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The smooth muscle pharmacology of maximakinin, a receptor-selective, bradykinin-related nonadecapeptide from the venom of the Chinese toad, *Bombina maxima*

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Abstract

Structural homologues of vertebrate regulatory peptides found in defensive skin secretions of anuran amphibians often display enhanced bioactivity and receptor binding when compared with endogenous mammalian peptide ligands. Maximakinin, a novel N-terminally extended bradykinin (DLPKINRKGPRPPGFSPFR) from the skin venom of a Chinese toad (*Bombina maxima*), displays such activity enhancement when compared with bradykinin but is additionally highly selective for mammalian arterial smooth muscle bradykinin receptors displaying a 50-fold increase in molar potency in this smooth muscle type. In contrast, a 100-fold decrease in molar potency was observed at bradykinin receptors in intestinal and uterine smooth muscle preparations. Maximakinin has thus evolved as a "smart" defensive weapon in the toad with receptor/tissue selective targeting. Natural selection of amphibian skin venom peptides for antipredator defence, through inter-species delivery by an exogenous secretory mode, produces subtle structural stabilisation modifications that can potentially provide new insights for the design of selectively targeted peptide therapeutics.

Keywords: Amphibian; Peptides; Bradykinin; Receptor

1. Introduction

The defensive secretions from the dermal granular, or poison glands of amphibians, particularly those of anurans, are complex molecular cocktails containing proteins, biogenic amines, alkaloids and a plethora of bioactive peptides [1–3]. Many skin peptides exhibit high degrees of structural similarity with endogenous vertebrate regulatory peptides but are usually more bioactive as a consequence of structural modifications occurring outside the conserved bioactive core sequence, an attribute produced by their natural selection for an exogenous delivery mode [1–3].

Many bradykinins found in the skin secretions of amphibians have been identified using the isolated uterus and ileum bioassay models that are highly sensitive to this peptide family [1]. Commonly, amphibian skin bradykinins are found to exhibit small changes in primary structure, with or without extensions at the N- or C-terminus of the nonapeptide [4]. Recently, novel bradykinins that are active on mammalian arterial and small intestinal smooth muscle have been structurally characterised and cloned from the skin of both *Bombina variegata* and *Bombina orientalis* [4,5]. Thus members of this group have a high potential for discovery of novel molecular variants of this peptide family.

The widespread occurrence of peptide-based defensive skin secretions in amphibians would suggest that this has been an effective anti-predator defence system for a long period of evolutionary time and which, by extrapolation, continues to be subject to natural selective pressures [2]. It is thus not surprising that a major biological investment in multiple peptide synthesis, with its associated metabolic requirements and consequences, should provide a substantial dividend in survival terms and that a requisite feature should be directed towards structural optimisation of components.

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Bioactive peptides are now known to be a major component of intercellular signalling, controlling many fundamental features of the life process including growth, differentiation, metabolism, neuromuscular activity and reproduction and consequently, are now regarded by the pharmaceutical industry as potential drug candidates of considerable importance [6]. Delivery and stability issues have long delayed the widespread use of such therapeutics and, while much progress has been made to this end, their potential remains unrealised.

Previously, we have described the isolation, structural characterisation and precursor cDNA cloning of a novel N-terminally extended bradykinin, maximakinin, from the defensive skin secretion of a Chinese toad (*Bombina maxima*) [7]. Here we describe in detail, for the first time, the highly selective pharmacological effects of this novel bradykinin using a range of rat smooth muscle preparations derived from the vasculature, small intestine and uterus. Despite the abundance and primary structural diversity of bradykinin-related peptides in defensive frog skin secretions [8–12], this study represents the first demonstration of tissue- and perhaps receptor-specific targeting of one of these peptides within another vertebrate taxon.

Table 1
Predicted b- and y-ion MS/MS fragment ion series (singly and doubly charged) of synthetic maximakinin

