Br. J. Pharmacol. (1991), **102**, 297–304

New, long-acting, potent bradykinin antagonists

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1 Three new bradykinin (BK) antagonists, D-Arg^0-Hyp^3-Thi^5-D-Tic^7-Oic^8-BK (compound I), D-Arg^0-Hyp^3-D-Tic^7-Oic^8-BK (compound II), and Arg(Tos)^1-Hyp^3-Thi^5-D-Tic^7-Oic^8-BK (compound III), were tested against the effects of BK in 9 bioassay preparations including visceral smooth muscles, vasoconstriction, plasma protein extravasation, release of prostaglandin E_2, bronchoconstriction, and stimulation of afferent C-fibre nociceptors. In some of these tests the effects of the new compounds were compared with those of the antagonist D-Arg^0-Hyp^2-Thi^{5,8}-D-Phe^7-BK (compound IV), described by Stewart & Vavrek (1987).

2 For all bioassays the general rank order of potency of the compounds was found to be I > II > III ⩾ IV. The new antagonists were long-acting; in some bioassays their effects outlasted the duration of the experiment.

3 The inhibitory effects of the new BK antagonists were specific for BK; actions of noradrenaline, angiotensin II, acetylcholine or histamine were unaffected by the antagonists. They did not stimulate the release of histamine or prostaglandins. An agonistic effect was observed only with very high concentrations of compounds I and II in the plasma protein extravasation test.

4 The long duration of action of the new BK antagonists is probably due to a high and long-lasting affinity to the BK receptors. A high resistance of the antagonists to enzymatic destruction may be another reason.

5 The new BK antagonists will be valuable tools for the investigation of the pathophysiological role of BK. In addition they may offer a potential for therapeutic applications.

Introduction

Following the synthesis of the first bradykinin (BK) antagonists (Regoli & Barabé, 1980) and the discovery of novel potent BK antagonists of broad spectrum by Vavrek & Stewart (1985), a number of further antagonists have been synthesized and are being explored at present from different angles. Although the specificity and high potency of these antagonists has been unequivocally established (Regoli *et al.*, 1986; Griesbacher & Lembeck, 1987; Schachter *et al.*, 1987; Steranka *et al.*, 1988; Francel *et al.*, 1989; Griesbacher *et al.*, 1989), their susceptibility to fast enzymatic degradation limited their usefulness (Griesbacher *et al.*, 1989). Now, new analogues of BK have been synthesized in which unusual amino acids have been inserted with the intention of making the antagonists more resistent to enzymatic cleavage. In the present investigation the BK antagonistic potency and duration of action of the following 3 new compounds were tested: D-Arg^0-Hyp^3-Thi^5-D-Tic^7-Oic^8-BK (compound I), D-Arg^0-Hyp^3-D-Tic^7-Oic^8-BK (compound II), and Arg(Tos)^1-Hyp^3-Thi^5-D-Tic^7-Oic^8-BK (compound III). They were compared with the BK antagonist D-Arg^0-Hyp^2-Thi^{5,8}-D-Phe^7-BK (compound IV) described earlier (Stewart & Vavrek, 1987; Francel *et al.*, 1989; Linz *et al.*, 1990).

In vitro and *in vivo* tests were carried out in several BK bioassay systems which had been used in our previous work on BK antagonists (Griesbacher & Lembeck, 1987; Griesbacher *et al.*, 1989). In addition, the BK-induced longitudinal contraction of the guinea-pig ileum (*in vitro*), and plasma protein extravasation in internal organs of the rat and bronchoconstriction in guinea-pigs (*in vivo*) were used.

Methods

Isolated visceral smooth muscle preparations

Rat uterus (in oestrous) Female Sprague-Dawley rats were injected with diethylstilboestrol (100 μg kg^{-1}, s.c.) 16 h before the uterus was used. The distal parts of the uterine horns were suspended at 32°C in De Jalón solution containing atropine (0.35 μM) and indomethacin (2.8 μM). Isotonic contractions were recorded under a resting tension of 0.5 g. BK (1.6 nM) was added to the organ bath at 5 min intervals and left in the organ bath for 1 min. Compounds I (0.25–2.5 nM), II (0.25–7.5 nM), III (0.75–25 nM), or IV (75–750 nM) were added to the bath 4 min before the 3rd dose of BK; thus, they were in contact with the tissue for a total period of 5 min.

Rat duodenum The proximal part (1.5 cm) of the duodenum of female Sprague-Dawley rats was suspended at 32°C in De Jalón solution containing atropine (0.35 μM) and indomethacin (2.8 μM). A resting tension of 2 g was applied to the tissue and isotonic relaxations in response to BK (3 nM) were recorded. Compounds I (1.25–5 nM), II (1.25–5 nM), III (1.25–50 nM), or IV (4–500 nM) were added 4 min before a subsequent addition of BK.

Guinea-pig ileum The ileum (1.5 cm) was suspended in Tyrode solution at 32°C. Isotonic contractions were recorded under a resting tension of 0.5 g. BK (73 nM) was applied repeatedly at intervals of 3 min. One min after each addition of BK the bath solution was replaced. Compounds I (3–100 nM), II (1–100 nM), III (3–300 nM), or IV (100–10000 nM) were added to the organ bath 2 min before the following dose of BK.

In all tissues a concentration-response curve to BK was established at the beginning of each experiment in order to insure that the test doses of BK used were submaximal. After the addition of the antagonists, the BK additions were continued for a period of 30 min to monitor the recovery of the BK response.

Vasoconstriction in the isolated perfused ear of the rabbit

Rabbits of either sex were killed by an i.v. injection of an overdose of pentobarbitone sodium. The auricular arteries of both ears were cannulated and the ears were then separated from the head as described earlier (Griesbacher & Lembeck, 1987). The ears were perfused with Tyrode solution (37°C) and the

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perfusion pressure was adjusted to give a flow rate of 5 ml min^{-1}. Then, the perfusion pressure (30–60 cmH_2O) was kept constant. Vasoconstriction was measured by recording the venous outflow with an electronic drop interval timer. BK (100 pmol) was injected into the arterial inflow cannula at intervals of 30 min. The antagonists were infused during the 5 min period preceding the third injection of BK, the subsequent doses of BK were injected as before. On several occasions angiotensin II (30 pmol) or noradrenaline (100 pmol) were injected between the BK injections to test the specificity of the antagonists.

