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Effects of trout bradykinin on the motility of the trout stomach and intestine: evidence for a receptor distinct from mammalian B_1 and B_2 subtypes

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1 Trout bradykinin ([Arg^0,Trp^5,Leu^8]-bradykinin; trout BK), recently isolated from kallikrein-treated trout plasma, produced sustained and concentration-dependent contractions of isolated longitudinal muscle from rainbow trout stomach ($pD_2 = 7.01 \pm 0.03$) and proximal small intestine ($pD_2 = 7.37 \pm 0.07$). The maximum responses were $85 \pm 2\%$ (stomach) and $101 \pm 35\%$ (intestine) of the corresponding responses to 10^{-5} M acetylcholine. Strips of circular smooth muscle from trout stomach and intestine did not contract in response to trout BK.

2 The potency of trout BK on gastric smooth muscle motility was significantly (5 fold; $P < 0.01$) reduced in the presence of the cyclo-oxygenase inhibitor, indomethacin (10^{-5} M) and by 4 fold ($P < 0.05$) in the presence of the lipoxygenase inhibitor, MK-886 (10^{-6} M), but there was no effect on the maximum response. Potency was also significantly reduced in the presence of 10^{-6} M methysergide (3 fold; $P < 0.02$) and 10^{-6} M tetrodotoxin (2 fold, $P < 0.05$) but atropine was without effect.

3 [Tyr^0,Trp^5,Leu^8]-BK was a full agonist but was approximately 50 fold less potent ($pD_2 = 5.35 \pm 0.08$) than trout BK, [Arg^0,Trp^5,Leu^8]des-Arg^9-BK was a partial agonist ($pD_2 = 6.80 \pm 0.03$; $56 \pm 7\%$ of the maximum response to trout BK) but [Trp^5,Leu^8]-BK, [Trp^5,Leu^8]-des-Arg^9-BK and mammalian BK produced no, or only very weak, contractions of the trout stomach.

4 The mammalian B_1 receptor antagonist, [Leu^8]des-Arg^9-BK was without effect on the response of the trout stomach to trout BK. The potent mammalian B_2 receptor antagonist Hoe 140 was a partial agonist ($pD_2 = 7.44 \pm 0.12$; $57 \pm 15\%$ of the maximum response to trout BK).

5 We conclude that the effects of trout BK on the motility of rainbow trout gastric smooth muscle are mediated through interaction with a receptor that has appreciably different ligand-binding properties than the mammalian B_1 and B_2 receptor subtypes. An involvement of arachidonic acid metabolites and 5-hydroxytryptaminergic nerves in the mechanism of action of the peptide is suggested.

Keywords: Bradykinin; B_1 receptor; B_2 receptor; gastric motility (trout); Hoe 140; [Leu^8] des-Arg^9-BK

Introduction

Activation of the kallikrein-kinin system of mammals involves the sequential activation of Factor XII (Hageman factor) and plasma prekallikrein and subsequent cleavage of high molecular mass kininogen to generate bradykinin (BK) (Colman, 1986). Evidence for the existence of a kallikrein-kinin system in the trout is accumulating. Using chromogenic substrates, Lipke & Olson (1990) showed that gill and kidney of the rainbow trout, *Oncorhynchus mykiss*, contained kallikrein activity and both kininogen and kininases were detected in trout plasma. Recently, we have demonstrated that incubation of heat-denatured trout plasma with porcine pancreatic kallikrein produced [Arg^0,Trp^5,Leu^8]-BK (trout BK) and that the peptide produced complex vasopressor and vasodepressor effects in the unanaesthetized trout (Conlon *et al*., 1996; Olson *et al*., 1996).

The actions of BK on mammalian smooth muscle are mediated through interaction with two well-characterized receptors, termed B_1 and B_2, that are differentiated pharmacologically by the rank orders of potencies of selected agonists and antagonists (reviewed in Farmer & Burch, 1992; Regoli & Gobeil, 1995). B_2 receptors are preferentially activated by BK and kallidin (Lys°-BK) and are specifically antagonized by Hoe 140 (Hock *et al*., 1991; Wirth *et al*., 1991), whereas des-Arg^9-BK and des-Arg^{10}-kallidin are selective B_1 receptor agonists and [Leu^8]-des-Arg^9-BK is a selective antagonist. The ability of BK and its analogues to contract isolated longitudinal smooth muscle from the guinea-pig ileum and cat ileum, 'classical' bioassays used in the original characterization of the peptide, are mediated through interaction with B_2 receptors (Regoli & Barabé, 1980) and receptor-binding studies have identified B_2 receptors in membrane vesicles from pig jejunal smooth muscle (Schäfer *et al*., 1986). However, more recent work has shown that contraction of the longitudinal muscle of the rat ileum is through the B_1 receptor (Meini *et al*., 1996). BK causes a biphasic relaxation followed by contraction of the rat isolated duodenum (Boschcov *et al*., 1984) and stomach fundus (Calixto & Medeiros, 1992). The aim of the present study was to investigate the effects and mechanism of action of trout BK on the motility of isolated smooth muscle from trout stomach and intestine.

