Br. J. Pharmacol. (1989), **98**, 619–629

Multiple mechanisms in the motor responses of the guinea-pig isolated urinary bladder to bradykinin

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1 Bradykinin (1 nM–1 μM) produced a contraction of bladder strips excised from the dome of the guinea-pig urinary bladder, an effect which was greatly enhanced by removal of the mucosal layer or by thiorphan (10 μM). All subsequent experiments were performed in mucosa-free strips and in the presence of thiorphan.

2 In carbachol (5 μM)-contracted strips, bradykinin produced a concentration (1 nM–1 μM)-dependent transient relaxation.

3 Kallidin was slightly more potent than bradykinin in producing a contraction and a relaxation of the carbachol-induced tone. By contrast, [des-Arg^9]-bradykinin, a selective B_1 receptor agonist was barely effective up to 1 μM.

4 The contractile response to bradykinin was: (a) unaffected by either tetrodotoxin (1 μM), *in vitro* capsaicin desensitization (10 μM for 30 min) or apamin (0.1 μM); (b) antagonized by indomethacin (5 μM), the prostaglandin receptor antagonist SC-19220 (100 μM) or the B_2 receptor antagonist [D-Arg^0, Hyp^3, Thi^{5,8}, Phe^7]-bradykinin (10 μM) and (c) almost abolished by nifedipine (1 μM).

5 The antagonism of the contractile response to bradykinin produced by indomethacin and SC-19220 was non-additive while that produced by indomethacin and the B_2 receptor antagonist was additive.

6 The relaxant response to bradykinin was unaffected by tetrodotoxin, *in vitro* capsaicin desensitization or indomethacin but antagonized in a competitive manner by the B_2 receptor antagonist. Further, this response was abolished by apamin (0.1 μM) but unaffected by glibenclamide (1 μM).

7 Bradykinin (10 μM) produced a consistent release of calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) but not substance P-LI from the guinea-pig bladder muscle. CGRP-LI release by bradykinin was greatly reduced in bladders exposed to indomethacin. [des-Arg^9]-bradykinin (10 μM) was ineffective.

8 We conclude that: (a) bradykinin-induced contraction involves activation of both B_2 receptors and prostanoid synthesis, via distinct mechanisms which act by inducing calcium influx via nifedipine-sensitive channels; (b) bradykinin-induced relaxation involves activation of B_2 receptors and opening of apamin-sensitive potassium channels; (c) bradykinin stimulates sensory nerves in this tissue largely via prostanoid production.

Introduction

Bradykinin and related kinins exert a variety of biological effects by stimulating or inhibiting smooth muscle tone in various organs (Regoli & Barabé, 1980; Regoli, 1987). Recently much attention has been directed to the study of pharmacological responses to bradykinin in various tissues and evidence has been provided indicating that multiple mechanisms mediate the smooth muscle responses to this autacoid. Regoli & Barabé (1980) recognized the existence of two distinct bradykinin receptors which were termed B_1 and B_2, respectively. Recently some evidence has been presented indicating that B_2 receptors might be heterogeneous (Rifo *et al.*, 1987; Field *et al.*, 1988; Braas *et al.*, 1988; see Plevin &

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Owen, 1988 for review). Further, bradykinin and related peptides might affect muscle motility through mechanisms not directly related to activation of B_1 or B_2 receptors. As an example, bradykinin stimulates the generation of prostanoids possibly by activating directly one of the enzymes of the arachidonic acid cascade (Rhaleb *et al.*, 1988).

An additional mechanism through which bradykinin could affect smooth muscle motility is related to activation of the 'efferent' function of capsaicin-sensitive sensory nerves (see Szolcsányi 1984, and Maggi & Meli 1988, for reviews). Bradykinin is, amongst the mediators of inflammation, one of the most potent stimulants of sensory nerves (Armstrong, 1970; Ferreira *et al.*, 1973). This action, responsible for the pain-producing action of bradykinin, also involves the release, from peripheral terminals of capsaicin-sensitive sensory nerves of several neuropeptides (Geppetti *et al.*, 1988; Manzini *et al.*, 1989) which may directly affect visceral motility.

Despite the obvious interest for its potential pathophysiological role in cystitis, relatively little information is available about the effects of bradykinin on the urinary bladder muscle. Bradykinin produces a contraction of the urinary bladder in several species, although with marked differences in sensitivity (Falconieri-Erspamer *et al.*, 1973). In the rabbit detrusor muscle the response to bradykinin was markedly reduced by cyclo-oxygenase inhibitors and biochemical evidence for prostaglandin E_2 (PGE_2) production was presented (Downie & Rouffignac, 1981; Nakahata *et al.*, 1987; Nakahata & Nakanishi, 1988). In the dog isolated urinary bladder the contractile response to bradykinin was mediated by B_2 receptors (Regoli *et al.*, 1986). Here we have addressed the question of the mechanisms responsible for the motor responses to bradykinin in the guinea-pig isolated urinary bladder. As recent evidence indicates that certain tissue components such as the bladder mucosa (Maggi *et al.*, 1987a) or metabolism via a thiorphan-sensitive mechanism (Devillier *et al.*, 1988; Dusser *et al.*, 1988) may exert a strong influence on the biological activity of exogenously administered peptides, we also investigated the possible influence of these variables on the response to bradykinin.

Methods

In vitro *experiments*

Male albino guinea-pigs (Dunkin-Hartley strain) weighing 240–300 g were used throughout the study. The animals were killed by cervical dislocation and exsanguinated. The dome of the urinary bladder was rapidly removed, placed in Krebs solution, opened along its longitudinal axis and pinned flat on a Petri dish. Bladders strips about 1 cm long and 3 mm wide were excised with fine scissors. In most strips the mucosa was separated from the muscle (see Results). The strips were placed in a 5 ml organ bath containing a standard Krebs solution gassed with 96% O_2 and 4% CO_2 of the following composition (mM): NaCl 119, KCl 4.7, $MgSO_4$ 1.5, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11.

A resting load of 5 mN was applied and the preparations were allowed to equilibrate for 60 min before commencing the experiments. Tension was recorded by means of an isotonic strain gauge connected to a Basile 7050 Unirecord. The contractile response to KCl (80 mM, added to the bath) was used as internal standard. The strips were electrically field stimulated (0.03 Hz, 60 V, 0.5 ms) by means of two wire platinum electrodes placed at the top and the bottom of the organ bath (GRASS S11 stimulator). Concentration-response curves to bradykinin were constructed in a cumulative manner, the next concentration being added when the effects of the preceding one had reached a steady state. Concentrations were increased on a log 10 basis scale starting from 1 nM. Preliminary experiments (see Results) had shown that no significant desensitization occurs with this protocol.