Release of prostaglandin E_2 from the rabbit ear

Rabbit isolated ears were prepared as described above. Perfusion with Tyrode solution at 37°C was carried out at a constant flow rate of 1.6 ml min^{-1} using a roller pump for at least 30 min before the tests were started. The BK antagonists (final concentration 20 nM) were then added to the perfusion medium of one ear for a period of 5 min. In each experiment a corresponding volume of a 154 mM solution of NaCl was infused into the contralateral control ear. Five min after the end of these infusions, BK (1 nmol) was injected into the arterial inflow cannula of each ear. The venous outflow was collected in 5 min fractions before and after the BK injections and stored at −20°C. The prostaglandin E_2 (PGE_2) content of these fractions was estimated by radioimmunoassay (Juan, 1977).

Plasma protein extravasation in rats

Female Sprague-Dawley rats (150–200 g) were anaesthetized with pentobarbitone sodium (50 mg kg^{-1}, s.c.). A jugular vein was cannulated and Evans blue dye (20 mg kg^{-1}) was injected i.v. to stain plasma proteins. Compound I or II (500 nmol kg^{-1}) was given i.v. 5 min later, followed by BK (500 nmol kg^{-1}) injected i.v. 3, 10 or 30 min after the BK antagonist. In 2 control groups no BK antagonists were used: in one of them BK was injected, in the second group physiological saline was used instead of BK. At the end of the experiments, 5 min after the last injections, the thorax was opened and the rats were perfused via the aorta with 50 ml of physiological saline. The trachea, the urinary bladder, and the duodenum (proximal 1.5 cm) were dissected, weighed and incubated for 48 h in formamide at 50°C (Saria *et al.*, 1983). The amount of Evans blue extracted was measured photometrically at 620 nm. Plasma protein extravasation was expressed as ng Evans blue mg^{-1} wet weight.

In order to establish whether a BK antagonist increased plasma protein extravasation when given on its own, compounds I, II or III were injected i.a. at the 10 fold higher dose of 5 μmol kg^{-1} and the experiment terminated 5 min later. To test whether histamine, acting via H_1 receptors, or prostaglandin release contributed to the observed BK-induced plasma protein extravasation, animals were pretreated with mepyramine maleate (10 mg kg^{-1}, s.c.) or indomethacin (10 mg kg^{-1}, s.c.) 30 min before the beginning of the experiment.

Bronchoconstriction in guinea-pigs

Guinea-pigs of either sex (400–700 g body weight) were anaesthetized with urethane (25% w/v in physiological saline, 7.5 ml kg^{-1}, i.p.). The trachea was cannulated and the animals were put on artificial respiration (stroke volume 10 ml kg^{-1}, 65 strokes min^{-1}). As an index of bronchoconstriction the pulmonary inflation pressure (PIP) was measured in a side arm of the tracheal cannula with a Statham pressure transducer (Jin *et al.*, 1989). One carotid artery was cannulated to measure the arterial blood pressure. BK (1 nmol) was injected into a jugular vein at intervals of 10 min. The BK antagonists I (1 nmol) or II (1 nmol) or III (2 nmol) were injected i.v. 1 min before a subsequent injection of BK. Compound IV (100 nmol) was injected i.v. immediately before the test dose of BK. To test the specificity of the antagonists, experiments were carried out in which histamine (10 nmol) was injected instead of BK. The injection volume of BK, the BK antagonists, and histamine was 0.1 ml.

Rabbit iris sphincter muscle

To test the antagonists in a system where BK acts on peptidergic afferent C-fibre nociceptors, the isolated sphincter muscle of the rabbit was used. In this preparation the BK-induced contractions of the smooth muscles are induced by the release of tachykinins from primary afferent trigeminal neurones (Bynke *et al.*, 1983; Ueda *et al.*, 1984; Håkanson *et al.*, 1987). Rabbits of either sex were killed by an i.v. injection of an overdose of pentobarbitone. The eyes were enucleated and the iris sphincter muscles were prepared as described by Kern (1970). Isotonic contractions were recorded under a resting tension of 0.5 g. The BK antagonists were added to the organ bath (Krebs solution, 37°C) 5 min before cumulative concentration-response curves to BK were established (Griesbacher & Lembeck, 1987); an appropriate amount of physiological saline was added to the organ bath of control tissues.

Stimulation of nociceptors in the rabbit ear

Rabbits of either sex (3–6 kg) were anaesthetized with pentobarbitone sodium (45 mg kg^{-1}, i.v.). The arterial blood pressure in a carotid artery was recorded by means of a Statham pressure transducer on a Grass Model 7B Polygraph. To maintain anaesthesia small amounts of pentobarbitone sodium (6 mg) were injected into a jugular vein; the stability of blood pressure and the corneal reflex were used to monitor anaesthesia. The central ear artery was cannulated and the ear separated from the head with the exception of the auricular nerve, which remained connected to the head (Griesbacher & Lembeck, 1987). The ear was perfused with Tyrode solution under constant pressure at a flow rate of 5 ml min^{-1}. PGE_1 (final concentration 30 nM) was added to the Tyrode solution in order to augment the responses to BK and acetylcholine (ACh) (Juan & Lembeck, 1974). BK (100 pmol) and ACh (60 nmol) were injected alternately into the arterial inflow cannula at intervals of 15 min; 26 min after the second injection of BK compound I (final concentration 1.5 or 15 nM) or compound III (final concentration 5 or 50 nM) were infused into the arterial inflow cannula of the ear for 4 min. Immediately after the end of this infusion the next dose of BK was injected. The reflex falls in carotid blood pressure induced by BK and ACh were then monitored over a period of 1 h.

Substances

Bradykinin (BK) was obtained from Bachem, Switzerland. The BK antagonists D-Arg^0-Hyp^3-Thi^5-D-Tic^7-Oic^8-BK (compound I), D-Arg^0-Hyp^3-D-Tic^7-Oic^8-BK (compound II), Arg(Tos)^1-Hyp^3-Thi^5-D-Tic^7-Oic^8-BK (compound III), and D-Arg^0-Hyp^2-Thi^{5,8}-D-Phe^7-BK (compound IV) (Thi = β-(2-thienyl)-L-alanine; D-Tic = D-(1,2,3,4-tetrahydroisoquinolin-2-yl-carbonyl); Oic = L-[(3aS,7aS)-octahydroindol-2-yl-carbonyl]; Arg(Tos) = N^G-tosyl-L-arginine) were synthesized by solid phase peptide synthesis by Hoechst AG.