Methods

Rainbow trout (400–600 g) were supplied by Anten trout farm (Alingsås, Sweden), and kept in aerated fresh water tanks at 10°C. The fish were killed by a sharp blow to the head and the stomach and intestine were dissected out. Pieces, approximately 2×10 mm, were cut in the longitudinal or circular direction from the cardiac part of the stomach and the proximal intestine. The muscle strip preparations were attached to Grass FT03 force displacement tranducers connected to a Grass polygraph model 7, and suspended in organ baths containing 7 ml of trout Ringer solution (10°C) bubbled with a gas mixture of 0.3% CO_2 in air. The composition of the Ringer solution used was (in mM) NaCl 140.0, KCl 2.5, $CaCl_2$ 1.5, $MgSO_4$ 0.8, $NaHCO_3$ 15.0, KH_2PO_4 1.0, HEPES 5.0 and

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glucose 10.0; pH 7.8. An initial tension of 10 mN was applied and 40–60 min was allowed for the preparations to obtain a steady basal tension before any drugs were added.

Acetylcholine (10^{-5} M) was added to test the viability of the preparations. Preparations showing no or a very weak contractile response to acetylcholine were not used for further experiments. The preparations were allowed to re-establish their basal tension and then trout BK was added in increasing concentrations until a maximum response was obtained. After washing and re-equilibration a second concentration-response curve was established with a BK analogue or with trout BK in the presence of antagonist (added at least 30 min before the experiment). As the magnitude of the response of muscle strips from the stomach was less variable than that of intestinal strips, mechanistic studies were carried out with gastric tissue.

Drugs

Trout BK (Arg-Arg-Pro-Pro-Gly-Trp-Ser-Pro-Leu-Arg), [Tyr^0,Trp^5,Leu^8]-BK, [Arg^0,Trp^5,Leu^8] des-Arg^9-BK, [Trp^5,Leu^8]-BK and [Trp^5,Leu^8]des-Arg^9-Bk were synthesized by Chiron Mimotopes (San Diego, CA) and their identities confirmed by automated Edman degradation and amino acid composition analysis. Mammalian BK was supplied by Novabiochem, Hoe 140 (D-Arg-[Hyp^3-Thi^5-D-Tic^7-Oic^8]-BK) by Research Biochemicals International, methysergide was a gift from Sandoz, MK-886 (3-[1-(*p*-chlorobenzyl)-5-(isopropyl)-3-*t*-butylthioindol-2-yl]-2,2-dimethylpropanoic acid) was from Calbiochem and other chemicals were from Sigma. Indomethacin, MK-886 and esculetin were dissolved in dimethylsulphoxide (DMSO) and diluted in phosphate buffer. Control experiments showed that 0.1% DMSO alone (the highest concentration used in the experiments) had no effect on the responses studied. All other drugs were dissolved in water and further diluted in 0.9% NaCl.

Data recording and statistics

Changes in tension recorded on the Grass polygraph were also sampled on a data acquisition software (AD/DATA; P.Thorén, Astra Hässle AB, Sweden). Concentration-response curves were established and pD_2 values ($-\log EC_{50}$) and maximum responses were calculated from the concentration-response curves by use of a linear regression analysis programme (GraphPad Prism, San Diego, U.S.A.). Wilcoxon matched pairs signed ranks test was used for statistical evaluation of the results. Differences where $P<0.05$ were regarded as statistically significant. Values are presented as means$\pm$s.e.mean from a minimum of 8 independent experiments.