In vitro capsaicin desensitization was achieved by prolonged (30 min) exposure of the strips to 10 μM capsaicin, followed by washing and re-equilibration, as described previously (Maggi *et al.*, 1987b; 1989).

Determination of substance P- and calcitonin gene-related peptide-like immunoreactivity by radioimmunoassay

Mucosa-free strips of bladder muscle were placed in 2 ml organ baths maintained at 37°C and superfused with standard Krebs solution, oxygenated with a mixture of O_2 and CO_2, containing 0.1% bovine serum albumin and 10 μM thiorphan (Sigma) at a rate of 2 ml min^{-1} by means of a peristaltic pump. Fractions were collected each min, in tubes containing acetic acid to a 2 N final concentration. At the end of the experiments the tissues were blotted 2–3 times on filter paper and weighed. Superfusates were freeze-dried, reconstituted with assay buffer (0.1 M, pH 7.4 phosphate buffer containing 0.9% NaCl, 0.01% NaN_3 and 0.1% bovine serum albumin). Substance P-like (SP-LI) and calcitonin gene-related peptide like activity (CGRP-LI) were measured by radioimmunoassay (RIA) as described previously (Maggi *et al.*, 1987c; 1988; Geppetti *et al.*, 1988).

For SP-LI determination (Maggi *et al.*, 1988; Geppetti *et al.*, 1988; Manzini *et al.*, 1989) the ^{125}I-Bolton and Hunter conjugated SP (Amersham, U.K.)

and No. 144 anti-SP serum (kind gift of Dr P. Pradelles, SPI-LERI, CEN/Saclay, 91191, Gif Sur Yvette, Cedex, France) were used. The sensitivity of RIA was 1.1 fmol/tube. The antiserum crossreacts to 1% with neurokinin A, 0.5% with neurokinin B and less than 0.1% with physalaemin and eledoisin.

CGRP-LI determination was made in the same samples as described previously (Geppetti *et al.*, 1988; Manzini *et al.*, 1989), by using ^{125}I-Bolton and Hunter conjugated human CGRP (Amersham) and anti human CGRP rabbit serum (Peninsula). The sensitivity of the RIA was 2.5 fmol/tube.

Statistical analysis

All data in the text are mean ± standard error of the mean (s.e.mean) of 5–7 experiments. Statistical analysis of the data was performed by means of the Student's *t* test for paired or unpaired data or by means of analysis of variance, when applicable. A P value < 0.05 was considered statistically significant. Regression analysis was made by means of the least squares method. EC_{50} and 95% confidence limits (c.l.) were calculated as the concentrations producing 50% of maximal contraction. pA_2 values were calculated as described by Van Rossum (1963).

Drugs

Drugs used were: bradykinin (Peninsula), indomethacin (Sigma), capsaicin (Sigma), nifedipine (Sigma), tetrodotoxin (Sankyo), carbachol HCl (Merck), apamin (Peninsula). SC-19220 (1-acetyl-2-(8-chloro-10,11-dihydrobenz[b,E][1,4]oxazepine-10-carbonyl)hydrazine) was a kind gift of Dr D.L. Hammond, Searle Company, Skokie, Illinois, U.S.A. [D-Arg^0, Hyp^3, Thi^{5,8}, Phe^7]-bradykinin and kallidin were a kind gift of Prof D. Regoli, Dept. of Physiology and Pharmacology, Sherbrooke University, Canada. Glibencalamide was a kind gift of Dr I. Cavero (Rhone-Poulenc, Paris, France).

Results

General

Strips excised from the dome of the guinea-pig urinary bladder responded to added KCl (80 mM) with a rapid phasic contraction the amplitude of which became reproducible after a 90–120 min equilibration period. Both in the presence or absence of the mucosa, electrical field stimulation (0.03 Hz, 0.5 ms, 60 V) induced twitches that were abolished by tetrodotoxin (1 μM). There was a striking difference in degree of spontaneous activity which averaged 40–80 and 10–30% of the response to electrical field stimulation in strips with and without mucosa, respectively.

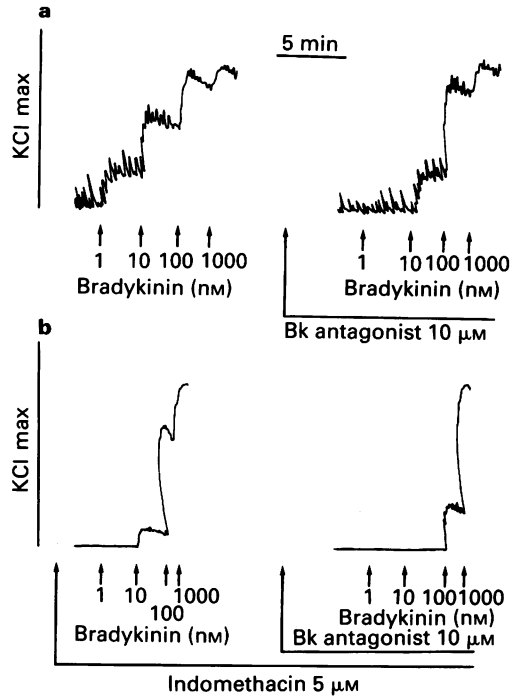

Figure 1 Typical tracings showing the contractile response of smooth muscle strips from the guinea-pig urinary bladder to cumulative addition of bradykinin (Bk) and its modification by the B_2 receptor antagonist [D-Arg^0, Hyp^3, Thi^{5,8}, Phe^7]-bradykinin (10 μM), in the absence (a) or presence (b) of indomethacin. Tension scale indicates the maximal contractile response to KCl.

Bradykinin produced a concentration-dependent contraction (Figure 1). In preliminary experiments we found no appreciable desensitization to cumulative addition of bradykinin (concentration increased on a log 10 scale, interval between doses 2–3 min), either in the presence or absence of mucosa. Thus, in strips without mucosa the response to 10 μM bradykinin amounted to 63 ± 6% of the response to KCl (80 mM) when the peptide was administered as a bolus and to 68 ± 6% when the same concentration of bradykinin was attained at the end of the cumulative concentration-response curve. Likewise, in strips with mucosa, the responses to 10 μM bradykinin were 46 ± 6 and 43 ± 8% of the response to KCl when the peptide was added to the bath as a bolus or at the end of cumulative concentration-response curves.

Effect of removal of the mucosa and thiorphan on the response to bradykinin

Removal of the mucosa increased significantly the contractile response to bradykinin which was about

Table 1 Effect of thiorphan and removal of the mucosa on the contractile response to bradykinin of the guinea-pig isolated bladder

	Without thiorphan (A)		*With thiorphan* (B)		A/B
With mucosa	6364	(835–12670)	82	(42–236)	78
Without mucosa	451	(173–2334)	26	(17–45)	17

Values are EC_{50} (nM) and 95% confidence limits (in parentheses).