Further substances used were: acetylcholine chloride (Becker, Austria); atropine sulphate (Merck Sharp & Dohme, F.R.G.); diethylstilboestrol, histamine dihydrochloride (Serva Feinbiochemie, F.R.G.); Evans blue, indomethacin, noradrenaline hydrochloride, prostaglandin (PG) E_1 (Sigma, U.S.A.); mepyramine maleate (Smith Kline & French, U.K.); pentobarbitone sodium (Ceva, F.R.G.); PGE_2 antiserum (BioMakor, Israel); [^{3}H]-PGE_2 (Amersham International, U.K.).

All peptides were dissolved in physiological saline containing 1 g l^{-1} gelatine and 25 mg l^{-1} cialit (sodium 2-

ethylmercuriothio-benzoxazole-5-carboxylate, Asid-Institut GesmbH, F.R.G.) to prevent absorption to glass and bacterial growth. Diethylstilboestrol was dissolved in and diluted with olive oil. Indomethacin was dissolved in 2% Na_2CO_3 and diluted with phosphate buffer (20 mM KH_2PO_4 and 50 mM Na_2HPO_4). PGE_1 was dissolved in 50% ethanol. All other substances were made up in physiological saline.

The compositions of the physiological salt solutions were (in mM): De Jalón: NaCl 154.0, KCl 5.6, $CaCl_2$ 0.54, $MgCl_2$ 0.05, $NaHCO_3$ 11.9, D-glucose 3.0; Krebs: NaCl 118.0, KCl 4.6, $CaCl_2$ 2.5, $MgSO_4$ 1.17, NaH_2PO_4 25.0, D-glucose 10.1; Tyrode: NaCl 136.9, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.15, NaH_2PO_4 0.42, $NaHCO_3$ 11.9, D-glucose 5.6; physiological saline: NaCl 154. All solutions used in *in vitro* experiments were gassed with a mixture of 95% O_2 and 5% CO_2.

Statistical analysis

In the smooth muscle preparations, the effects of BK before and after the addition of the antagonists were compared by the Quade test (Conover, 1980). To quantify the potency of the antagonists, the % inhibition, transformed to logits as a linearising transformation, was plotted against the negative logarithm of the antagonist concentrations and log-logit regression analysis was used to calculate IC_{50} values. Log-logit regression analysis was used to test the concentration-response curves to BK on the rabbit isolated iris sphincter muscle for non-identity and non-parallelism. Where the concentration-response curves in the absence and presence of an antagonist proved not to deviate significantly from parallelism, the horizontal displacement to the right was calculated (Geigy, 1980) to determine the concentration-ratio.

Vasoconstriction in rabbit isolated perfused ears was analyzed by performing multiple nonparametric comparisons (Zar, 1984) of the BK-induced effect in antagonist-treated ears compared with that in the saline-treated control ears. The Mann-Whitney U test was used to compare the magnitude of the release of PGE_2 from rabbit ears that had been perfused with an antagonist with the release of PGE_2 from control ears. Dunnett's t test was employed to compare results obtained in individual groups in the plasma protein extravasation test. The effects of the BK antagonists *in vivo* on BK-induced bronchoconstriction in guinea-pigs and on BK-induced nociceptor stimulation in the perfused rabbit ear were analyzed by comparing the effects of BK before and after the administration of the antagonists with the Quade test.

Values calculated by regression analysis are given with 95% confidence intervals. All other values are given as means ± s.e.mean.

Results

Isolated visceral smooth muscle preparations

All BK antagonists tested inhibited the contractile effects of BK on the rat uterus and on the guinea-pig ileum, as well as the relaxant effects of BK on the rat duodenum in a concentration-dependent manner. However, the concentrations of compound IV that were needed to inhibit effectively the actions of BK were about 2–3 orders of magnitude higher than those of compounds I, II and III (Table 1).

In addition, there was a difference in the duration of the action of the antagonists in these preparations (Figure 1 shows the results from the rat uterus). Compound IV reduced the effects of BK only while it was present in the organ bath; the next application of BK already produced a response as before the addition of compound IV. The new antagonists I, II and III inhibited BK for a much longer time, despite the repeated washing procedures after each addition of BK. A period of 30 min was required to restore the full potency of BK when these antagonists were used in concentrations which almost abolished the effect of the first dose of BK added to the organ bath while the antagonist was still present (see Figure 1).

Table 1 Inhibition of the bradykinin (BK)-induced contractions of the isolated rat uterus and the guinea-pig ileum, and of the BK-induced relaxations of the isolated rat duodenum

	$-log\ IC_{50}$		
Antagonist	*Rat uterus*	*Rat duodenum*	*Guinea-pig ileum*
I	9.3 (8.6–10.0)	8.7 (8.3–9.3)	8.1 (7.4–8.8)
II	9.2 (8.5–10.0)	8.5 (7.9–9.1)	7.9 (7.0–8.8)
III	8.4 (7.3–9.5)	8.1 (7.4–8.7)	7.8 (6.8–8.8)
IV	6.2 (5.3–7.1)	7.1 (5.3–8.8)	6.3 (5.5–7.1)

BK was applied to the tissues at concentrations that gave approximately 70% of the maximum effect. The inhibition by the BK antagonists was plotted against the antagonist concentration and $-\log IC_{50}$ values were calculated by log-logit regression analysis; 95% confidence intervals are given in parentheses. Each value was obtained from a total of 24–30 experiments.

Vasoconstriction in the rabbit isolated perfused ear

Intra-arterial injections of BK (100 pmol) at intervals of 30 min elicited, under control conditions, consistent decreases of the venous outflow between 60 and 70%. Short infusions (5 min) of the BK antagonists reduced the effect of subsequent injections of BK in a concentration-dependent manner (Figure 2). Compound I was the most potent antagonist: in a concentration of 5 nM it reduced the effects of BK significantly ($P < 0.05$); a concentration of 15 nM blocked the BK-induced vasoconstriction completely ($P < 0.001$). Three times higher concentrations of compound II and compound III were required to obtain comparable inhibitory effects. The inhibition of the BK-induced vasoconstriction by these antagonists persisted during the whole duration of the experiment, i.e. up to 3 h (see Figure 2). In 2 ears the antagonistic effect of compound I (15 nM) was monitored for a period of 6 h during which the responses to BK did not recover.