Results

Effect of trout BK on longitudinal muscle from trout stomach and intestine

Trout BK produced sustained and concentration-dependent contractions of strips of longitudinal muscle from the cardiac part of the stomach ($pD_2=7.01\pm0.03$) and proximal small intestine ($pD_2=7.37\pm0.07$) of adult rainbow trout. The maximum responses were 85 ± 2% (stomach) and 101 ± 35% (intestine) of the corresponding responses to 10^{-5} M acetylcholine. This concentration of acetylcholine produces an approximately half-maximal contraction. Representative responses to cumulative additions of the peptide in a single experiment are shown in Figure 1. In four independent experiments, strips of circular smooth muscle from trout stomach and intestine did not contract in response to trout BK. As shown in Figure 2a, the pD_2 values (7.01 ± 0.03 and 6.89 ± 0.04) and maximum responses to trout BK were not significantly different when two successive concentration-response studies were carried out on the same strip of gastric smooth muscle after an interval of 2 h, demonstrating that the preparation was not subject to tachyphylaxis.

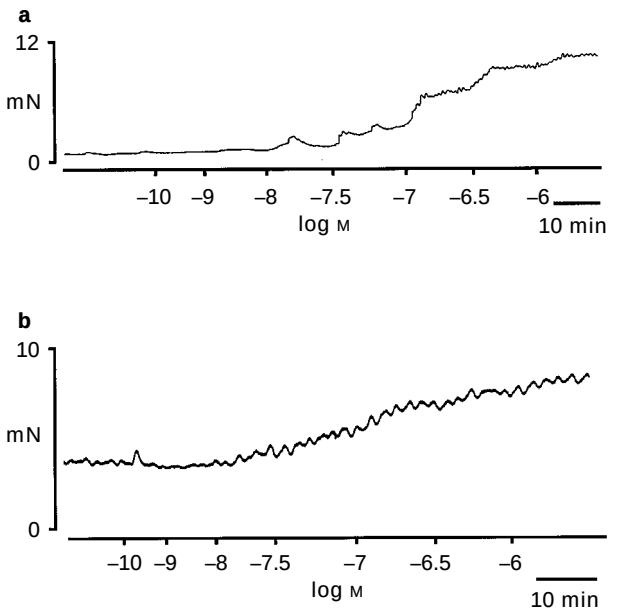

Figure 1 Effect of cumulative additions of trout BK ([Arg^0, Trp^5,Leu^8]-bradykinin) on the tension of longitudinal smooth muscle strips from (a) cardiac stomach and (b) proximal intestine of rainbow trout. The responses from a single experiment are shown.

Effect of inhibitors on the trout BK-induced contraction of longitudinal smooth muscle from trout stomach

Incubation of the gastric longitudinal muscle strips with the cyclo-oxygenase inhibitor, indomethacin (10^{-5} M) had no effect on the maximum contractile response produced by trout BK but there was a significant (5 fold; $P<0.01$) decrease in potency (Table 1). In the presence of 10^{-4} M indomethacin, the relative decrease in the potency of trout BK was 8 fold ($P<0.01$) (Table 1). The potency of trout BK, but not the maximum response, for contraction of gastric smooth muscle was significantly (3 fold; $P<0.02$) reduced in the presence of the relatively non-specific 'redox-based' lipoxygenase inhibitor, esculetin (10^{-5} M). The potency of trout BK was decreased by 13 fold ($P<0.01$) in the presence of 10^{-4} M esculetin (Table 1). The potency of trout BK for contraction of trout gastric smooth muscle was also significantly reduced ($P<0.05$) in the presence of 10^{-6} M and 10^{-5} M MK-886 but the agent had no effect upon the maximum contractile response (Table 1). MK-886 inhibits leukotriene biosynthesis by binding to 5-lipoxygenase-activating protein and, unlike esculetin, has no effect upon cyclo-oxygenases (Ford-Hutchinson, 1991).

The potency of trout BK was decreased by approximately 2 fold ($P<0.05$) when gastric tissue was incubated with the sodium channel blocker, tetrodotoxin (10^{-6} M) and by approximately 3 fold ($P<0.02$) in the presence of methysergide (10^{-6} M), an antagonist of 5-hydroxytryptamine (5-HT) that has been shown to be effective in the trout intestine (Jensen & Holmgren, 1991). (Table 1). Neither agent had a significant effect upon the maximum response to trout BK. The muscarinic receptor antagonist, atropine (10^{-6} M) had no effect on the contractile action of trout BK but the agent completely abolished the response to acetylcholine (10^{-5} M).