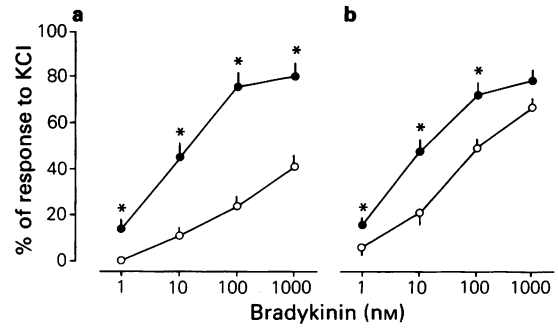

Figure 2 Effect of removal of the mucosa (b) and thiorphan (10 μM) on the contractile response of smooth muscle strips from the guinea-pig urinary bladder to bradykinin; (○) control; (●) plus thiorphan. Each value is mean of at least 6 experiments, with s.e. shown by vertical bars. Significantly different from control, $*P < 0.05$.

14 times more potent in the absence than in the presence of the mucosa (Table 1). Thiorphan (10 μM), a well known inhibitor of endopeptidase 24.11, produced a small (< 10% of KCl maximum) contraction *per se* which faded to baseline within 15 min in about 50% of preparations while it had no effect on the remainder. In the presence of thiorphan, the response to bradykinin was markedly enhanced (Figure 2). This potentiation was larger in the presence (78 times) than in the absence (17 times) of the mucosa (Table 1; Figure 2).

Mucosa-free bladder strips treated with thiorphan (10 μM, 15 min before) were then used in all the remaining experiments, unless otherwise specified.

Relaxant responses to bradykinin

To assess whether bradykinin may produce relaxation, bladder strips (mucosa-free, thiorphan 10 μM in the bath) were exposed to carbachol (5 μM). This produced a large phasic contraction followed by a steady tonic response which approached 80–90% of the response to KCl. In these conditions, bradykinin produced a concentration-dependent transient relaxation (Figure 3).

The maximal relaxant response to 10 μM bradykinin added as a bolus (48 ± 4%, $n = 4$) did not significantly differ from that observed when the same concentration was attained at the end of a cumulative concentration-response with a tenfold increase in concentration for each dose (42 ± 6%, $n = 6$). On the other hand when the relaxant response produced by a given concentration of bradykinin had faded, a second addition of the same dose produced a relaxant response much smaller (70–80% reduction) than that produced by the first dose. Bradykinin (0.1 μM) had no relaxant effect on the tonic contraction produced by KCl (80 mM, $n = 3$).

Effect of kallidin and [des-Arg^9]-bradykinin

Kallidin (1 nM–1 μM) produced motor responses similar to those produced by bradykinin, that is a concentration-dependent contraction of the unstimulated bladder and a concentration-dependent relaxation of the carbachol-induced tone (Figure 4). Kallidin was as effective as bradykinin in producing a contraction and slightly more effective in producing the relaxation (Figure 4).

[des-Arg^9]-bradykinin had little or no contractile activity nor did it induce relaxation of carbachol-

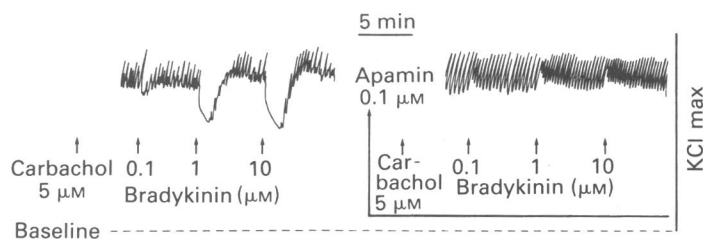

Figure 3 Typical tracings showing the relaxation produced by cumulative addition of bradykinin of smooth muscle strips from the guinea-pig urinary bladder (tone elevated by 5 μM carbachol) and blockade of this response by apamin. Tension scale indicates the maximal contractile response to KCl.

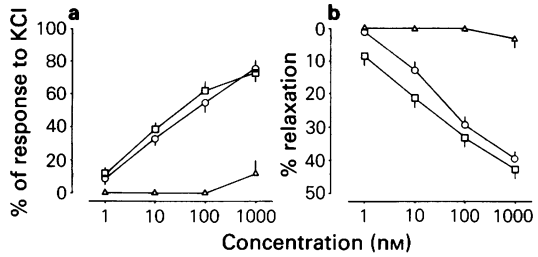

Figure 4 Contractile (a) and relaxant (b) responses to bradykinin (○), kallidin (□) and [des-Arg^9]-bradykinin (△) of smooth muscle strips from the guinea-pig urinary bladder. To study relaxations, tension was elevated by addition of 5 μM carbachol to the bath. Each value is mean of at least 6 experiments; s.e.mean shown by vertical lines.

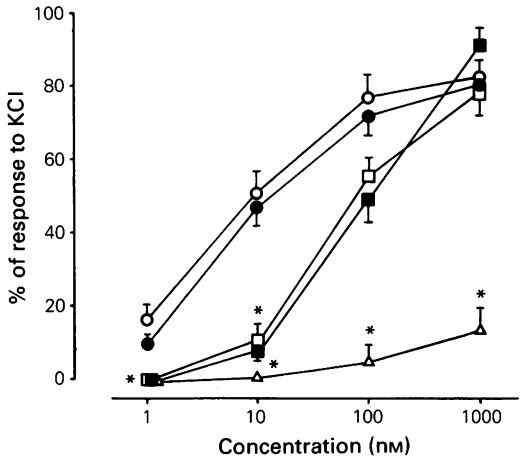

Figure 5 Effect of tetrodotoxin (1 μM, ●), indomethacin (5 μM, □), SC-19220 (100 μM, ■) or nifedipine (△) on the contractile response of smooth muscle strips from the guinea-pig urinary bladder to bradykinin; (○) control. Each value is mean of at least 6 experiments, s.e. shown by vertical bars. Significantly different from control, $*P < 0.05$.

induced tone up to 1 μM (Figure 4). In some experiments, to assess the possible involvement of a *de novo* synthesis of B_1 receptors during prolonged *in vitro* incubation (see Regoli & Barabé, 1980), the effect of the B_1 receptor agonist was investigated 4–5 h from the beginning of the experiment. Even in this case ($n = 3$) there was little or no response to [des-Arg^9]-bradykinin.