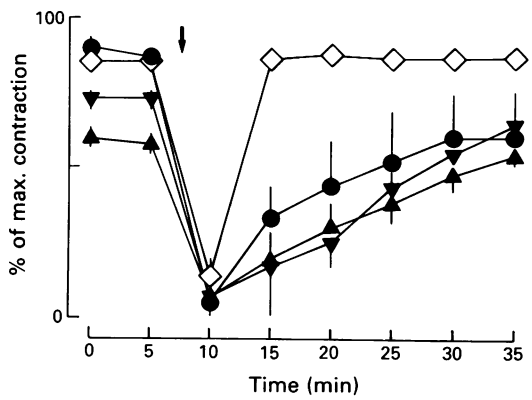

Figure 1 Inhibition of the effect of bradykinin (BK) on the rat uterus: BK (1.6 nM) was added to the organ bath at intervals of 5 min and the medium (De Jalón solution) was changed 1 min later. Ordinate scale: contractions of the uterus as % of the maximum BK-induced response. Abscissa scale: time in min. At the time indicated by the arrow, the BK antagonists compound I (2.5 nM, ●), compound II (2.5 nM, ▲), compound III (25 nM, ▼), or compound IV (2500 nM, ◇) were added. The effect of the subsequent dose of BK was, in all cases, nearly abolished ($P < 0.001$). The next response to BK was again normal when compound IV had been used; in the case of the other antagonists the BK response stayed significantly decreased for at least 25 min. Symbols represent mean values ($n = 6$); vertical lines show s.e.mean. Where no s.e.mean is given it was smaller than the symbol.

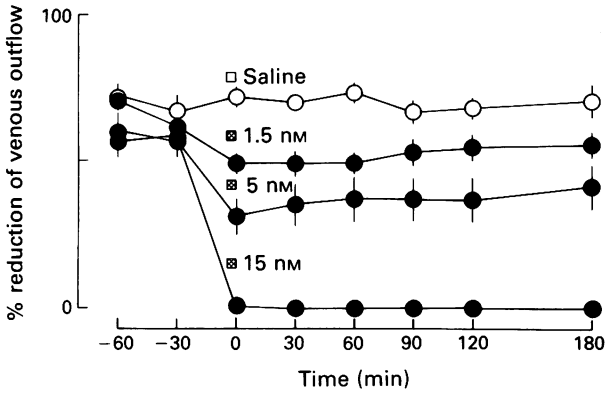

Figure 2 Rabbit isolated ears were perfused under constant pressure with Tyrode solution at an initial flow rate of 5 ml min^{-1}. Bradykinin (BK, 100 pmol) was injected i.a. at intervals of 30 min. The BK-induced vasoconstriction was quantified as % reduction of the venous outflow from the ear. Compound I was infused into the arterial inflow (final concentrations 1.5 nM, 5 nM, and 15 nM, ●) during a period of 5 min prior to the third injection of BK (cross-hatched squares); control animals (○) received a corresponding volume of physiological saline (open square). Subsequent effects of BK were either completely blocked (15 nM, $P < 0.001$) or partly inhibited (5 nM, $P < 0.05$) by compound I. Each antagonist concentration was tested in 5–7 ears. Symbols represent mean values; vertical lines show s.e.mean. Where no s.e.mean is given it was smaller than the symbol.

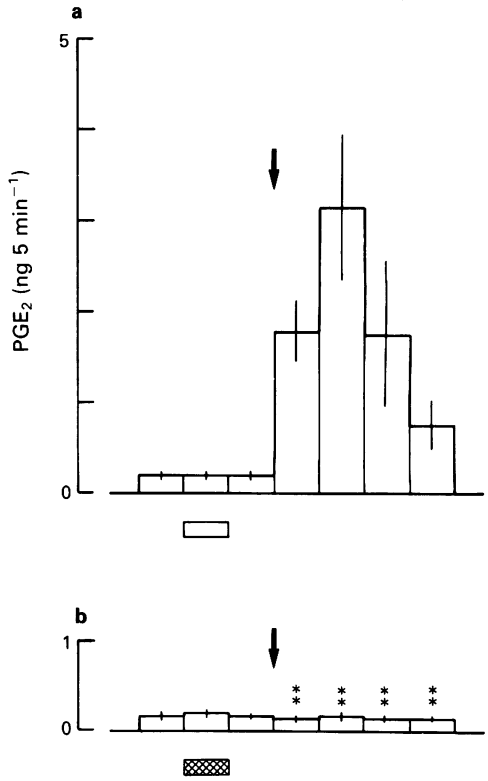

Figure 3 Release of prostaglandin E_2 (PGE_2, in ng 5 min^{-1}) from the rabbit isolated perfused ear (Tyrode solution, constant flow of 1.6 ml min^{-1}): bradykinin (1 nmol) was injected i.a. as a bolus (indicated by the arrow). Compound I (final concentration 20 nM) was present in the Tyrode solution for 5 min (indicated by cross-hatched bar below abscissa scale in b). In control ears a corresponding volume of physiological saline (indicated by open bar below abscissa scale, a) was infused. The PGE_2 content of the venous effluent, collected in 5 min fractions, was determined by radioimmunoassay. Columns represent mean values with s.e.mean shown by vertical lines; $n = 6$. Significance of difference from the corresponding fractions of control ears: ** $P < 0.01$.

Compound IV infused at concentrations of 50, 150, and 500 nM reduced, but did not abolish, BK-induced vasoconstriction; 30 min after the infusion the degree of vasoconstriction induced by BK was again the same as in the control ears.

The order of potency of the antagonists was I $\geqslant$ II $\approx$ III $\gg$ IV (Table 2). Vasoconstrictions elicited by i.a. injections of angiotensin II (30 pmol) or noradrenaline (100 pmol) were not affected by the infusions of the BK antagonists ($n = 4$; data not shown).

Release of prostaglandin E_2 from the rabbit ear

The basal PGE_2 release from rabbit isolated ears, perfused at a constant flow rate of 1.6 ml min^{-1}, was 0.2 ± 0.05 ng 5 min^{-1} ($n = 22$). Injection of BK (1 nmol, i.a.) caused a release of a further 8.4 ± 0.9 ng PGE_2 during a period of 20 min (compare Figure 3, upper panel). All antagonists were infused i.a. (final concentration 20 nM) over a period of 5 min. No increase of the PGE_2 content of the venous outflow was observed during these infusions. Five min after the infusion of compound I the PGE_2 release normally induced by an injection of 1 nmol BK was completely abolished (Figure 3b). Infusions of compound II or compound III also abolished the effect of BK ($n = 5$–6; results not shown). When compound IV was infused, the PGE_2 content of the venous outflow was not reduced; it amounted to 9.4 ± 1.7 ng during 20 min ($n = 6$) and did not differ from the values obtained in the corresponding control ears (10.1 ± 2.7 ng).