Effect of analogues of trout BK on longitudinal muscle from trout stomach

[Tyr^0,Trp^5,Leu^8]-BK was a full agonist but was approximately 50 fold less potent ($pD_2=5.35\pm0.08$) than trout BK (Figure 2c). [Arg^0,Trp^5,Leu^8]des-Arg^9-BK was a partial agonist (56 ± 7% of the maximum response to trout BK) but was only 5 fold less potent than trout BK ($pD_2=680\pm0.03$) (Figure 2d).

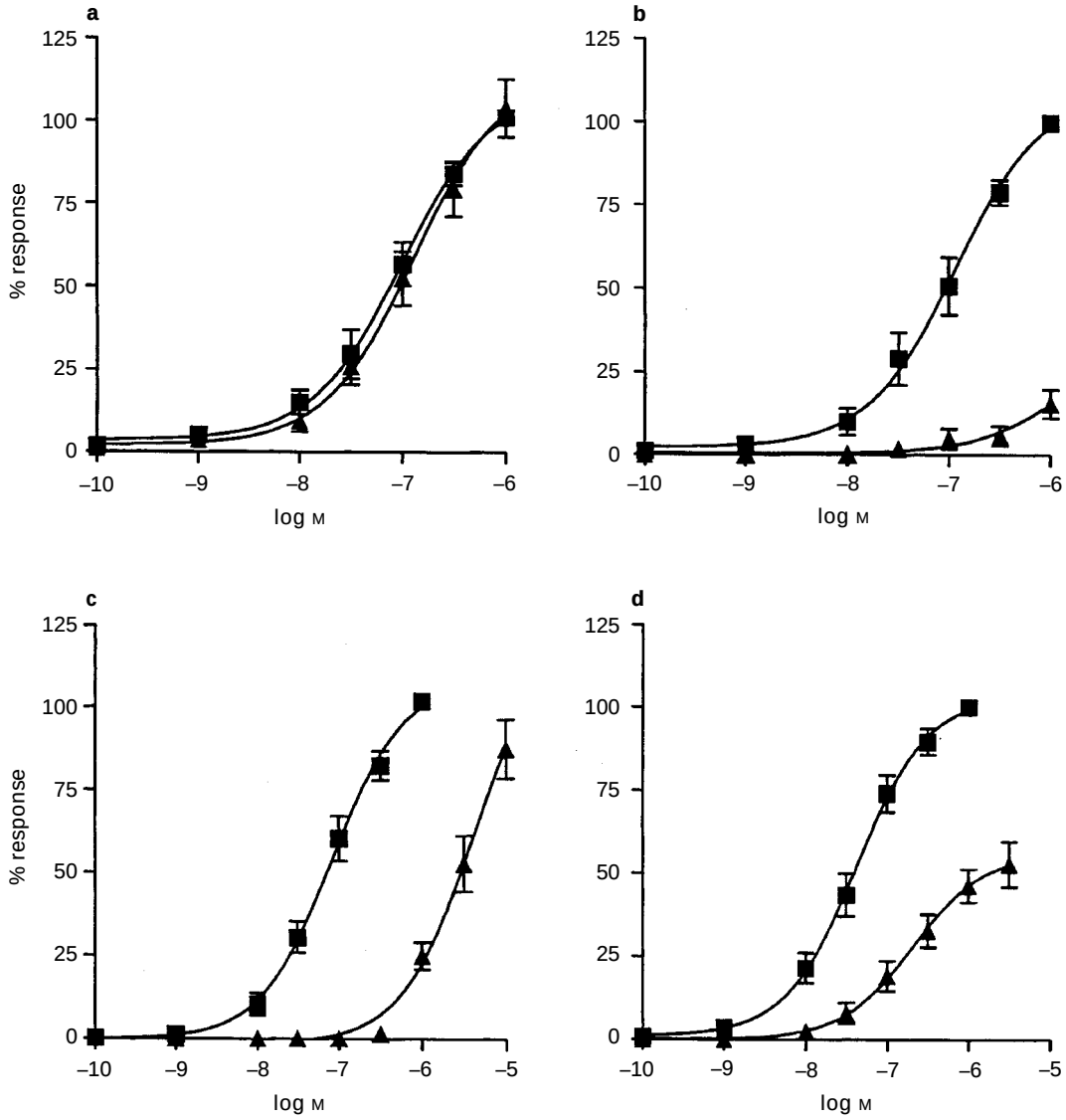

Figure 2 Agonistic action of bradykinin analogues on the contraction of isolated longitudinal muscle from trout stomach. Data are shown as mean for at least 8 experiments; vertical lines show s.e.mean. Sequential concentration-response curves were generated on the same strip by [Arg^0,Trp^5,Leu^8]-bradykinin (■) followed by (a) [Arg^0,Trp^5,Leu^8]-bradykinin (▲), (b) [Trp^5,Leu^8]-bradykinin (▲), (c) [Tyr^0,Trp^5,Leu^8]-bradykinin (▲) and (d) [Arg^0,Trp^5,Leu^8]des-Arg^9-bradykinin (▲). The interval between successive stimulations was 2 h.