Effect of in vitro *capsaicin desensitization on the contractile response to bradykinin*

Previous exposure to capsaicin (10 μM for 30 min) did not significantly affect the contractile response to bradykinin, the EC_{50} and 95% confidence limits of this latter being 23 (7–45) and 22 (8–36) nM in controls and in preparations exposed to capsaicin, respectively. The same occurred in the absence of thiorphan, the EC_{50} of bradykinin being 251 (73–2334) and 143 (33–1815) nM in controls and in preparations exposed to capsaicin, respectively.

Effect of tetrodotoxin, indomethacin, nifedipine or SC-19220 on the contractile response to bradykinin

Tetrodotoxin (1 μM for 15 min) had a slight and not statistically significant inhibitory effect on the response to bradykinin (Figure 5, Table 2). Either indomethacin (5 μM for 90 min, Figure 1) or the prostaglandin antagonist SC-19220 (100 μM for 15 min) significantly antagonized the response to low concentrations of bradykinin (1–10 nM) while having no effect on the response to the higher concentrations (0.1–1 μM) (Figure 5). The EC_{50} of bradykinin was increased by about 10 times in the presence of either indomethacin or SC-19220 (Table 2). In other experiments the effect of both indomethacin (5 μM for 90 min) and SC-19220 (100 μM for 15 min) on the response to bradykinin was investigated. As shown in Table 2, the combined treatment with these two agents was not more effective than that produced by either of them when administered alone, as the EC_{50} of bradykinin was increased by about 13 times as compared to controls.

At the concentration tested, SC-19220 produced a slight enhancement of electrical field stimulation-induced contractions while having no effect against the response to KCl ($n = 4$). The bradykinin-induced

Table 2 Effects of various pretreatments on the contractile response to bradykinin of the guinea-pig isolated urinary bladder (mucosa-free, thiorphan 10 μM in the bath)

Treatment	EC_{50} *and 95% confidence limits* (nM)	
Controls	26	17–45
Tetrodotoxin 1 μM	36	13–90
Indomethacin 5 μM	259	64–810
SC-19220 100 μM	258	150–315
Indomethacin 1 μM plus SC-19220 100 μM	330	218–456
B_2 antagonist 10 μM	179	158–207
B_2 antagonist 10 μM plus indomethacin 5 μM	1760	1189–2347
Nifedipine 1 μM	$\geqslant$ 10000	–
Apamin 0.1 μM	26	16–53

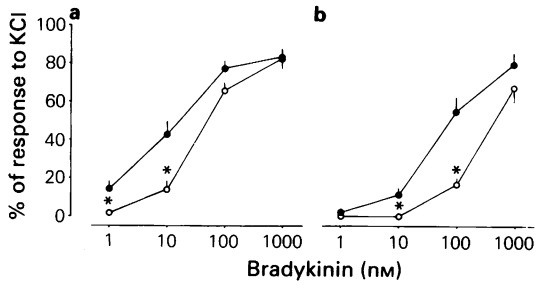

Figure 6 Effect of the bradykinin antagonist, [D-Arg^0, Hyp^3, Thi^{5,8}, Phe^7]-bradykinin (10 μM), on the contractile response of smooth muscle strips from the guinea-pig urinary bladder to bradykinin in the absence (a) or presence (b) of indomethacin: (●) control; (○) B_2-antagonist. Each value is mean of at least 6 experiments, s.e. shown by vertical lines. * Significantly different from control, *$P < 0.05$.

contraction was almost abolished by nifedipine (1 μM, 30 min before, $n = 4$, Figure 5).

Effect of [D-*Arg*^{0}, *Hyp*^{3}, *Thi*^{5,8}, *Phe*^{7}]-*bradykinin on the contractile response to bradykinin*

The B_2 receptor antagonist, [D-Arg^0, Hyp^3, Thi^{5,8}, Phe^7]-bradykinin (10 μM) itself produced either a small potentiation (5–10%) or no effect on twitches produced by electrical field stimulation and did not modify the contractile response to KCl, either in absence or presence of indomethacin ($n = 8$). This antagonist (10 μM, 15 min before) produced a rightward shift of the concentration-response curve to bradykinin which, however, attained a similar maximal response, both in the absence or presence of indomethacin (Figure 1 and 6). The B_2 antagonist increased the EC_{50} of bradykinin by 6.9 and 6.8 times in the absence and presence of indomethacin, respectively. The corresponding apparent pA_2 values, calculated as described by Van Rossum (1963), were 5.33 and 5.69 in the absence and presence of indomethacin, respectively.

Effect of tetrodotoxin, capsaicin desensitization, indomethacin, or [D-*Arg*^{0}, *Hyp*^{3}, *Thi*^{5,8}, *Phe*^{7}]-*bradykinin on bradykinin-induced relaxation*

Neither tetrodotoxin (1 μM) nor indomethacin (5 μM) had any effect on bradykinin-(1 μM) induced relaxation which was slightly but not significantly enhanced after *in vitro* capsaicin desensitization (Figure 7).

By contrast, the bradykinin-induced relaxation was antagonized, in a competitive manner by [D-Arg^0, Hyp^3, Thi^{5,8}, Phe^7]-bradykinin (10 μM) (Figure

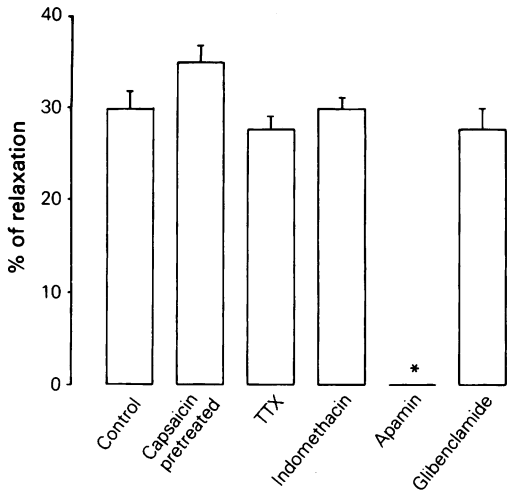

Figure 7 Effect of *in vitro* capsaicin desensitization, tetrodotoxin (1 μM), indomethacin (5 μM), apamin (0.1 μM) or glibenclamide (1 μM) on the relaxant response produced by 1 μM bradykinin on carbachol-induced tone of smooth muscle strips from the guinea-pig urinary bladder. Each column is mean of 4–6 experiments; vertical bars show s.e.mean. Significantly different from control *$P < 0.05$.