Table 2 Inhibition of bradykinin (BK)-induced vasoconstriction in the isolated perfused ear of the rabbit

Antagonist	$-log\ IC_{50}$
I	8.5 (7.9–9.1)
II	8.2 (7.1–9.1)
III	8.2 (7.5–9.0)
IV	6.9 (5.7–8.2)

BK (100 pmol) was injected into the arterial inflow cannula at intervals of 30 min. The antagonists were infused i.a. during the 5 min period preceding the third injection of BK. $-log\ IC_{50}$ values were calculated by log-logit regression analysis; 95% confidence intervals are given in parentheses. Each value was obtained from a total of 15–28 experiments.

Plasma protein extravasation in rats

Intravenous injections of BK (500 nmol kg^{-1}) in anaesthetized rats caused a significant increase ($P < 0.01$) in plasma protein extravasation over control levels in the trachea, the urinary bladder and the duodenum. It was most pronounced in the duodenum. When compound I (500 nmol kg^{-1}) or compound II (500 nmol kg^{-1}) had been given i.v. 3, 10, or 30 min before the BK injection, BK-induced plasma protein extravasation was completely abolished in all organs (Table 3). The effect of BK was totally unaffected by pretreatment with either mepyramine (10 mg kg^{-1}, s.c.) or indomethacin (10 mg kg^{-1}, s.c.).

Figure 4 shows plasma protein extravasation in the duodenum induced by i.v. injections of BK antagonists injected alone in doses 10 times higher than those used in the experiments above. Only compound I (5 μmol kg^{-1}) elicited plasma protein extravasation to an extent comparable to that induced by BK (500 nmol kg^{-1}). This effect was seen in duodenum, trachea and bladder. Compound II caused plasma protein extravasation only in the trachea, whereas compound III showed no agonist activity in any of the organs tested.

Bronchoconstriction in guinea-pigs

BK (1 nmol) injected i.v. into anaesthetized guinea-pigs at 10 min intervals elicited reproducible increases in PIP between

Table 3 Plasma protein extravasation in the duodenum, trachea and urinary bladder of anaesthetized rats (pretreated i.v. with Evans blue)

	Duodenum	*Trachea*	*Bladder*
Saline	37 ± 8	25 ± 7	10 ± 1
BK	140 ± 22**	90 ± 15**	74 ± 12**
BK + I			
3 min	52 ± 19	28 ± 5	14 ± 2
10 min	36 ± 5	30 ± 5	12 ± 2
30 min	26 ± 6	20 ± 3	10 ± 2
BK + II			
3 min	34 ± 4	30 ± 5	11 ± 2
10 min	34 ± 8	28 ± 4	18 ± 6
30 min	38 ± 8	18 ± 3	8 ± 1

Bradykinin (500 nmol kg^{-1}) was injected i.v. either alone (BK) or 3–30 min after the i.v. injection of compound I or II. Control animals were injected with physiological saline instead of BK. Evans blue content of the tissues (in ng mg^{-1} wet weight) was determined 5 min after the injection of BK or physiological saline. Means ± s.e.mean; $n = 4$–10. Significance of difference from saline controls: ** $P < 0.01$.

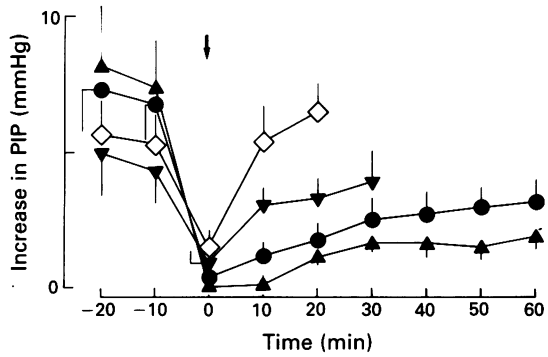

Figure 5 Changes of pulmonary inflation pressure (PIP, in mmHg) in anaesthetized guinea-pigs *in vivo*: bradykinin (BK, 1 nmol) was injected i.v. at intervals of 10 min. At the time indicated by the arrow an i.v. injection of compound I (1 nmol, ●), compound II (1 nmol, ▲), compound III (2 nmol, ▼), or compound IV (100 nmol, ◇) was made. The response to the next dose of BK was nearly abolished by all compounds ($P < 0.001$). The effects of subsequent 6 doses of BK were significantly ($P < 0.01$) inhibited by compounds I and II. Symbols represent mean values; vertical lines give s.e.mean. $n = 6$–9.

5 and 8 mmHg (Figure 5). The BK antagonists were injected i.v. 1 min before the third injection of BK. The response to this BK injection was completely blocked when compound I (1 nmol) or compound II (1 nmol) had been given; 60 min later the effect of BK was still reduced to 51% and 23%, respectively. Compound III (2 nmol) reduced BK-induced bronchoconstriction to 16% of the control value; 30 min later the BK response was back to 70% of its original value. To block BK-induced bronchoconstriction with compound IV, a dose of 100 nmol had to be given and the response to BK given 10 min later was already back to normal.

None of the antagonists had a significant effect on the increase in PIP induced by i.v. injections of histamine (10 nmol, $n = 5$).

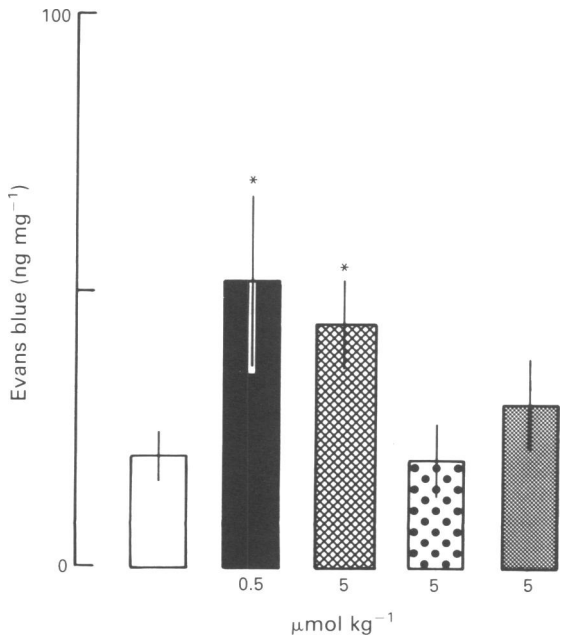

Figure 4 Plasma protein extravasation in the duodenum of anaesthetized rats (pretreated i.v. with Evans blue): column heights represent Evans blue content of the duodenum (in ng mg^{-1} wet weight) of rats 5 min after the i.v. injection of physiological saline (open columns), bradykinin (BK, solid column), or of the BK antagonists on their own (compound I: cross-hatched column; compound II: stippled column; compound III: shaded column). The doses of BK and the BK antagonists are given below the columns (in μmol kg^{-1}). Significance of difference from saline controls: * $P < 0.05$. Means with s.e.mean shown by vertical lines; $n = 6$.

