouch using a cumulative-dosing protocol with a random order of peptides between hamsters. Maximal dilator responses to tachykinins developed within 1–2 min of drug application, so each concentration of peptide was applied for 2 min. In experiments with SR140333, following determination of a control concentration-response curve to substance P methyl ester, concentration-responses curves were obtained every 20 min in the presence of increasing concentrations of SR140333 (1, 3 and 10 nM; 10 min incubation see Emonds-Alt *et al.*, 1993; test hamsters). In a separate series of experiments investigating the reproducibility of agonist responses, full cumulative concentration-response curves were obtained every 20 min for substance P methyl ester for 2.5 h (the duration of the longest experiment). In a separate series of experiments, SR140333 (10 nM) was tested against submaximal responses to human alpha CGRP (αCGRPh; 1 nM). Substance P (100 nM) and neurokinin A (100 nM) were also tested for effects on basal blood vessel diameter in cheek pouches that were not superfused with endothelin-1.

Nitric oxide studies

Preparations were superfused with Krebs solution containing N^G-nitro-L-arginine methyl ester (L-NAME; 10 μM) or endothelin-1 (at concentrations to give equivalent vasoconstriction). Submaximal responses were obtained to carbachol (0.1–1.0 μM), substance P (10 nM–100 nM), αCGRPh (0.1 nM) and sodium nitroprusside (10 nM) using a 20 min dose-cycle and a randomized order of dosing. Responses to carbachol and substance P were maximal within 2 min whereas those to αCGRPh and sodium nitroprusside were maximal after 3 min, so these agents were applied for 2 and 3 min respectively.

Source of drugs

Drugs were obtained as follows: sodium pentobarbitone (Sagatal; Rhône Mérieux Ltd, U.K.), human endothelin-1, substance P, neurokinin A, substance P methyl ester, [β-Ala^8]-NKA(4-10), senktide, bradykinin (Bachem, U.K.). Carbamylcholine chloride (carbachol), N^G-nitro-L-arginine methyl ester, sodium nitroprusside (Sigma, U.K.). SR140333 ((S)1-{2-[3(3,4-dichlorophenyl)-1-(3-iso-propoxyphenylacetyl)piperidin-3-yl] ethyl}-4-phenyl-1-azoniabicyclo[2.2.2]octone, hydrochloride) was a gift from Drs Brelière and Emonds-Alt, Sanofi Recherche, France. All salts were of analytical grade and were obtained from B.D.H., U.K. Senktide and [β-Ala^8]-NKA(4-10) were dissolved in dimethyl sulphoxide, all other peptides and other agents were dissolved in distilled water. Peptides were stored at −20°C.

Expression of results and statistical analysis

Vasodilatation was calculated as % maximal increase in arteriolar diameter (when compared with basal or preconstricted tone). Data from individual experiments were combined, and results are expressed as mean ± s.e.mean of experiments carried out in at least 4 hamsters for each study. One experiment only was carried out in each hamster. Tests for significant differences between means were made using Student's paired or unpaired *t* tests as appropriate. The apparent pK_B values were determined from individual dose-ratios using the Gaddum-Schild equation $pK_B = -\log K_B = \log(x\text{-}1) - \log[A]$; where x is the dose-ratio and [A] the antagonist concentration.

Results

Tachykinin receptor studies

In the absence of endothelin-1 in the superfusing Krebs solution, substance P (100 nM) and neurokinin A (100 nM) gave small, reversible arteriolar dilator responses (by 12.2 ± 2.6% and 14.6 ± 5.6% respectively; $n = 7$). In preparations where arterioles were preconstricted with endothelin-1, all tachykinins and analogues produced a rapid, concentration-related and rapidly reversible arteriolar dilatation, but were without significant effect on venule diameter. The relative activities of the various peptides, however, differed markedly. Thus, substance P was the most potent dilator being approximately 10 fold more active than both neurokinin A and the NK_1 receptor-selective agonist, substance P methyl ester. The NK_2 receptor-selective agonist [β-Ala^8]-NKA(4-10) was active only at high concentrations, and the NK_3 receptor-selective agonist, senktide, was vir-

tually inactive (Figure 1). In experiments with no antagonist, the lateral position and maximal response of the log concentration-response curve to substance P methyl ester did not change with time (2.5 h; $n = 4$; data not shown).

In cheek pouches superfused with endothelin-1, SR140333 (1–10 nM) had no effect on arteriolar diameter *per se*, but caused a concentration-related shift to the right of the log concentration-response curve of the NK_1 receptor-selective agonist, substance P methyl ester (Figure 2). The position of the log concentration-response curve to substance P methyl ester in control hamsters did not change with time ($n = 4$; data not shown). A full kinetic analysis of these data was not carried out (see Discussion); however, for the purpose of comparison, an estimate of antagonist potency (apparent pK_B) was determined. The apparent pK_B was estimated as 9.9 ± 0.2 ($n = 10$ estimates; $n = 5$ hamsters). SR140333 (10 nM) did not inhibit submaximal responses to αCGRPh ($n = 4$; $P > 0.05$; Figure 2), and in these latter experiments as a control, SR140333 (10 nM) significantly inhibited submaximal responses to substance P methyl ester ($n = 4$; $P < 0.01$; Figure 2).