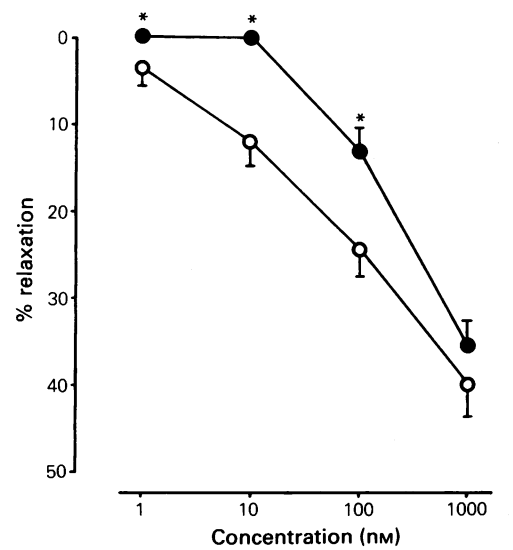

Figure 8 Effect of the bradykinin antagonist, [D-Arg^0, Hyp^3, Thi^{5,8}, Phe^7]-bradykinin (10 μM) on the bradykinin-induced relaxation of carbachol-induced tone of smooth muscle strips from the guinea-pig urinary bladder: (○) control; (●) B_2 receptor antagonist. Each value is mean of 6 experiments with s.e. shown by vertical bars. * Significantly different from control, *$P < 0.05$.

8). The corresponding pA_2 value, calculated as described by Van Rossum (1963) was 5.56.

Effect of apamin

Apamin (0.1 μM 15 min before), the peptide extracted from bee venom which blocks certain Ca-activated K channels (see Cook, 1988 for review), completely prevented the bradykinin-induced relaxations of carbachol-contracted strips (Figure 3). By contrast, glibenclamide (1 μM for 15 min) a potent blocker of ATP-sensitive K channels (Schmit-Antomarchi *et al.*, 1987) had no effect on bradykinin-induced relaxations ($n = 4$). The effect of apamin on the contractile response to bradykinin was examined. Estimation of the effect of low concentrations of bradykinin in the presence of apamin was difficult because apamin itself produced rhythmic contractile activity, the amplitude of which was 10–20% of the response to KCl. In spite of this, the estimated ED_{50} of bradykinin in the presence of apamin (0.1 μM, 15 min before) was similar to that observed in controls (Table 2).

Activation of capsaicin-sensitive nerves by bradykinin

As shown in Figure 9, bradykinin (10 μM) produced a consistent increase in CGRP-LI outflow from mucosa-free muscle strips from the guinea-pig urinary bladder. By contrast, no significant SP-LI outflow was induced (Figure 9). We have shown previously that CGRP-LI is stored and released from capsaicin-sensitive sensory nerves in this organ (Maggi *et al.*, 1988). In subsequent experiments CGRP-LI outflow was measured as an index of activation of capsaicin-sensitive fibres in this tissue. [des-Arg^9]-bradykinin (10 μM) failed to elicit any increase in CGRP-LI outflow while subsequent administration of bradykinin (10 μM) on the same samples had a consistent releasing effect ($n = 4$, data not shown). The bradykinin (10 μM)-induced CGRP-LI release was abolished by indomethacin (5 μM for 30 min, Figure 10).

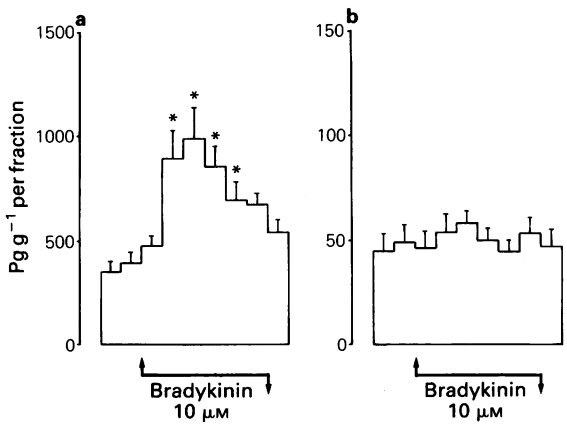

Figure 9 Effect of 10 μM bradykinin on calcitonin gene-related peptide-like immunoreactivity (a) and substance P-like immunoreactivity (b) outflow from superfused muscle of the guinea-pig urinary bladder. Each value is mean of 4 experiments; vertical bars show s.e.mean. Significantly different from baseline, $*P < 0.05$.

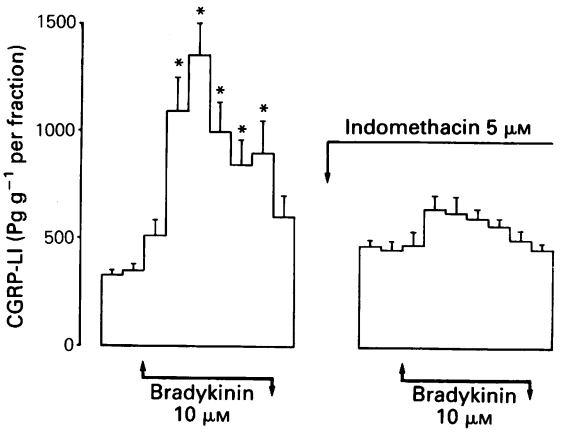

Figure 10 Effect of indomethacin (5 μM) on bradykinin (10 μM)-induced calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) released from superfused muscle strips of the guinea-pig isolated urinary bladder. These experiments were performed in parallel by comparing the effect of bradykinin in absence or presence of indomethacin on muscle strips from the same bladders. Each value is mean of 4 experiments; s.e. shown by vertical bars. Significantly different from baseline, $*P < 0.05$.

Discussion

Effect of removal of the mucosa and thiorphan

The present findings provide further evidence indicating that estimates of the biological activity of bradykinin may be strongly influenced by peptide degradation (Erdös, 1979; Regoli, 1987; Dusser *et al.*, 1988). Several proteases are able to hydrolyze kinins (Erdös, 1979; Regoli, 1987). Recently Dusser *et al.* (1988) reported that phosphoramidon and leucine-thiorphan, two inhibitors of endopeptidase 24.11, markedly enhance the contractile response to

bradykinin in the ferret isolated trachea. Here we show that thiorphan produced a marked potentiation of the response to bradykinin. Biochemical investigations have shown that bradykinin can be cleaved by endopeptidase 24.11 (Turner, 1987). We have not directly assessed whether this enzymatic activity is present in the guinea-pig urinary bladder and, for this reason, we cannot exclude that thiorphan produced its effect by blocking other enzymes capable of degrading bradykinin.

The present data indicate that the thiorphan-sensitive activity which limits the expression of the full biological activity of bradykinin resides in both the mucosal and the muscle layer of the guinea-pig urinary bladder. Indeed removal of the mucosal layer itself increased the potency of bradykinin, a finding which recalls similar observations with SP in this same organ (Maggi *et al.*, 1987a). We cannot exclude the possibility that the mucosal layer acted as a diffusion barrier toward exogenous peptides, but the observation that enhancement of bradykinin activity by thiorphan was greater in the presence (78 times) than in the absence (17 times) of the mucosa suggests that the latter plays a role in the inactivation of bradykinin, e.g. the thiorphan-sensitive activity is more concentrated in the mucosa than in the muscle.