Sequence	#	Single charged ions			Sequence	#	Double charged ions		
		b (*m*/*z*)	y (*m*/*z*)				b (*m*/*z*)	y (*m*/*z*)	
D	1	116.1	2180.5	19	D	1	58.5	**1090.8**	19
L	2	229.2	**2065.5**	18	L	2	115.1	1033.2	18
P	3	**326.4**	1952.3	17	P	3	163.7	**976.7**	17
K	4	**454.5**	**1855.2**	16	K	4	227.8	928.1	16
L	5	**567.7**	1727.0	15	L	5	**284.4**	**864.0**	15
N	6	**681.8**	1613.8	14	N	6	**341.4**	807.4	14
R	7	**838.0**	**1499.7**	13	R	7	**419.5**	**750.4**	13
K	8	966.2	**1343.6**	12	K	8	**483.6**	672.3	12
G	9	**1023.2**	**1215.4**	11	G	9	512.1	**608.2**	11
P	10	1120.3	1158.3	10	P	10	**560.7**	579.7	10
R	11	**1276.5**	**1061.2**	9	R	11	**638.8**	531.1	9
P	12	1373.6	**905.0**	8	P	12	**687.3**	**453.0**	8
P	13	1470.8	807.9	7	P	13	735.9	**404.5**	7
G	14	1527.8	**710.8**	6	G	14	764.4	355.9	6
F	15	**1675.0**	**653.7**	5	F	15	**838.0**	327.4	5
S	16	1762.1	**506.6**	4	S	16	881.5	**253.8**	4
P	17	**1859.2**	419.5	3	P	17	930.1	**210.2**	3
F	18	**2006.4**	**322.4**	2	F	18	**1003.7**	161.7	2
R	19	2162.5	175.2	1	R	19	**1081.8**	88.1	1

Observed ions are indicated in bold typeface.

2. Materials and methods

2.1. Synthesis of maximakinin

Maximakinin (DLPKINRKGPRPPGFSPFR) was synthesised by solid-phase fmoc chemistry using an Applied Biosystems 433 automated peptide synthesiser. Following deprotection and cleavage from the resin, the peptide was purified by reverse-phase HPLC. The purification was monitored by LC/MS (Thermo Electron LCQ electrospray ion-trap mass spectrometer, Thermo-Electron, San Jose, CA, USA) and by MALDI-TOF mass spectroscopy (Perseptive Biosystems, MA, USA). The purity of the final product and its identity with the natural peptide were assessed by MS/MS fragmentation sequencing using the same instrument (Table 1).

2.2. Smooth muscle bioassays

2.2.1. Arterial smooth muscle

Male albino Wistar rats (200–350 g) were euthanised by asphyxiation followed by cervical dislocation. The tail artery was prepared as previously described [13]. Incubation buffer was 95% O_2/5% CO_2 oxygenated Kreb's solution (NaCl 118 mM, KCl 4.7 mM, $NaHCO_3$ 25 mM, NaH_2PO_4 1.15 mM, $CaCl_2$ 2.5 mM, $MgCl_2$ 1.1 mM, glucose 5.6 mM). Constriction/dilation of the arterial smooth muscle preparation was detected by an increase or decrease in pressure generated by water column displacement using pressure transducers connected to a MacLab System (AD Instruments, Australia). Data were displayed graphically on a Macintosh computer. Viability was determined using a range of bolus phenylephrine (5 μM–1 mM) exposures and the endothelial layer of the artery was removed by bubbling with oxygen for 10 s. Absence of the endothelial layer was confirmed by the lack of relaxation in response to a 30 min perfusion of acetylcholine (50 μM) after preconstriction with phenylephrine (10 μM).

2.2.2. Small intestinal smooth muscle

For intestinal smooth muscle preparations, 1 cm thick rings of ileum were carefully placed onto the pins of a MacLab force transducer, one pin acting as a stationary fixed point while the second pin was free, permitting application of tension to the smooth muscle. The muscle rings were gradually exposed to 0.1*g* increments in resting tension until the spontaneous contractions originated from a resting tension of 0.5*g*. The contracting muscle preparations were allowed to stabilise for 25 min before the application of peptides. The dose responses for both maximakinin and bradykinin, as determined by relative changes in muscle tension, were determined following application of both peptides in separate experiments, in the range of 1×10^{-5}–1×10^{-10} M.

2.2.3. Uterine smooth muscle

Virgin female Wistar rats (200–250 g) were injected with 0.1 mg kg^{-1} β oestradiol benzoate, 24 h prior to sacrifice. The animals were asphyxiated in a carbon dioxide atmosphere and death was confirmed by cervical dislocation. The

dorsal fur was trimmed to expose the skin and the abdomen opened to expose the uterus. The entire uterine horns were removed and placed in ice-cold De Jalon's solution which was vigorously aerated with carbogen mixture (95% CO_2; 5% O_2). Each uterine horn was halved and individual strips mounted in a 2 ml organ bath, perfused with De Jalon's solution at 30 °C for 10 min with no tension. The uterus strips were gradually exposed to increasing tension until 0.5*g* was reached and maintained. The preparation was allowed to equilibrate for a further 10 min before the first peptide challenge. Changes in tension across the muscle strip were measured using MacLab force transducers and data were recorded using a MacLab System (AD Instruments). After a second equilibration period of 10 min, peptide dose responses were performed and recorded as described above.