Nitric oxide studies

Superfusion of the cheek pouch with L-NAME (10 μM) caused a gradual arteriolar constriction which stabilized after approximately 20 min at $51.3 \pm 5.4\%$ reduction in arteriole diameter. Control preparations were superfused with endothelin-1 to a similar degree of constriction (by $48.0 \pm 5.6\%$). Concentrations of substance P that caused significant dilatation in endothelin-1 constricted preparations gave markedly reduced dilator responses in preparations superfused with L-NAME (Figure 3). Dilator responses to carbachol were similarly significantly smaller when measured in the presence of L-NAME, whereas submaximal vasodilator responses to αCGRPh (Figure 3) and sodium nitroprusside (not shown) were similar in both groups of hamsters.

Discussion

This study demonstrates that tachykinin-evoked arteriolar vasodilatation in the hamster cheek pouch microvasculature results from NK_1 receptor stimulation, and is dependent, at least in part, on the release of nitric oxide. Further, endogenous nitric oxide release contributes to basal arteriolar tone in the hamster cheek pouch microvasculature. The recently described non-peptide tachykinin NK_1 receptor antagonist, SR140333, is a high affinity and selective antagonist when applied topically in this preparation.

NK_1 receptors

Tachykinin receptors are divided into three types, NK_1, NK_2 and NK_3 (see Guard & Watson, 1991; Maggi *et al.*, 1992, for reviews). In the vasculature, NK_1 receptors account for the majority of reported effects of tachykinins (see Holzer, 1992; Hall, 1994). Consistent with this, in the present study on tachykinin-evoked arteriolar dilatation, the relative activities of the endogenous tachykinins and receptor-selective analogues, and the high affinity of the NK_1 receptor-selective antagonist, SR140333, indicate the involvement of NK_1 receptors in vasodilatation. Thus, as previously described (Raud *et al.*,1991), substance P caused arteriolar dilatation and, in this present study, was found to be 10 fold more potent than neurokinin A. The NK_1 receptor-selective agonist, substance P methyl ester, was also a potent arteriolar vasodilator whereas the NK_2 receptor agonist, [β-Ala^8]-NKA(4-10) was only weakly active at high concentrations and the NK_3 selective agonist senktide was virtually inactive. The vasodilator effect seen with high concentrations of [β-Ala^8]-NKA(4-10) is likely to result from an interaction

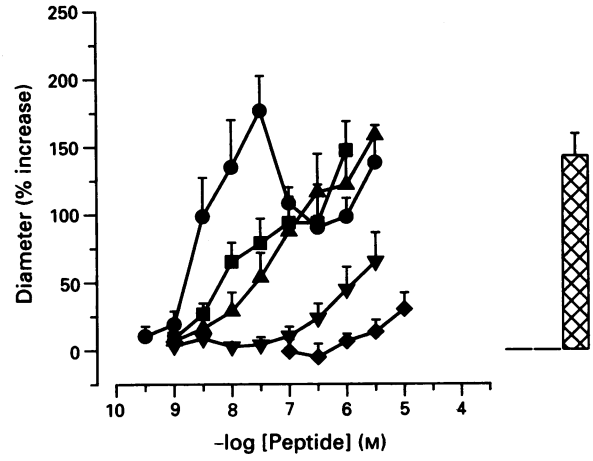

Figure 1 Log concentration-response curves showing vasodilator responses for tachykinins and analogues in the hamster cheek pouch. Arterioles were preconstricted by ca. 50% by endothelin-1, and arteriolar dilatation is expressed as % increase from this preconstricted level. Tachykinins and analogues were applied by superfusion at each concentration for 2 min and results are shown for substance P (●), neurokinin A (▲), substance P methyl ether (■), [β-Ala^8]-NKA(4-10) (▼) and senktide (◆). For comparison, the vasodilator response to sodium nitroprusside (100 nM; cross-hatched column) is shown. Responses are mean ± s.e.mean for experiments carried out on 8 hamsters.