In contrast, [Trp^5,Leu^8]-BK (Figure 2b) produced only a very weak contraction of the trout stomach that was not significantly different from that produced by [Trp^5,Leu^8]des-Arg^9-BK. Mammalian BK at concentrations up to 10^{-5} M was without effect on tension. These results are summarized in Table 1.

Effect of antagonists of mammalian B_1 and B_2 receptors on the trout BK-induced contraction of trout stomach

The mammalian B_1 receptor antagonist, [Leu^8]des-Arg^9-BK, alone had no effect on the tension of longitudinal muscle strips from trout stomach. Pre-incubation of the strips with [Leu^8]des-Arg^9-BK (10^{-5} M) had no significant effect on the maximum response to trout BK and no significant effect on its potency ($pD_2 = 6.67 \pm 0.21$ in the presence and $pD_2 = 6.99 \pm 0.10$ in the absence of the antagonist) (Figure 3). The potent mammalian B_2 receptor antagonist, Hoe 140 was a partial agonist ($57 \pm 15\%$ of the maximum response to trout BK) and was approximately equipotent with trout BK ($pD_2 = 7.44 \pm 0.12$ compared with $pD_2 = 6.95 \pm 0.05$ for the effect of trout BK in the same preparations of trout stomach) (Figure 3).

Discussion

This study, by demonstrating that endogenous BK is active upon trout gastrointestinal smooth muscle, has provided further evidence for the existence of a functioning kallikrein-kinin system in teleost fish. This result complements earlier work (Conlon *et al.*, 1996) showing that bolus intra-arterial injections of trout BK into unanaesthetized trout produce a triphasic pressor-depressor-pressor response on arterial blood pressure. Although our data and those of earlier workers (Lipke & Olson, 1990) indicate that trout tissues contain BK receptors and synthesize kallikrein, kininogen and kinases, we have not yet demonstrated that BK is generated in response to a physiological stimulus. Trout plasma does not appear to contain an enzyme analogous to mammalian Factor XII (Conlon *et al.*, 1996) and so the mechanism of activation of prekallikrein and the precise structure of the endogenous trout kinin remains to be elucidated.

The properties of the receptor(s) mediating the contractile response to trout BK in trout gastrointestinal smooth muscle are appreciably different from the mammalian receptor subtypes. The most striking illustration of this is the fact that

Table 1 Agonistic effects of trout bradykinin ([Arg^0,Trp^5,Leu^8]-BK) and its analogues on the contraction of the trout stomach

Peptide	*pD_2*
[Arg^0,Trp^5,Leu^8]-BK	6.89±0.04 (7.01±0.03)
[Tyr^0,Trp^5,Leu^8]-BK	5.37±0.08 (7.08±0.04)
[Arg^0,Trp^5,Leu^8,des-Arg^9]-BK	6.69±0.02 (7.37±0.03)
[Trp^5,Leu^8]-BK	Very weak agonist
[Trp^5,Leu^8,des-Arg^9]-BK	Very weak agonist
Hoe 140	7.37±0.14 (6.95±0.05)
BK	No effect
[Arg^0,Trp^5,Leu^8]-BK + atropine 10^{-6} M	6.68±0.10 (6.97±0.04)
[Arg^0,Trp^5,Leu^8]-BK + tetrodotoxin 10^{-6} M	6.64±0.11 (7.00±0.08)*
[Arg^0,Trp^5,Leu^8]-BK + methysergide 10^{-6} M	6.59±0.06 (7.03±0.04)*
[Arg^0,Trp^5,Leu^8]-BK + indomethacin 10^{-5} M	6.47±0.04 (7.19±0.07)**
[Arg^0,Trp^5,Leu^8]-BK + indomethacin 10^{-4} M	6.15±0.13 (7.04±0.07)**
[Arg^0,Trp^5,Leu^8]-BK + esculetin 10^{-5} M	6.62±0.04 (7.05±0.02)*
[Arg^0,Trp^5,Leu^8]-BK + esculetin 10^{-4} M	5.95±0.07 (7.08±0.11)**
[Arg^0,Trp^5,Leu^8]-BK + MK-886 10^{-6} M	6.46±0.20 (7.09±0.12)*
[Arg^0,Trp^5,Leu^8]-BK + MBK-886 10^{-5} M	6.58±0.12 (7.27±0.09)*