3. Results

3.1. Chemical synthesis of maximakinin

The synthesis of maximakinin was successful yielding 180 mg of peptide following purification to >99% purity as demonstrated by peak absorbance and symmetry following reverse phase HPLC fractionation and by electrospray MS/MS (Fig. 1).

3.2. Smooth muscle bioassays

The relative potencies of bradykinin and maximakinin, expressed as comparative EC_{50} values with that of bradykinin normalised to one arbitrary unit, in arterial, small intestinal and uterine smooth muscle preparations, are illustrated in Fig. 2a–c. Actual EC_{50} values (mean ± standard error, $n=6$) for each peptide in each tissue are given in the figure legend. In the artery preparation, maximakinin was found to be 49.7-fold more potent than bradykinin. In contrast, in both small intestinal and uterine preparations, maximakinin displayed approximately 0.01–0.02 of the potency of bradykinin. The arterial smooth muscle bradykinin receptor thus appears to be differentiated from the small intestinal and uterine smooth muscle receptors by maximakinin. Comparative analyses of the time course of the effects of maximakinin and bradykinin on each smooth muscle preparation are shown in Figs. 3–5. In the arterial smooth muscle preparation (Fig. 3), maximakinin achieved maximal relaxation some 10 min before bradykinin, although the response curves were very similar. In contrast, the temporal patterns of response to maximakinin and bradykinin in both small intestinal and uterine smooth muscle preparations were quite different. In the small intestinal preparation (Fig. 4), the response to bradykinin was transient and biphasic and occurred within the first 5 min of application, whereas the response to maximakinin was later and prolonged until termination of the experiment at 30 min. In the uterine preparation (Fig. 5), the response to bradykinin was rapid (within 1 min of application) and the induced increase in tone slowly decayed over the time course of the experiment (up to 30 min). In contrast, the response to applied maximakinin was observed after 2 min and was followed by a series of rhythmic relaxations and

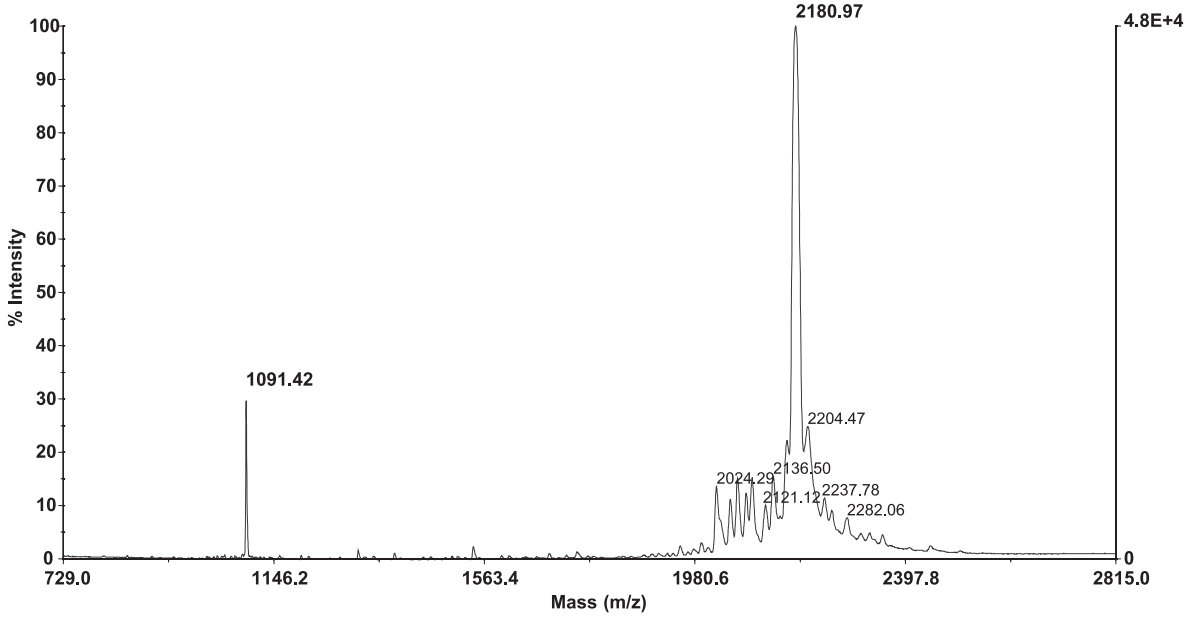

Fig. 1. MALDI-TOF mass spectrogram of deprotected, cleaved reaction mixture indicating major authentic product ion (*m/z* 2180.97 ($M+H^+$) and minor product ion (*m/z* 1091.42 ($M+2H^+$)), corresponding to synthetic maximakinin.