Rabbit iris sphincter muscle

Cumulative concentration-response curves to BK were shifted to the right when the antagonists were present in the bathing solution of the preparations. The maximum contraction obtained with BK in the presence of the antagonists was not different from that seen with control tissues and was 70–90% of the contraction induced by a maximal concentration of carbachol (10 μM). The concentrations of the antagonists were chosen so as to give a rightward shift by approximately 0.5 to 1 log unit. The regression lines for the concentration-response relations proved not to differ significantly from parallelism. Therefore, concentration-ratios were calculated by regression analysis (Table 4). When Compound I was employed at the same concentration as compound II and compound III (5 nM) the highest BK concentration that could be used (5 μM) yielded only 42 ± 11% of the carbachol-induced maximum. Since no information on the upper regions of the concentration-response curve could be obtained, no regression line was calculated for this concentration of compound I. However, the data available suggest a shift of the concentration-response curve by 2–2.5 log units. Compound IV was about 1000 times less potent than compound I and about 100 times less potent than compounds II and III.

Stimulation of nociceptors in the rabbit ear

Basal systemic arterial blood pressure was 90–110 mmHg and remained constant throughout the course of the experiment.

Table 4 Bradykinin (BK)-induced contractions of the rabbit isolated iris sphincter muscle

Antagonist	*Concentration* [nM]	*Concentration-ratio for BK*
I	0.5	7.3 (5.3–10.0)
II	5	4.5 (3.8–5.5)
III	5	10.2 (7.7–13.5)
IV	500	4.2 (3.3–5.3)

Cumulative concentration-response curves to BK were established in the absence or presence of the BK antagonists. Log-logit regression analysis was used to calculate regression lines for the BK responses. All antagonists shifted the concentration-response curves to the right. No significant deviation from parallelism could be detected. The horizontal displacement of the regression lines was used to calculate concentration-ratios for BK with 95% confidence intervals (given in parentheses). For each antagonist 5–6 pairs of tissue preparations were used.

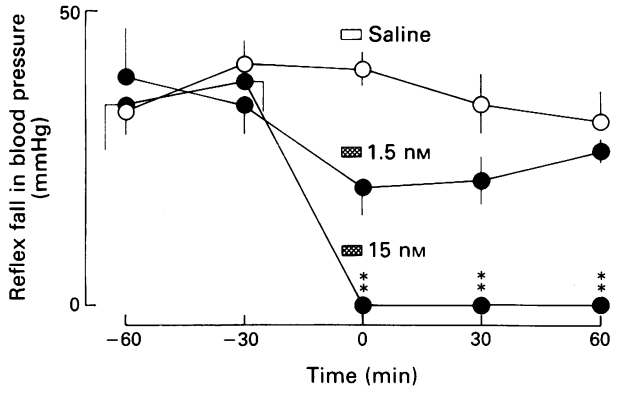

Figure 6 Nociceptor stimulation in the rabbit ear: bradykinin (BK, 100 pmol) was injected into the arterial inflow of isolated perfused ears (left in contact with the head only by the intact auricular nerve) at intervals of 30 min. The ensuing reflex falls in systemic blood pressure (in mmHg) were measured in a carotid artery. In two groups of rabbits, compound I (final concentrations 1.5 nM or 15 nM, ●) was added to the perfusion of the ears during a period of 5 min (cross-hatched bars) prior to the 3rd injection of BK. Control animals (○) received a corresponding volume of physiological saline (open bar). Symbols represent mean values, vertical lines show s.e.mean. For each concentration of compound I (0–15 nM) 3 animals were used. Significance of difference from values before the antagonist: ** $P < 0.01$.

Stimulation of paravascular nociceptors by injections of BK (100 pmol) at intervals of 30 min into the arterial inflow cannula of the 'reflex ear' preparation (Juan & Lembeck, 1974) caused short-lasting reflex falls in blood pressure by 35–40 mmHg (Figure 6). When compound I was present in the perfusion medium (final concentration 15 nM, $n = 3$) over a period of 5 min, the effect of subsequent injections of BK were completely abolished ($P < 0.01$); in a concentration of 1.5 nM compound I significantly attenuated ($P < 0.05$) the actions of BK. As with the results on vasoconstriction in the rabbit isolated perfused ear (see Figure 2), the antagonism was not reversible during the time of observation (1 h). Similar results were obtained when higher concentrations of compound III were used: a concentration of 5 nM reduced the BK-induced fall in blood pressure from 34 ± 6 mmHg to 12 ± 10 mmHg, and 50 nM abolished the effect of BK. The complete block of the effect of BK seen after compound III (50 nM) also lasted throughout the course of the experiment ($n = 3$). However, a reflex fall in blood pressure could still be elicited when much higher doses of BK (1 and 10 nmol) were injected. The nociceptor stimulation by ACh (60 nmol, not shown in Figure 6) was completely unaffected by compounds I or III. Compounds II and IV were not tested in this preparation.

Discussion

Isolated smooth muscles

The actions of four new BK antagonists were studied on BK-induced contractions of the guinea-pig ileum and the rat uterus. The former preparation has often been used in earlier investigations of this type of drugs (Vavrek & Stewart, 1985; Regoli *et al.*, 1986; Schachter *et al.*, 1987; Stewart & Vavrek, 1987; Steranka *et al.*, 1988), and the latter has been employed because of its high sensitivity to BK. In addition, the rat duodenum was used because it responds to BK with a relaxation by a mechanism which is not yet fully understood, but involves activation of BK receptors (Boschcov *et al.*, 1984; Griesbacher *et al.*, 1989; Pereira & Paiva, 1989; Altinkurt & Öztürk, 1990). The order of potency of the antagonists was similar with all three preparations: I ≈ II > III ⩾ IV (Table 1). Compound I was 100–1000 times more potent than compound IV.

Furthermore, a distinction between the BK antagonists regarding their duration of action was demonstrated on the rat uterus. Compound IV (2500 nM) caused a complete inhibition of the response to BK by this tissue when it was present in the organ bath. Much lower concentrations of the other antagonists (e.g. 2.5 nM for compounds I and II) showed an inhibitory effect for a much longer period of time. In spite of repeated changes of the bath fluid, the inhibition by compounds I, II and III did not fully disappear even 20–30 min after their washout, indicating that the new antagonists have a long duration of action (Figure 1).