The values in parentheses show the pD_2 value of [Arg^0,Trp^5,Leu^8]-BK on the same tissue preparation. *$P<0.05$, **$P<0.01$ vs the effect of [Arg^0,Trp^5,Leu^8]-BK.

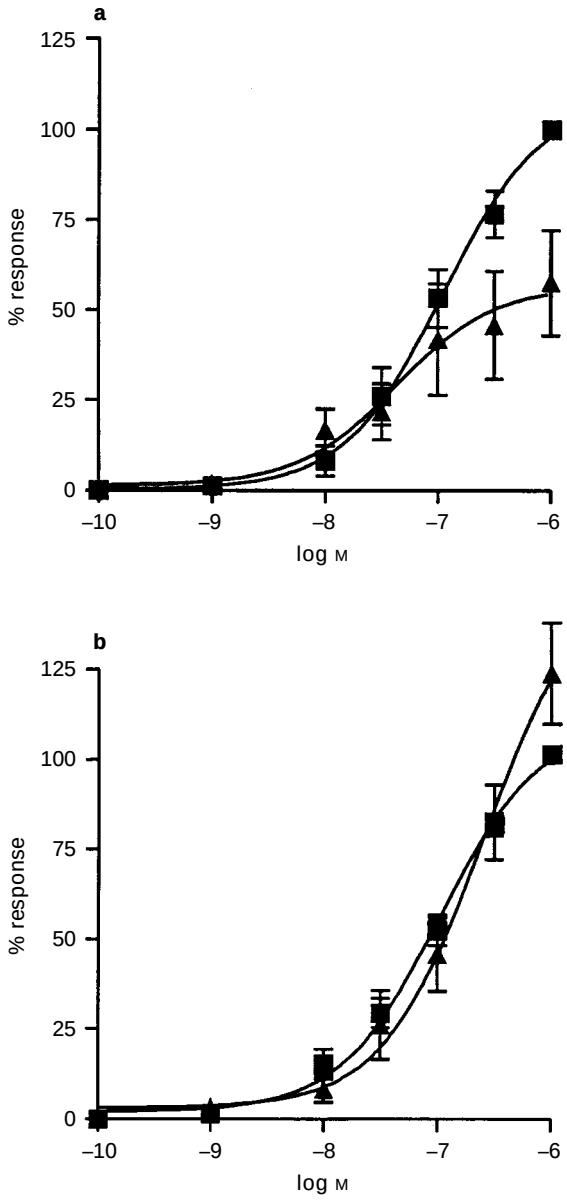

Figure 3 (a) Agonistic effect of the mammalian B_2 receptor antagonist, Hoe-140 (▲) compared with the effects of [Arg^0,Trp^5,Leu^8]-bradykinin (■) and (b) effects of the mammalian B_1 receptor antagonist, [Leu^8]des-Arg^9-bradykinin (10^{-5} M) (▲) on the log concentration-response curve of [Arg^0,Trp^5,Leu^8]-bradykinin (■) in trout gastric longitudinal smooth muscle. Data are shown as mean for 8 experiments; vertical lines indicate s.e.mean.