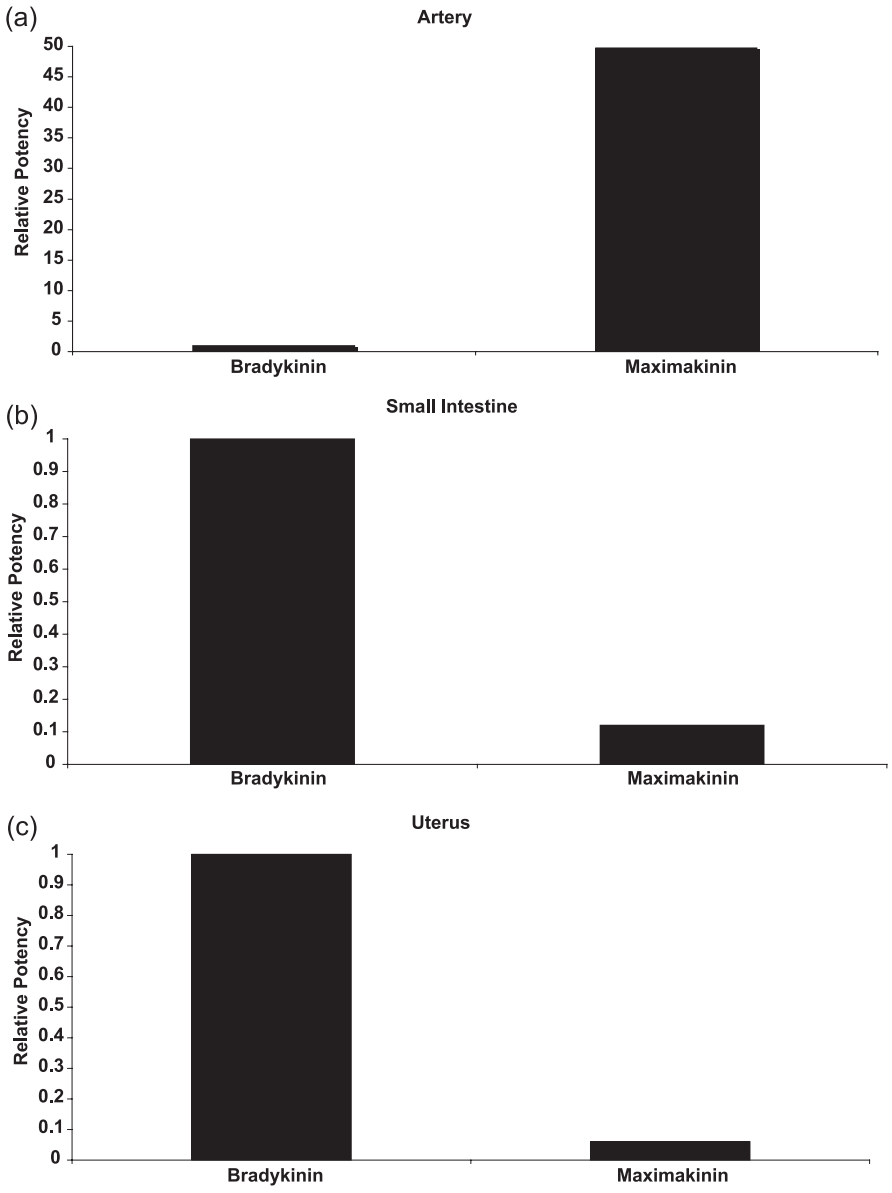

Fig. 2. Relative molar potencies of bradykinin and maximakinin on isolated smooth muscle preparations. Effect on rat tail artery (a), small intestine (b) and uterus (c). Expressed as comparative EC_{50} values for each peptide with bradykinin normalized to one arbitrary unit in each case.

contractions with regular periodicity but decreasing amplitude. Maximakinin responses in each tissue were analysed in the presence of a specific bradykinin B_1-receptor antagonist (desArg-HOE 140) [14], a specific bradykinin B_2-receptor antagonist (HOE 140) [15], or both in combination (Fig. 6). The results from the arterial and small intestinal smooth muscle preparations showed that each antagonist significantly reduced the responses to maximakinin and, that in combination, they were more effective. In contrast, in the uterine preparation, the B_1 receptor antagonist marginally reduced the maximakinin response and the B_2-receptor antagonist had no observable effect on this tissue's responsiveness to maximakinin. In combination, there was likewise no observable effect.