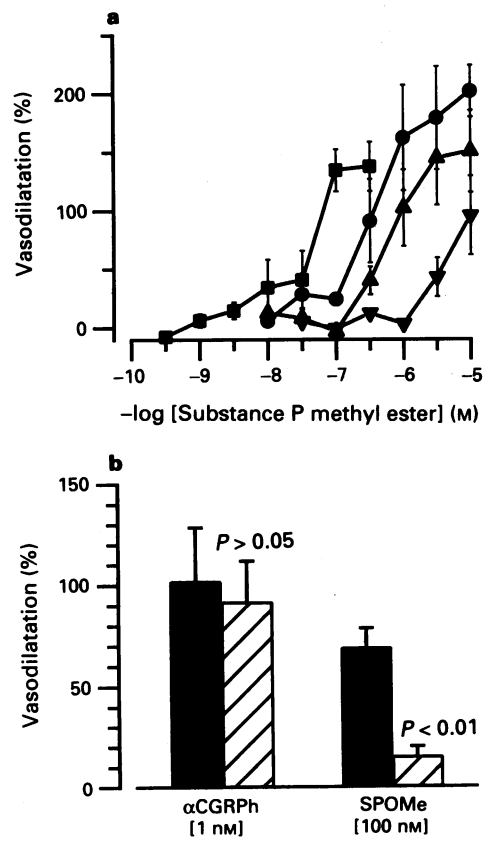

Figure 2 SR140333 selectively inhibits NK_1 receptor-mediated vasodilatation in hamster cheek pouch. In (a), concentration-response curves were obtained for substance P methyl ester either in the absence (■) or presence of SR140333 at 1 nM (●), 3 nM (▲) or 10 nM (▼). Selectivity of SR140333 for NK_1 receptor-mediated vasodilatation is shown in (b). Submaximal vasodilator responses to human alpha calcitonin gene-related peptide (αCGRPh; 1 nM) are unaffected by SR140333 whereas responses to substance P methyl ester (SPOMe, 100 nM) are significantly inhibited. Solid columns show control vasodilator responses; hatched columns, responses obtained in the presence of SR140333 (10 nM). Mean responses ± s.e.mean as shown for 4–5 hamsters.

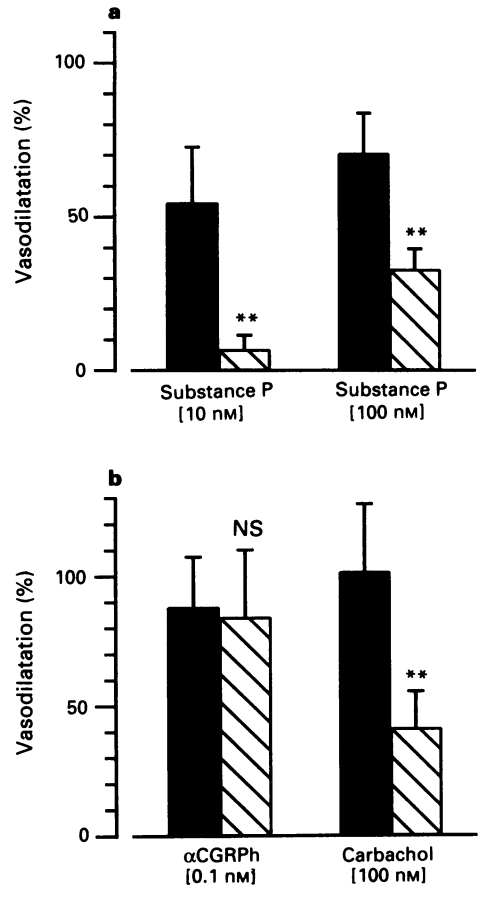

Figure 3 N^G-nitro-L-arginine methyl ester (L-NAME) inhibits vasodilator responses to substance P in hamster cheek pouch microvasculature. L-NAME (10 μM) produced a 51.3 ± 5.4% reduction in basal arteriole diameter. Vasodilator responses obtained in the presence of L-NAME (10 μM; hatched columns) are compared with those obtained in preparations preconstricted to a similar degree (48.0 ± 5.6%) by endothelin-1 (solid columns). In (a) L-NAME significantly inhibited vasodilator responses to substance P (10 and 100 nM), and in (b), vasodilator responses to carbachol (100 nM) were similarly inhibited whereas vasodilator responses to human alpha calcitonin gene-related peptide (α-CGRPh; 0.1 nM) were similar in both groups of hamsters. Responses are shown as mean ± s.e.mean for 5 hamsters. $^{**}P<0.05$.

of this agonist with NK_1 receptors as we have previously suggested in other preparations *in vitro* (Hall *et al.*, 1993b).