mammalian BK is without effect on the tension of trout tissues suggesting that the substitutions (Phe^5 → Trp) and/or (Phe^8 → Leu) in trout BK have pronounced effects on binding of the peptide to its receptor. Previous structure-activity studies have shown that Trp^5-BK is 50 fold and Leu^8-BK is 700 fold less potent than BK for contraction of the cat ileum (Regoli & Barabé, 1980). Additionally, we have shown that removal of the N-terminal arginyl residue from trout BK results in a marked diminution in both potency and effectiveness of the ligand. In contrast, BK and Lys^0-BK (kallidin) are equipotent in contracting the cat ileum (Regoli & Barabé, 1980) and in inhibiting binding of [^{3}H]-BK to B_2 receptors in the pig jejunum (Schafer *et al.*, 1986). The importance of the Arg^0 residue in trout BK for recognition by the trout BK receptor is confirmed by the fact that [Tyr^0,Trp^5,Leu^8]-BK was approximately 50 fold less potent than [Arg^0,Trp^5,Leu^8]-BK in the contraction of trout stomach. Similarly, the potent B_2 receptor antagonist Hoe 140, which inhibits BK-induced contractions of the guinea-pig ileum with an IC_{50} value of 1.1×10^{-8} M (Hock *et al.*, 1991), behaved as a partial agonist in the trout stomach preparation. However, it should be pointed out that in certain mammalian smooth muscle preparations, such as the sheep femoral artery without endothelium (Félétou *et al.*, 1994), Hoe 140 acts as an agonist. Evidence that the putative BK receptor in trout stomach differs in ligand-binding properties from the mammalian B_1 receptor subtype is provided by the observations that, removal of the C-terminal arginyl residue from trout BK resulted in a 5 fold decrease in potency and a reduction in the maximum response, and that [Trp^5,Leu^8]des-Arg^9-Bk was a very weak agonist. In contrast, des-Arg^9-BK ($pD_2=8.27$) was appreciably more potent than BK ($pD_2=6.69$) in inducing the B_1 receptor-mediated contraction of the longitudinal muscle of the rat ileum (Meini *et al.*, 1996). The mammalian B_1 receptor antagonist, [Leu^8]des-Arg^9-BK, was without significant effect on the trout BK-induced contraction of trout stomach.

The inability of B_1 and B_2 receptor antagonists to inhibit responses in certain mammalian smooth muscle preparations, such as guinea-pig and sheep trachea (Farmer *et al.*, 1989; Farmer & DeSiato, 1994), has led to the hypothesis that these tissues contain a novel B_3 receptor. However, the availability of cloned mammalian BK receptors for pharmacological studies has made it apparent that there are appreciable differences in properties between the same receptor subtype from different species. For example, the cloned mouse B_2 receptor, transfected into Chinese hamster ovary cells, has a 60 fold higher affinity for the antagonist [D-Arg^0,Hyp^3,D-Phe^7]-BK than its cloned human homologue (Hess *et al.*, 1994). Thus, it is premature to claim that the trout receptor identified in the present study is a novel receptor subtype rather than the piscine homologue of a mammalian receptor that has appreciable different ligand-binding properties. The amino acid sequence of the human B_1 receptor is 36% identical to the amino acid sequence of the human B_2 receptor (Menke *et al.*, 1994) indicating that the receptors are probably homologous. The trout is phylogenetically closely related to the earliest teleost stock and it is tempting to speculate that the receptor identified

in the trout gastrointestinal tract has similar properites to the ancestral receptor from which the B_1 and B_2 receptors are derived.

The contractile effects of BK on mammalian gastrointestinal tissues are in part the result of a direct action on smooth muscle and, in part, due to synthesis and release of stimulant prostaglandins (Terragno & Terragno, 1979). In these preparations, the concentration-response curve of BK is shifted to the right in the presence of the cyclo-oxygenase inhibitor, indomethacin. Despite the different ligand binding properties of the BK receptor in the trout stomach, our data have shown that prostaglandin production plays a role in mediating the contractile action of trout BK. Similarly, the decrease in potency in the presence of esculetin, a relatively non-specific inhibitor of 5- and 12-lipoxygenase of the 'redox' type (Neichi *et al*., 1983) and in the presence of MK-866, a more specific inhibitor of 5-lipoxygenase activation (Ford-Hutchinson, 1991), indicates a possible involvement of trout BK-stimulated leukotriene synthesis in the trout stomach. We have previously shown that bolus intra-arterial injections of trout BK into unanaesthetized trout produce an elevation in the circulating concentrations of prostaglandin E_2 and leukotriene C_4 (Olson *et al*., 1996). The effects of BK on either the guinea-pig or cat ilea are not attenuated by the 5-HT receptor antagonist, methysergide (Regoli & Barabé, 1980). In contrast, the significant decrease in potency of trout BK in the presence of both methysergide and tetrodotoxin suggest the effects of the peptide may be mediated, in part, by the release of 5-hydroxytryptamine from gastrointestinal neurones. It has been shown that the exitatory action of substance P on the trout stomach involves release of 5-hydroxytryptamine from intrinsic 5-hydroxytryptaminergic nerves (Holmgren *et al*., 1985).

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