4. Discussion

Maximakinin, a novel N-terminally extended bradykinin, that represents a major peptide in the defensive skin secretion of the Chinese large-webbed bell toad (*B. maxima*), was found to be a potent bradykinin agonist with a high degree of selectivity for rat arterial smooth muscle receptors. Despite the abundance and primary structural diversity of bradykinin-related peptides in defensive frog skin secretions [8–12], this is the first demonstration of tissue- and perhaps receptor-specific targeting of these amphibian peptides within another species.

The time courses of observed responses to maximakinin at half-maximal effective concentration, in a range of mammalian smooth muscle preparations, differ from those

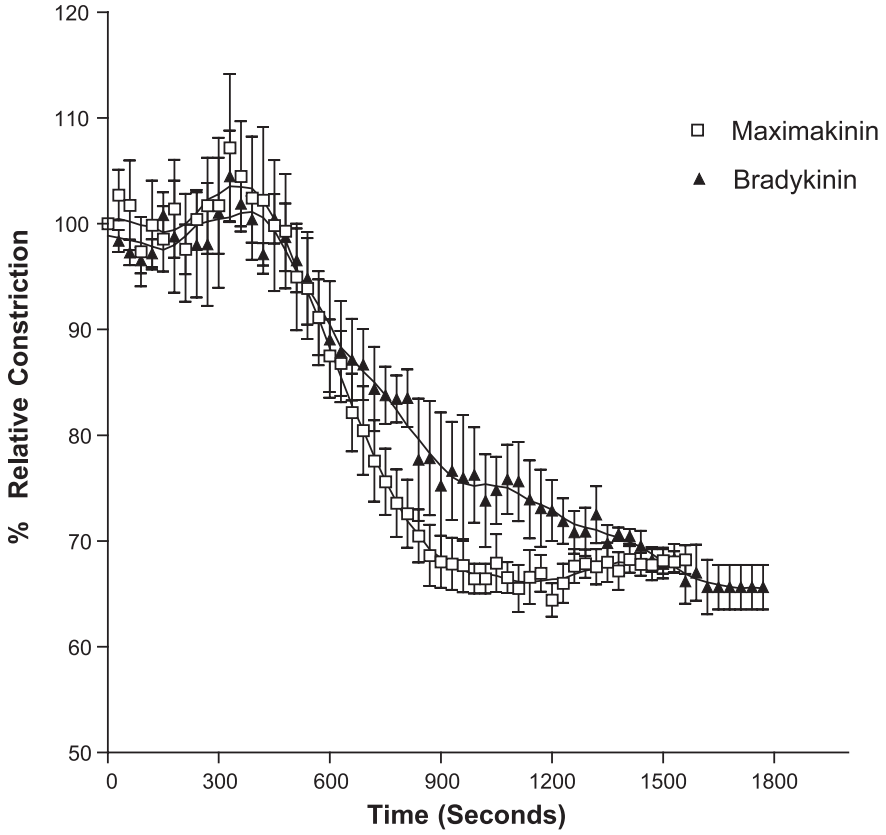

Fig. 3. The time response curves of dilation induced by maximakinin and bradykinin in phenylephrine pre-constricted isolated rat tail artery smooth muscle. Both peptides employed at EC_{50} concentrations ($n=4$).