The involvement of NK_1 receptors in evoking arteriolar vasodilatation in the hamster cheek pouch was confirmed by our studies with the recently described non-peptide receptor antagonist, SR140333 (Emonds-Alt *et al.*, 1993; Jung *et al.*, 1994). Topical application of SR140333 inhibited arteriolar dilator responses to the NK_1 receptor-selective agonist, substance P methyl ester in a concentration-related manner, but was without effect on submaximal vasodilator responses evoked by αCGRPh. In our experiments on the hamster cheek pouch, we did not think it appropriate to analyse the nature of the antagonism in detail since these experiments were carried out *in vivo*. Further, in the guinea-pig ileum *in vitro*, we found the antagonism of responses to substance P methyl ester by high antagonist concentrations was apparently non-competitive (unpublished observations), possibly as a result of its very high affinity; as also reported by Emonds-Alt *et al.* (1993). However, a comparison of apparent pK_B values indicates that the affinity of SR140333 in the hamster cheek pouch (apparent pK_B 9.9) is similar to its affinity in the guinea-pig ileum (apparent pK_B 9.4; Hall *et al.*, 1994). The antagonist therefore does not appear to distinguish between hamster and guinea-pig NK_1 receptors (see Hall *et al.*, 1993a).

Our results demonstrating NK_1 receptor-mediated arteriolar dilatation in the hamster cheek pouch are consistent with studies in the microvasculature of other species. Thus, topically applied substance P was found to increase blood flow in rat knee joint and this was partially inhibited by the NK_1 receptor antagonist, CP-96,345 (Lam & Ferrell, 1993). In guinea-pig pial arterioles, perivascular administration of the NK_1 receptor-selective agonist, substance P methyl ester, caused vasodilatation, an effect inhibited by the NK_1 receptor antagonist GR82334 (Beattie *et al.*, 1993), and the vasodilatation resulting from nerve stimulation in rat cremaster skeletal muscle microvasculature was inhibited by a tachykinin receptor antagonist (Brock & Joshua, 1993). It should be noted, however, that evidence for the involvement of endogenous tachykinins to sensory nerve-evoked blood flow is limited; and, at least in rat and rabbit skin, evidence favours a role for CGRP (Hughes & Brain, 1991; Delay-Goyet *et al.*, 1992; Escott & Brain, 1993). For example, the NK_1 receptor antagonist, RP67580, does not inhibit the increased blood flow in skin evoked by stimulation of the saphenous nerve in the rat (Delay-Goyet *et al.*, 1992), though the increased blood flow is inhibited by the CGRP receptor antagonist, $CGRP_{8-37}$ (Escott & Brain, 1993). However, basal blood flow in rat extrapulmonary airways is increased by capsaicin, an effect mimicked by substance P, but not CGRP (Piedimonte *et al.*, 1992) suggesting a role for tachykinins in blood flow control in this tissue.

Mechanism of NK_1 receptor evoked vasodilatation

Vasodilator responses to substance P were significantly inhibited by the nitric oxide synthase inhibitor, L-NAME (10 μM), which itself produced a ca. 50% basal arteriolar constriction. This suggests that NK_1 receptor activation results in the release of nitric oxide which is responsible for the observed arteriolar vasodilatation. Nitric oxide-dependent arteriolar vasodilatation has previously been demonstrated in large blood vessels in response to NK_1 receptor activation (see Holzer, 1992; Hall, 1994), in substance P-evoked vasodilatation in certain rat oral microvessels (Kerezoudis *et al.*, 1993), and in substance P and neurokinin A-evoked vasodilatation in rabbit tenuissimus muscle microvasculature (Persson *et al.*, 1991). The observation that L-NAME significantly reduced basal arteriolar diameter in the hamster cheek pouch suggests that tonic release of nitric oxide contribute to basal arteriolar tone, as has been reported previously in rat skin (Lawrence & Brain, 1992), and in other microvasculature beds including the rat gastric mucosa (Tepperman & Whittle, 1992).

In conclusion, we have demonstrated that NK_1 receptor stimulation in the hamster cheek pouch results in arteriolar dilatation. The dilator effect of tachykinins was inhibited by the nitric oxide synthase inhibitor, L-NAME, which also reduced arteriolar diameter in this preparation. These results are consistent with the idea that in certain microvascular beds, endogenous tachykinins, in addition to CGRP, could potentially contribute to neurogenic vasodilatation of arterioles; and that in the case of the tachykinins, such neurogenic vasodilatation may be mediated via nitric oxide release. The possible contribution of the tachykinins to control of blood flow is of interest in that it is well established that the tachykinins act to increase microvascular permeability at the post-capillary venule, leading to oedema formation. The present study therefore indicates that tachykinins can potentially contribute to neurogenic inflammation by more than one NK_1 receptor-mediated acute pro-inflammatory mechanism. SR140333 inhibited NK_1 receptor-mediated vasodilatation, and since this antagonist is active following topical application *in vivo*, it may be a valuable tool with which to investigate the role of NK_1 receptors in neurogenic inflammatory processes.

We thank Drs Brelière and Emonds-Alt, Sanofi Recherche, Montpellier, France for the gift of SR140333 and Dr Ian K.M. Morton for preparing the figures. This study was supported by the Wellcome Trust.

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(Received April 25, 1994
Revised May 25, 1994
Accepted June 2, 1994)