Rabbit isolated perfused ear

This preparation has previously been shown to be useful for testing the inhibition of the BK-induced vasoconstriction, and the BK-induced release of PGE_2 by BK antagonists (Griesbacher & Lembeck, 1987). The reduction in venous outflow induced by BK results from venoconstriction (Guth *et al.*, 1966). To inhibit BK-induced venoconstriction the antagonists corresponded in potency to the rank order found in the isolated smooth muscle preparations: I > II ≈ III ⩾ IV (Table 2). The duration of action of the new compounds on the ear veins was remarkable (Figure 2). The complete inhibition of this BK response by compound I exceeded 3 h and in 2 preparations lasted longer than 6 h. This is in contrast to the effect of compound IV. Even at a concentration of 500 nM the inhibition was incomplete and terminated within 30 min. Thus, compounds I, II and III appear to have an unusually high affinity for the BK receptor and/or are resistent to enzymatic cleavage.

The antagonists I, II, III and IV (20 nM) did not evoke a release of PGE_2 by themselves, and thus did not act like BK agonists in this test. The BK-evoked PGE_2 release could be blocked completely by compound I (Figure 3); it is likely that such an effect could have been achieved by a concentration much lower than 20 nM. A complete inhibition of the BK-evoked PGE_2 release was also obtained with compounds II and III. Compound IV was inactive at this concentration. The related BK antagonist B4310 (Stewart & Vavrek, 1987) was found to inhibit the BK-induced PGE_2 release only at a concentration of 800 nM (Griesbacher & Lembeck, 1987). The mechanism involved in the BK-induced synthesis and release of PGE_2 is unknown. The present results suggest that the mode of action involves specific BK receptors.

Plasma protein extravasation

BK-induced plasma protein extravasation has usually been tested in the skin of rats, guinea-pigs and rabbits. In a previous study, the combined injection of BK and BK antagonists in rabbit skin demonstrated that this response can be inhibited (Griesbacher & Lembeck, 1987), but the method did not seem to be very reliable. Therefore, in the present study, plasma protein extravasation was measured in several internal organs following i.v. injection of BK (Saria *et al.*, 1983). Plasma protein extravasation induced by BK was most prominent in the duodenum, the urinary bladder and the trachea. This effect was unaffected by mepyramine or by indomethacin. Thus, the release of histamine to act on H_1 receptors, or prostaglandins is not involved in the plasma protein extravasation induced by BK. Compounds I and II blocked BK-induced plasma protein extravasation for at least 30 min (Table 3). When the antagonists I, II and III were injected i.v. alone in a dose that was 10 times as large as that required for a full antagonistic effect (and 10 times as large as the dose of BK employed in this test), only compound I caused plasma protein extravasation (Figure 4). This indicates an agonistic action of compound I only at extremely high doses.

Bronchoconstriction

Changes in pulmonary inflation pressure were used to assess the action of the new antagonists on BK-induced broncho-

constriction *in vivo*. All of the antagonists inhibited the BK-induced bronchoconstriction within 1 min and the order of potency was I ≈ II > III ⩾ IV. Similar differences were found when the durations of inhibition were compared. The effect of compound IV lasted for less than 10 min whereas an inhibitory effect of compounds I and II was still detectable after 60 min (Figure 5). The new antagonists, therefore, also have a high potency and long duration of action in the guinea-pig *in vivo*. The bronchoconstriction induced by histamine was not reduced by any of the four compounds, which demonstrates their specificity. No bronchoconstriction occurred after the injection of the antagonists on their own; thus, agonistic effects at the BK receptor in the bronchi, or release of histamine by the antagonists can be excluded.

Farmer *et al.* (1989) have reported that, in a similar experimental setup, a bradykinin analogue similar to compound IV (D-Arg-[Hyp^3-D-Phe^8]-BK) was only a very weak inhibitor of BK, and other related antagonists were virtually inactive. These findings, together with the results from binding studies, have been interpreted by suggesting a B_3 BK receptor subtype. It is therefore remarkable that our new compounds are very potent BK antagonists also in the guinea-pig airways *in vivo*.

Effects of bradykinin on afferent neurones

Two models of BK-induced stimulation of afferent neurones have been used to test the new BK antagonists. BK is the most potent stimulator known, of paravascular nociceptors in the 'rabbit reflex ear' preparation *in vivo* (Juan & Lembeck, 1974). In addition, the BK-induced contractions of the isolated iris sphincter muscle of the rabbit *in vitro* are due to the release of tachykinins from peripheral terminals of afferent neurones (Bynke *et al.*, 1983; Ueda *et al.*, 1984; Håkanson *et al.*, 1987).

In an earlier investigation (Griesbacher & Lembeck, 1987), in which the BK antagonist B4310 was used, concentrations of 50–500 nM had been necessary to block the BK-induced nociceptor stimulation in the rabbit ear *in vivo*. Moreover, this effect was only present during the infusion of the antagonist. In contrast, in the present investigation, the BK effect was completely inhibited for at least 1 h after a 5 min infusion of compound I in a concentration of 15 nM (Figure 6). Compound III was less potent (by a factor of about 3). Thus, this test provided evidence for the high potency and long duration of action of the new antagonists on BK-induced nociceptor stimulation.

The test on the rabbit iris sphincter muscle allowed an evaluation of the antagonism by regression analysis. It confirmed the extremely high antagonistic potency of compound I which, in a concentration as low as 0.5 nM, shifted the concentration-response curve of BK to the right by a factor of 7. Compounds II and III were slightly less potent. In contrast, compound IV was approximately 1000 times less potent than compound I (Table 4).

In summary, the present experiments have demonstrated, both *in vitro* and *in vivo*, that compound I is an extremely potent antagonist of BK in a number of different preparations. Compounds II and III were slightly less active. The antagonistic effect was specific for BK, as responses to acetylcholine, noradrenaline, and histamine were unaffected. It was not intended, however, to determine whether the new antagonists were specific for any BK receptor subtype. To investigate receptor subtype specificity quite different methods would be needed. An agonistic effect was observed only in the plasma protein extravasation test with very high concentrations of compound I, and with compound II in only one of the organs tested. The long duration of the antagonists is especially important. In some tests the inhibition of the BK response was almost irreversible. BK is metabolized by a variety of enzymes (Regoli & Barabé, 1980). A role of peptidases in the inactivation of the new BK antagonists is possible but remains to be investigated. At present it is most likely that the antagonists have a high affinity for BK receptors and that this is the major reason for their long duration of action.