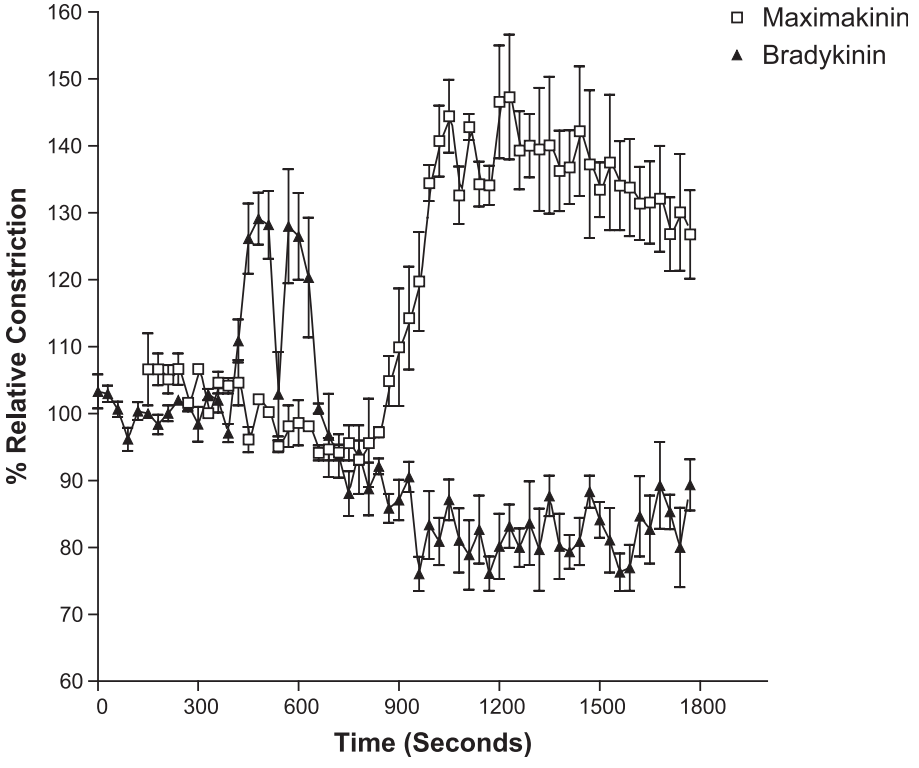

Fig. 4. The time response curves of constriction induced by maximakinin and bradykinin in isolated rat small intestinal (ileal) smooth muscle rings. Both peptides employed at EC_{50} concentrations ($n=4$).

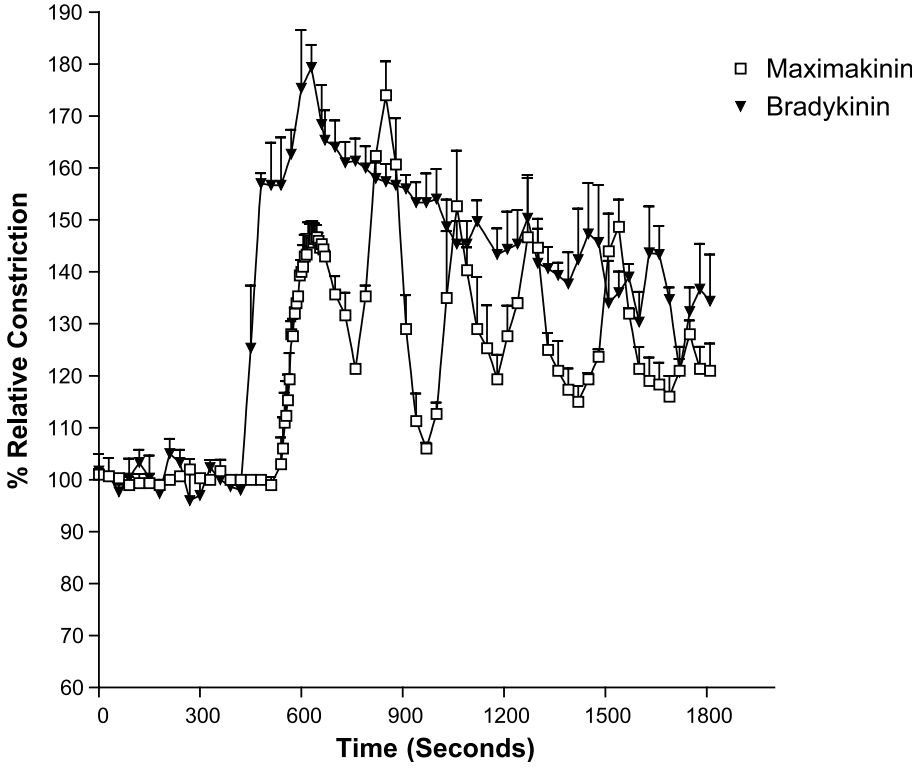

Fig. 5. The time response curves of contractions induced by maximakinin and bradykinin in isolated rat uterine smooth muscle strips. Both peptides employed at EC_{50} concentrations ($n = 4$).