Further studies are needed to establish the mechanism(s) whereby removal of the mucosa and addition of thiorphan enhanced the response to bradykinin. Nevertheless, these experiments allowed us to select proper experimental conditions (mucosa-free muscle strips exposed to 10 μM thiorphan) for studying the mechanisms underlying the motor responses to bradykinin.

Motor responses to bradykinin

The present findings indicate that bradykinin exerts two opposite motor effects in the guinea-pig urinary bladder muscle. These two effects can be better assessed by a further selection of the experimental conditions, that is a contraction at resting tone and a relaxation when the tone was raised by carbachol. Both effects are largely if not totally tetrodotoxin-resistant suggesting that intramural nerves do not play a key role in their genesis. The same applies to the intramural capsaicin-sensitive nerves. Although bradykinin is capable of stimulating the 'efferent' function of these nerve endings (see below), both the contraction and the relaxation produced by this peptide were unaffected by a previous exposure of the tissues to a high concentration of capsaicin, a procedure shown to produce a long-lasting blockade of sensory nerves (Maggi *et al.*, 1987b; 1989; Barthó *et al.*, 1987) (sensory neurone blocking action of capsaicin, as defined by Szolcsányi, 1984; 1989). Either tachykinins or CGRP can affect motility of the guinea-pig urinary bladder (Maggi *et al.*, 1988) but, conceivably, the motor effects of bradykinin are strong enough to overcome those produced by released tachykinins or CGRP.

The present findings suggest that B_1 receptors play no role in the action of bradykinins, as [des-Arg^9]-bradykinin, a selective B_1 receptor agonist (Regoli & Barabé, 1980) was virtually ineffective. The contractile response to bradykinin apparently involves both B_2 receptor activation and prostanoid synthesis along the cyclo-oxygenase pathway. The two mechanisms are apparently independent, because the inhibitory actions of indomethacin and the B_2 receptor antagonist were additive. By contrast, the actions of indomethacin and SC-19220 a PGE_2 antagonist (Drower *et al.*, 1987) were not additive. This conclusion agrees with the findings of Rhaleb *et al.* (1988) who proposed that activation of prostanoid synthesis by bradykinin may occur independently from B_1 or B_2 receptor activation. In any case, influx of extracellular calcium via nifedipine-sensitive calcium channels seems a likely final event for the genesis of the contractile response. B_2 receptors are apparently responsible for the bradykinin-induced relaxation which was observed when the tone was raised with carbachol but not when tone was raised by KCl. Presumably this implies that bradykinin relaxes smooth muscle cells by hyperpolarization due to opening of K channels. This could explain the sensitivity of the bradykinin-induced relaxation to apamin, a well known blocker of certain calcium-activated K channels, while glibenclamide, a blocker of certain ATP-sensitive K channels was ineffective (Schmit-Antomarchi *et al.*, 1987; Cook, 1988). These findings are reminiscent of the apamin-sensitive relaxation produced by bradykinin in the guinea-pig taenia caeci preparation (Carter *et al.*, 1987; Den Hertog *et al.*, 1988) which have been shown to involve activation of B_2 receptors. Likewise, bradykinin was shown to produce a transient rise in intracellular free neuronal calcium associated with transient hyperpolarization due to activation of an apamin-sensitive outward K current (Miller, 1987).

It is interesting to note that the B_2 receptor antagonist used in this study had a similar pA_2 value (about 5.5) when tested against bradykinin-induced contraction and relaxation. Thus the same receptor could mediate both responses in the guinea-pig bladder. [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7] bradykinin has been shown to be quite a potent ($pA_2 > 6.5$) bradykinin antagonist in some systems (rabbit jugular vein, rat vas deferens) (Rifo *et al.*, 1987; Whalley *et al.*, 1987) while in other tissue (guinea-pig taenia caeci, Field *et al.*, 1988; guinea-pig urinary bladder, this study) it showed a consistently lower

affinity for B_2 receptors (pA_2 about 5.5). Further studies are needed to assess which subtype of the B_2 receptor (see Plevin & Owen, 1988 for review) mediates the motor responses to this peptide in the guinea-pig urinary bladder.

Activation of sensory nerves by bradykinin

There is ample evidence indicating that bradykinin is a potent stimulant of sensory nerves and this action produces not only pain and activation of reflex responses (Lembeck *et al.*, 1976; Kumazawa *et al.*, 1980; Hori *et al.*, 1986; Dray *et al.*, 1988a,b; Rioux *et al.*, 1987; Staszewska-Woolley *et al.*, 1988) but also neuropeptide release from peripheral endings of capsaicin-sensitive nerves which exert a variety of motor and inflammatory actions. As discussed above, the motor effects produced by neuropeptides released by bradykinin in the guinea-pig urinary bladder are probably obscured by the motor effects produced by bradykinin itself (B_2 receptor activation) and prostanoid generation. It is however possible that peptides released from sensory nerves (Saria *et al.*, 1984; Markowitz *et al.*, 1987) play a role in the inflammatory response produced by bradykinin in the urinary tract. In a previous study (Maggi *et al.*, 1988) we demonstrated a simultaneous release of both SP-LI and CGRP-LI by capsaicin from mucosa-free muscle strips of the guinea-pig urinary bladder. Tissue content of both peptides is depleted (>90% reduction) by systemic capsaicin desensitization. Therefore we can reasonably assume that CGRP-LI released by bradykinin in the present experiments originated from peripheral endings of these primary sensory neurones. The reason why no SP-LI release was detected in response to bradykinin is at present unclear. However, the CGRP-LI release induced by bradykinin is only a fraction (<20%) of the response to capsaicin (unpublished data). When looking at the response to capsaicin in the guinea-pig urinary bladder, the released CGRP-LI/SP-LI ratio lies between 12–16/1 (Maggi *et al.*, 1988). It is therefore conceivable that a putative SP-LI release by bradykinin might have been too low to be detected with RIA.

Using different experimental models, evidence has been presented that bradykinin might excite sensory fibres both directly and through the release of prostanoids (Lembeck *et al.*, 1976; Hori *et al.*, 1986). In some instances evidence has been presented indicating the involvement of B_2 receptors (Dray *et al.*, 1988a; Staszewska-Woolley *et al.*, 1988).

The present findings indicate that SP-LI and CGRP-LI release by bradykinin in the guinea-pig urinary bladder is largely indirect, via prostanoid production. A similar conclusion was drawn about the SP-LI and CGRP-LI release from sensory nerves in the guinea-pig heart (Manzini *et al.*, 1989). When looking at the contractile response produced by bradykinin in the guinea-pig bladder (cf. Rhaleb *et al.*, 1988) one might speculate that B_2 receptors play a minor role in activating sensory fibres in this preparation, but further studies are needed to elucidate this point.