Finally, the high potency and the long duration of action of the new antagonists will be of great value in the investigation of the pathophysiological role of BK. Furthermore, the lack of agonist effects should offer the possibility of therapeutic applications.

The authors wish to thank Mr W. Schluet for expert technical assistance and for preparing the drawings. The investigation was supported by the Pain Research Commission of the Austrian Academy of Sciences, the Austrian National Bank (grant No. 2811), and the Austrian Funds for Scientific Research (grant No. P5616).

References

ALTINKURT, O. & ÖZTÜRK, Y. (1990). Bradykinin receptors in isolated rat duodenum. *Peptides*, **11**, 39–44.

BOSCHCOV, P., PAIVA, A.C.M., PAIVA, T.B. & SHIMUTA, S.I. (1984). Further evidence for the existence of two receptor sites for bradykinin responsible for the diphasic effect in the rat isolated duodenum. *Br. J. Pharmacol.*, **83**, 591–600.

BYNKE, G., HÅKANSON, R., HÖRIG, J. & LEANDER, S. (1983). Bradykinin contracts the pupillary sphincter and evokes ocular inflammation through release of neuronal substance P. *Eur. J. Pharmacol.*, **91**, 467–475.

CONOVER, W.J. (1980). *Practical Nonparametric Statistics*. 2nd edition. pp. 295–299. New York: John Wiley & Sons.

FARMER, S.G., BURCH, R.M., MEEKER, S.A. & WILKINS, D.E. (1989). Evidence for a pulmonary B_3 bradykinin receptor. *Mol. Pharmacol.*, **36**, 1–8.

FRANCEL, P.C., KEEFER, J.F. & DAWSON, G. (1989). Bradykinin analogs antagonize bradykinin-induced second messenger production in a sensory cell line. *Mol. Pharmacol.*, **35**, 34–38.

GEIGY (1980). *Wissenschaftliche Tabellen Geigy (Statistik)*. 8th edition. p. 216. Basle: Ciba-Geigy.

GRIESBACHER, T. & LEMBECK, F. (1987). Actions of bradykinin antagonists on bradykinin-induced plasma extravasation, venoconstriction, prostaglandin E_2 release, nociceptor stimulation and contraction of the iris sphincter muscle of the rabbit. *Br. J. Pharmacol.*, **92**, 333–340.

GRIESBACHER, T., LEMBECK, F. & SARIA, A. (1989). Effects of the bradykinin antagonist B4310 on smooth muscles and blood pressure in the rat, and its enzymatic degradation. *Br. J. Pharmacol.*, **96**, 531–538.

GUTH, P.S., BOBBIN, R., CANO, G. & AMARO, J. (1966). Venoconstriction induced by bradykinin in the rabbit ear. In *Hypotensive Peptides*. ed. Erdös, E.G., Back, N. & Sicuteri, F. pp. 396–406. Berlin, Heidelberg, New York: Springer.

HÅKANSON, R., BEDING, B., EKMAN, R., HEILIG, M., WAHLESTEDT, C. & SUNDLER, F. (1987). Multiple tachykinin pools in sensory nerve fibres in the rabbit iris. *Neuroscience*, **21**, 943–950.

JIN, L.S., SEEDES, E., PAGE, C.P. & SCHACHTER, M. (1989). Inhibition of bradykinin-induced bronchoconstriction in the guinea-pig by a synthetic B_2 receptor antagonist. *Br. J. Pharmacol.*, **97**, 598–602.

JUAN, H. (1977). Mechanism of action of bradykinin-induced release of prostaglandin E_2. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **300**, 77–85.

JUAN, H. & LEMBECK, F. (1974). Action of peptides and other algesic agents on paravascular pain receptors of the isolated perfused rabbit ear. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **283**, 151–164.

KERN, R. (1970). Die adrenergischen Rezeptoren der intraoculären Muskeln des Menschen. *Albrecht v. Graefes Arch. Klin. Exp. Ophthal.*, **180**, 231–248.

LINZ, W., MARTORANA, P.A., GRÖTSCH, H., BEI-YIN, Q. & SCHÖLKENS, B.A. (1900). Antagonizing bradykinin (BK) obliterates the cardioprotective effects of bradykinin and angiotensin-converting enzyme (ACE) inhibitors in ischemic hearts. *Drug Dev. Res.*, **19**, 393–408.

PEREIRA, C.C. & PAIVA, T.B. (1989). Characterization of the receptors responsible for the diphasic effect of bradykinin in rat duodenum. *Brazil. J. Med. Biol. Res.*, **22**, 1137–1140.

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'ORLÉANS-JUSTE, P. & BARABÉ, J. (1986). The actions of kinin antagonists on B_1 and B_2 receptor systems. *Eur. J. Pharmacol.*, **123**, 61–65.

SARIA, A., LUNDBERG, J.M., SKOFITSCH, G. & LEMBECK, F. (1983). Vascular protein leakage in various tissues induced by substance P, capsaicin, bradykinin, serotonin, histamine and by antigen challenge. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **324**, 212–218.

SCHACHTER, M., UCHIDA, Y., LONGRIDGE, D.J., LABEDZ, T., WHALLEY, E.T., VAVREK, R.J. & STEWART, J.M. (1987). New synthetic antagonists of bradykinin. *Br. J. Pharmacol.*, **92**, 851–855.

STERANKA, L.R., MANNING, D.C., DeHAAS, C.J., FERKANY, J.W., BOROSKY, S.A., CONNOR, J.R., VAVREK, R.J., STEWART, J.M. & SNYDER, S.H. (1988). Bradykinin as a pain mediator: Receptors are localized to sensory neurons, and antagonists have analgesic actions. *Proc. Natl. Acad. Sci., U.S.A.*, **85**, 3245–3249.

STEWART, J.M. & VAVREK, R.J. (1987). Bradykinin competitive antagonists: design and activities. *Adv. Biosci.*, **65**, 73–80.

UEDA, N., MURAMATSU, I. & FUJIWARA, M. (1984). Capsaicin and bradykinin-induced substance P-ergic responses in the iris sphincter muscle of the rabbit. *J. Pharmacol. Exp. Ther.*, **230**, 469–473.

VAVREK, J.M. & STEWART, R.J. (1985). Competitive antagonists of bradykinin. *Peptides*, **6**, 161–164.

ZAR, J.H. (1984). *Biostatistical Analysis*. 2nd edition. pp. 199–201. Englewood Cliffs: Prentice Hall.

(Received July 3, 1990
Revised September 6, 1990
Accepted September 19, 1990)