observed with the endogenous ligand, bradykinin. In the arterial smooth muscle, maximakinin achieves effective plateau dilatation more rapidly than bradykinin but the effects are broadly comparable. In contrast, small intestinal and uterine smooth muscle effects of both peptides were strikingly different. In small intestinal smooth muscle, bradykinin evoked a rapid but transient biphasic contraction, whereas maximakinin induced a later but prolonged contractile response that was still evident upon termination of the experiment. This may be due to maximakinin's higher intrinsic resistance to endogenous, enzymatically mediated termination of activity as a consequence of the N-terminal extension—a factor that would lead to an extended functional presence in the system and would add evidence to our claim of biotransformation resistance. In uterine smooth muscle, the effects of both peptides displayed the most dramatic differences. Bradykinin induced a typical rapid increase in muscle tone [16] that slowly decayed over the time course of the experiment. In contrast, maximakinin induced a series of regular rhythmic contraction/relaxation events that were not observed in any other smooth muscle preparation employed. It could be that the biotransformation of maximakinin by cell surface peptidases on these smooth muscle cells produces catabolites with either complementary agonist/antagonist activity or that these interact with different affinities at receptor subtypes. Of interest is the fact that maximakinin was less potent in these latter smooth muscle preparations when compared with bradykinin—a factor that could easily have masked their differing pharmacological response profiles.

While the biological actions of bradykinin in mammalian tissues are thought to be mediated by just two different receptor sub-types, designated as B_1 and B_2 [17,18], their different pharmacological profiles with fragments and antagonists and their different species-specific tissue distributions remain unclear with several conflicting reports in the literature [17,18]. In terms of rank potency of both peptides, the data presented in this study would imply that rat uterine/small intestinal bradykinin receptors are similar but that the receptor on arterial smooth muscle is different. For these reasons, we have referred to maximakinin responses as mediated through receptors defined by tissue localisation. This we believe to be prudent as the results from specific antagonist studies would substantiate. The data obtained for each smooth muscle type, stimulated by maximakinin in the presence of specific B_1- and B_2-receptor antagonists (des Arg-HOE 140 and HOE 140), would tend to suggest that in rat artery and small intestine there are both receptor subtypes. However, in the uterus, there is a bradykinin receptor that is not antagonized by either antagonist and hence is representative of a putative novel subtype. It should be noted that the properties of each bradykinin receptor antagonist have been deduced in models using the endogenous bradykinin nonapeptide as competitive ligand and that maximakinin, due yet again to its N-terminal extension, may not parallel bradykinin in this respect.