Conclusions

The present findings indicate that bradykinin produces multiple responses in the bladder which involve activation of both B_2 receptors and prostanoid synthesis, apparently via independent mechanisms. The consequences of mucosal removal and addition of thiorphan suggest that potent endogenous mechanism(s) operate to limit the activity of this peptide. When produced in biologically relevant quantities during an inflammatory process such as cystitis (cf. Marceau *et al.*, 1980) the effects of bradykinin would probably be such as to affect reflex regulation of bladder motility, by stimulation of capsaicin-sensitive nerves which regulate the afferent branch of reflex micturition in this species (Maggi *et al.*, 1987c). This may occur through stimulation of sensory nerves (either directly or via prostanoid production) and the motor response to the peptide which could itself activate bladder mechanoreceptors. Thus, bradykinin might contribute significantly to the genesis of symptoms such as urinary frequency in addition to sustaining neurogenic inflammation.

This work was in part supported by IMI, Rome (Progetto di Ricerca: Farmaci per il trattamento a lungo termine della incontinenza urinaria: VES, grant # 46287) and CNR, Rome (Progetto Finalizzato Chimica Fine e Secondaria).

References

ARMSTRONG, D. (1970). Pain. *Handbook Exp. Pharmacol.*, **25**, 434–481.

BARTHÓ, L., PETHÓ, G., ANTAL, A., HOLZER, P. & SZOLCSÁNYI, J. (1987). Two types of relaxation due to capsaicin in the guinea-pig isolated ileum. *Neurosci. Lett.*, **81**, 146–150.

BRAAS, K.M., MANNING, D.C., PERRY, D.C. & SNYDER S.H. (1988). Bradykinin analogues: differential agonist and antagonist activities suggesting multiple receptors. *Br. J. Pharmacol.*, **94**, 3–5.

CARTER, T.D., HALL, J.M., McCABE, D., MORTON, I.K.M. & SCHACHTER, M. (1987). Biphasic actions of bradykinin

in the guinea-pig taenia caeci preparation, *Br. J. Pharmacol.*, **90**, 137P.

COOK, N.S. (1988) The pharmacology of potassium channels and their therapeutic potential. *Trends Pharmacol. Sci.*, **9**, 21–28.

DEN HERTOG, A.D., NELEMANS, A. & VAN DEN AKKER, J. (1988). The multiple action of bradykinin on smooth muscle of guinea-pig taenia caeci. *Br. J. Pharmacol.*, **151**, 357–363.

DEVILLIER, P., ADVENIER, C., DRAPEAU, G., MARSAC, J. & REGOLI, D. (1988) Comparison of the effects of epithelium removal and of an enkephalinase inhibitor on the neurokinin-induced contractions of guinea-pig isolated trachea. *Br. J. Pharmacol.*, **94**, 675–684.

DOWNIE, J.W. & ROUFFIGNAC, S. (1981). Response of rabbit detrusor muscle to bradykinin. *Life Sci.*, **28**, 603–608.

DRAY, A., BETTANEY, J., FORSTER, P. & PERKINS, M.N. (1988a). Activation of a bradykinin receptor in peripheral nerve and spinal cord in the neonatal rat in vitro. *Br. J. Pharmacol.*, **95**, 1008–1010.

DRAY, A., BETTANEY, J., FORSTER, P. & PERKINS, M.N. (1988b). Bradykinin-induced stimulation of afferent fibres is mediated through protein kinase C. *Neurosci. Lett.*, **91**, 301–307.

DROWER, E.J., STAPELFELD, A., MUELLER, R.A. & HAMMOND, D.L. (1987). The antinociceptive effects of prostaglandin antagonists in the rat. *Eur. J. Pharmacol.*, **133**, 249–255.

DUSSER, D.J., NADEL, J.A., SEKIZAWA, K., GRAF, P.D. & BORSON, D.B. (1988). Neutral endopeptidase and angiotensin converting enzyme inhibitors potentiate kinin-induced contraction of ferret trachea. *J. Pharmacol. Exp. Ther.*, **244**, 531–536.

ERDÖS, E.G. (1979). Kininases. *Handb. Exp. Pharmacol.*, **25**, 427–487.

FALCONIERI-ERSPAMER, G., NEGRI, L. & PICCINELLI, D. (1973). The use of preparations of urinary bladder smooth muscle for bioassay of and discrimination between polypeptides. *Naunyn Schmiedebergs Arch. Pharmacol.*, **279**, 61–74.

FERREIRA, S.H., MONCADA, S. & VANE, J.R. (1973). Prostaglandins and the mechanism of analgesia produced by aspirin-like drugs. *Br. J. Pharmacol.*, **49**, 86–97.

FIELD, J.L., FOX, A.J., HALL, J.M., MAGBAGBEOLA, A.O. & MORTON, I.K.M. (1988). Multiple bradykinin B_2 receptor subtypes in smooth muscle preparations. *Br. J. Pharmacol.*, **93**, 284P.

GEPPETTI, P., MAGGI, C.A., PERRETTI, F., FRILLI, S. & MANZINI, S. (1988). Simultaneous release by bradykinin of substance P- and calcitonin gene-related peptide immunoreactivities from capsaicin-sensitive structures in guinea-pig heart. *Br. J. Pharmacol.*, **94**, 288–290.

HORI, Y., KATORI, M., HARADA, Y., UCHIDA, Y. & TANAKA, K. (1986). Potentiation of bradykinin-induced nociceptive response by arachidonate metabolites in dogs. *Eur. J. Pharmacol.*, **132**, 47–52.

KUMAZAWA, T. & MIZUMURA, K. (1980). Chemical responses of polymodal receptors of the scrotal contents in dogs. *J. Physiol.*, **299**, 219–231.

LEMBECK, F., POPPER, H. & JUAN, H. (1976). Release of prostaglandins by bradykinin as an intrinsic mechanism of its algesic effect. *Naunyn Schmiedebergs. Arch Pharmacol.*, **294**, 69–73.

MAGGI, C.A. & MELI, A. (1988). The sensory-efferent function of capsaicin-sensitive sensory neurons. *Gen. Pharmacol.*, **19**, 1–43.

MAGGI, C.A., GIULIANI, S., MANZINI, S. & MELI, A. (1989). $GABA_A$ receptor-mediated positive inotropism in guinea-pig isolated left atria: evidence for the involvement of capsaicin-sensitive nerves. *Br. J. Pharmacol*, **97**, 103–110.