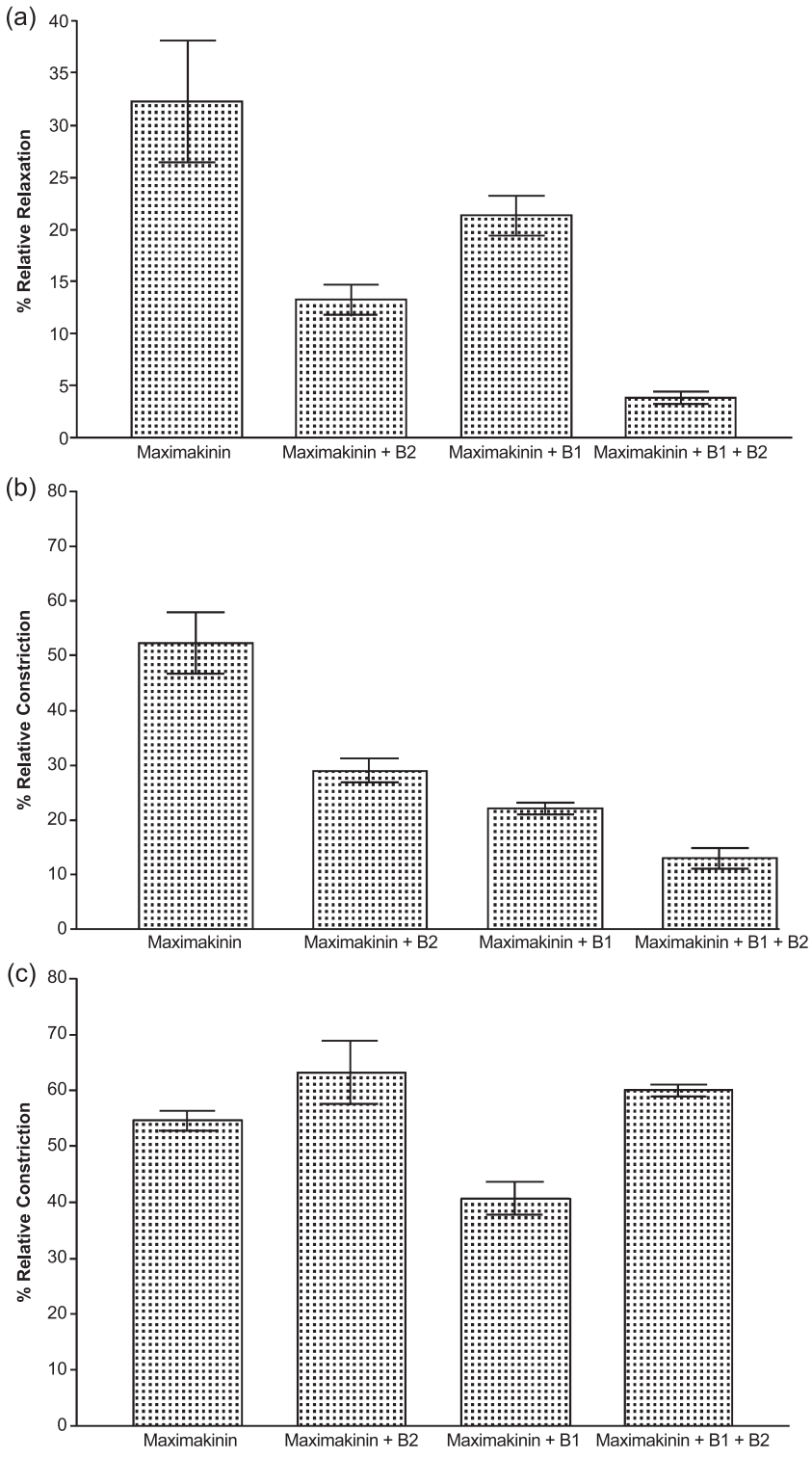

Fig. 6. Effects of selective bradykinin receptor antagonists on maximakinin smooth muscle activities. (a) The effect of bradykinin antagonists HOE 140 (B_2 receptor antagonist—0.3 μM) and desArg HOE 140 (B_1 receptor antagonist—0.3 μM) on maximakinin (0.1 μM) induced dilation of phenylephrine (10 μM) pre-constricted rat tail artery smooth muscle ($n=6$). (b) The effect of the above bradykinin antagonists on maximakinin (1.0 μM) induced constriction of isolated rat small intestinal (ileal) smooth muscle rings under 0.5*g* of resting tension ($n=6$). (c) The effect of the above bradykinin antagonists on maximakinin (0.1 μM) induced contraction of isolated rat uterine smooth muscle strips under 0.5*g* of resting tension ($n=6$).

Hence, established highly characterised receptor antagonists may prove to be of little use in assigning receptor subtype interaction or specificity when using a substantially modified, yet biologically active analogue. Such ligands, from phylogenetically distinct species, may prove invaluable in the molecular pharmacological dissection of novel receptor

subtypes in mammals in a manner not facilitated by simplistic structural homology profiling. Thus frog skin-derived structural analogues of endogenous mammalian regulatory peptides may interact with autologous receptors in a plethora of ways that produce quantitative and qualitative differences in biological responses as a consequence of structural modifications selected for in the biosphere over long periods of time.

We conclude that this element of the survival strategy of amphibians can provide new insights and leads in the development of novel peptide drugs. Here we provide experimental evidence for structural modifications that not only enhance bioactivity compared to normal endogenous analogues but which can lead to a high degree of receptor subtype selectivity. The features of structural modification found in this bradykinin-related peptide, maximakinin, produce significantly altered activity in different receptive tissues. Evolutionary design of this frog skin bradykinin receptor ligand has thus resulted in a molecule of superior efficiency in arterial smooth muscle relaxation. A closer study of molecular variants of endogenous vertebrate regulatory peptides, especially those present in venoms or defensive secretions, is thus warranted in the light of the emerging principle that structural variations do not simply represent phylogenetic differences. Rather, these peptides may be naturally selected products of molecular evolution, directed to the generation of receptor ligands with superior potency. Exendin-4, a glucagon-like peptide-1 (GLP-1) structural analogue and receptor agonist found in Gila monster (*Heloderma suspectum*) lizard venom, has recently been shown to have a significantly longer half-life in rat plasma than GLP-1 and that this is largely independent of administration route [19]. Although the venom that contains this peptide is normally injected, nevertheless it illustrates the previous point most effectively. In the case of the frog skin defensive peptide, maximakinin, described here, superior potency and receptor selectivity has resulted from natural, genetically engineered and selected structural features. Such a system, honed by natural selection over many millions of years, has the potential to provide the raw materials that could produce fundamental insights into the design of novel peptide therapeutics and to aid in the identification of novel receptor variants or structure/activity profiles of existing receptors that are known or putative drug targets.

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