MAGGI, C.A., GIULIANI, S., SANTICIOLI, P., ABELLI, L., GEPPETTI, P., SOMMA, V., RENZI, D. & MELI, A. (1987c). Species-related variations in the effects of capsaicin on urinary bladder functions: relation to bladder content of substance P-like immunoreactivity. *Naunyn Schmiedebergs Arch. Pharmacol.*, **336**, 546–555.

MAGGI, C.A., MELI, A. & SANTICIOLI, P. (1987b). Four motor effects of capsaicin on guinea-pig distal colon. *Br. J. Pharmacol.*, **90**, 651–660.

MAGGI, C.A., SANTICIOLI, P., PARLANI, M., ASTOLFI, M., PATACCHINI, R. & MELI, A. (1987a). The presence of mucosa reduces the contractile response of the guinea-pig urinary bladder to substance P. *J. Pharm. Pharmacol.*, **39**, 653–655.

MAGGI, C.A., SANTICIOLI, P., PATACCHINI, R., GEPPETTI, P., GIULIANI, S., ASTOLFI, M., BALDI, E., PARLANI, M., THEODORSSON, E., FUSCO, B. & MELI, A. (1988). Regional differences in the motor response to capsaicin in the guinea-pig urinary bladder: relative role of pre- and postjunctional factors related to neuropeptide-containing sensory nerves. *Neuroscience*, **27**, 675–688.

MANZINI, S., PERRETTI, F., DE BENEDETTI, L., PRADELLES, P., MAGGI, C.A. & GEPPETTI, P. (1989). A comparison of bradykinin and capsaicin-induced myocardial and coronary effects in isolated perfused guinea-pig hearts. Involvement of substance P and CGRP release. *Br. J. Pharmacol.*, **97**, 303–312.

MARCEAU, F., BARABÉ, J., ST PIERRE, S. & REGOLI, D. (1980). Kinin receptors in experimental inflammation. *Can. J. Physiol. Pharmacol.*, **58**, 536–542.

MARKOWITZ, S., SAITO, K. & MOSKOWITZ, M.A. (1987). Neurogenically mediated leakage of plasma protein occurs from blood vessels in dura mater but not brain. *J. Neurosci.*, **7**, 4129–4136.

MILLER, R.J. (1987). Bradykinin highlights the role of phospholipid metabolism in the control of nerve excitability. *Trends Neurosci.*, **10**, 226–228.

NAKAHATA, N. & NAKANISHI, H. (1988). Bradykinin-induced contraction is inhibited by tiaramide an anti-inflammatory drug with an inhibition of increase of intracellular free calcium, *J. Pharmacol. Exp. Ther.*, **246**, 635–640.

NAKAHATA, N., ONO, T. & NAKANISHI, H. (1987). Contribution of PGE_2 to bradykinin-induced contraction in rabbit urinary detrusor. *Jpn. J. Pharmacol.*, **43**, 351–359.

PLEVIN, R. & OWEN, P.J. (1988). Multiple B_2 kinin receptors in mammalian tissues. *Trends Pharmacol. Sci.*, **9**, 387–389.

REGOLI, D. (1987). Kinins. *Br. Med. Bull.*, **43**, 270–284.

REGOLI, D. & BARABÉ, J. (1980). Pharmacology of bradykinin and related kinins. *Pharmacol. Rev.*, **32**, 1–46.

REGOLI, D., DRAPEAU, G., ROVERO, P., DION, S., D'ORLEANS-JUSTE, P. & BARABÉ, J. (1986). The actions of kinin antagonists on B_1 and B_2 receptor systems. *Br. J. Pharmacol.*, **123**, 61–65.

RHALEB, N.E., DION, S., D'ORLEANS-JUSTE, P., DRAPEAU,

G., REGOLI, D. & BROWN, R.G. (1988). Bradykinin antagonism: differentiation between peptide antagonists and antiinflammatory agents. *Eur. J. Pharmacol.*, **151**, 275–279.

RIFO, J., POURRAT, M., VAVREK, R.J., STEWART, J.M. & HUIDOBRO-TORO, J.P. (1987). Bradykinin receptor antagonists used to characterize the heterogeneity of bradykinin-induced responses in rat vas deferens. *Eur. J. Pharmacol.*, **142**, 305–312.

RIOUX, F., BACHELARD, E., ST PIERRE, S. & BARABÉ, J. (1987). Epicardial application of bradykinin elicits pressor effects and tachycardia in guinea-pigs. Possible mechanisms. *Peptides*, **8**, 863–868.

SARIA, A., LUNDBERG, J.M., SKOFITSCH, G., HUA, X. & LEMBECK, F. (1984). Neurogenic plasma extravasation in various organs in relation to capsaicin-sensitive substance P neurons. In *Antidromic Vasodilatation and Neurogenic Inflammation*. ed. Chahl, L.A., Szolcsányi, J. & Lembeck, F., pp. 245–258. Budapest: Akademiai Kiado.

SCHMIT-ANTOMARCHI, H., DE WEILLE, J., FOSSET, M. & LAZDUNSKI, M. (1987). The receptor for diabetic sulfonylureas controls the activity of the ATP-modulated K channel in insulin secreting cells. *J. Biol. Chem.*, **262**, 15840–15844.

STASZEWSKA-WOOLLEY, J., WOOLLEY, G. & REGOLI, D. (1988). Specific receptors for bradykinin-induced cardiac sympathetic chemoreflex in the dog. *Eur. J. Pharmacol.*, **156**, 309–314.

SZOLCSÁNYI, J. (1984). Capsaicin-sensitive chemoceptive neural system with dual sensory-efferent function. In *Antidromic Vasodilatation and Neurogenic Inflammation*. ed. Chahl, L., Szolcsányi, J. & Lembeck, F. pp. 26–52, Budapest: Akademiai Kiado.

SZOLCSÁNYI, J. (1989). Capsaicin, irritation and desensitization, In *Chemical Irritation of the Nose and Mouth*, ed. Green, B. & Mason, J.R. May and Baker (in press).

TURNER, A.J. (1987). Endopeptidase 24.11. In *Neuropeptides and their Peptidases*, ed. Turner, A.J. pp. 183–199. Chichester: Ellis Horwood.

VAN ROSSUM, J.M. (1963). Cumulative dose-response curves II. Technique for making the dose-response curves in isolated organs and the evaluation of drug parameters. *Arch. Int. Pharmacodyn. Ther.*, **143**, 299–330.

WHALLEY, E.T., NWATOR, I.A., STEWART, J.M. & VAVREK, R.J. (1987). Analysis of the receptors mediating vascular actions of bradykinin. *Naunyn Schmiedebergs Arch. Pharmacol.*, **336**, 430–433.

(Received March 14, 1989
Revised May 15, 1989
Accepted May 24, 1989)