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The pharmacology of T-kinin and des-Arg^{11}-T-kinin in primary cultures of rat bladder smooth muscle cells

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Abstract

T-kinin and its putative carboxypeptidase product des-Arg^{11}-T-kinin are members of the kinin family that are unique to the rat. Primary cultures of rat bladder smooth muscle cells were used to investigate the pharmacology of these peptides. Calcium imaging experiments showed that rat bladder smooth muscle cells responded to both bradykinin and des-Arg^{9}-bradykinin with an increase in $[Ca^{2+}]_i$ and responses to both agonists could be observed in the same cell. A more detailed pharmacological characterisation with a range of bradykinin receptor agonists and antagonists using $^{45}Ca^{2+}$ efflux confirmed the presence of both B_1 and B_2 bradykinin receptors. Using this cellular model, we confirm that T-kinin is a bradykinin B_2 receptor agonist and show for the first time that des-Arg^{11}-T-kinin is a potent and selective bradykinin B_1 receptor agonist. In addition, using cells expressing the cloned rat and human bradykinin B_2 receptors plus the Ca^{2+}-sensitive protein aequorin, T-kinin was shown to be selective for the rat over the human bradykinin B_2 receptor.

Keywords: Bradykinin; Bradykinin receptor; T-kinin; Des-Arg^{11}-T-kinin; Bladder, rat; Smooth muscle cell

1. Introduction

The effects of the inflammatory mediators bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) and kallidin (Lys-bradykinin), and their carboxypeptidase metabolites des-Arg^{9}-bradykinin and des-Arg^{10}-kallidin have been extensively investigated; however, less is known about the pharmacology of the rat-specific T-kinin (Ile-Ser-bradykinin) and the potential role of its putative carboxypeptidase breakdown product des-Arg^{11}-T-kinin has not been investigated.

In most species, bradykinin is formed by the action of plasma kallikrein on high molecular weight kininogen, and kallidin is formed from high or low molecular weight kininogens by tissue kallikrein. In the rat, there are two differences in the kinin generating system. Due to a minor difference in the sequence of the cleavage site, tissue kallikrein generates bradykinin from kininogens and, therefore, kallidin is not generated in the rat. Secondly, a third type of kininogen known as T-kininogen, coded for by a separate gene, is found in the rat. This is broken down by T-kininogenase leading to the production of T-kinin (Ile-Ser-bradykinin). T-kininogen is thought to have a role in inflammatory processes in the rat, as its expression is increased following an inflammatory insult (Barlas et al., 1985a,b).

Bradykinin and kallidin are subject to degradation by proteases, cleavage by carboxypeptidases removes the terminal arginine to form des-Arg^{9}-bradykinin and des-Arg^{10}-kallidin, respectively (Bhoola et al., 1992). T-kinin is also subject to degradation and can be converted to bradykinin by the action of an aminopeptidase. It is also susceptible to carboxypeptidases in vitro (Passaglio and Vieira, 1996), and it is likely that the potential carboxypeptidase metabolite of T-kinin, des-Arg^{11}-T-kinin, occurs naturally.

The effects of bradykinin and kallidin are mediated by the bradykinin B_2 receptor. The bradykinin B_1 receptor, whose expression is regulated by inflammatory mediators, is activated by des-Arg^{9}-bradykinin and des-Arg^{10}-kallidin. In the rat, T-kinin is thought to act on the bradykinin B_2 receptor (Yamawaki et al., 1995). The activity of des-Arg^{11}-T-kinin has not been explored but it might be expected to be a bradykinin B_1 receptor agonist. Recently, it has been shown that des-Arg^{11}-T-kinin can displace

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[^{3}H]des-Arg^{10}-kallidin binding to the cloned rat bradykinin B_1 receptor (Jones et al., 1999); however, its pharmacological actions have not been investigated in detail.

Rat bladder has been reported to express both B_1 and B_2 bradykinin receptors (Lecci et al., 1995), and the expression of the bradykinin B_1 receptor is increased following inflammatory insults (Belichard et al., 1999; Jones et al., 1999). The pharmacology of bradykinin receptors in rat bladder smooth muscle cells has not, however, been studied. In this report, we show that isolated rat bladder smooth muscle cells express both B_1 and B_2 bradykinin receptors and characterise the responses to T-kinin and the novel kinin des-Arg^{11}-T-kinin.

2. Methods

2.1. Preparation of bladder smooth muscle cells

Bladder smooth muscle cells were prepared by a modification of the method of Levesque et al. (1993). The urinary bladder was removed from female Sprague–Dawley rats weighing about 150–170 g and washed in growth medium (Medium 199 containing 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin plus 10% foetal calf serum (FCS)). After chopping into approximately 2 × 2 mm pieces, the tissue was digested with 1 mg/ml elastase (from porcine pancreas) and 3 mg/ml collagenase (Type 4) for 90 min on a rotary shaker at 37 °C. The digested tissue was spun at 300 × *g* for 5 min, the pellet was resuspended in growth medium and then triturated gently. The resulting cell suspension was passed through a 90-μm muslin filter to remove undigested clumps of tissue and the cells transferred to 80-cm^2 tissue culture flasks in growth medium and grown in 6% CO_2 at 37 °C. The medium was changed after 1 day to remove any debris. The cells multiplied in culture and were split by a ratio of 1:2 every 3–4 days using a solution containing 1 mM EDTA and 10 mM HEPES in Ca^{2+}- and Mg^{2+}-free Hanks Balanced Salt Solution (HBSS) to detach the cells. All experiments were carried out on cells that had been in culture for 7–9 days.

In culture, the isolated rat bladder smooth muscle cells showed the classic "hill and valley" phenotype typical of smooth muscle cells (Chamley-Campbell et al., 1979). To determine the proportion of the cell population that were smooth muscle, a monoclonal antibody to the smooth muscle form of actin was used. The cells were plated onto poly-D-ornithine-coated glass coverslips (22 mm) at approximately 15,000 cells per coverslip in growth medium and grown overnight. The cells were fixed (methanol and glacial acetic acid (95%/5% v/v) for 30 min), washed in phosphate-buffered saline (PBS), pH 7.2, incubated in blocking solution (5% sheep serum in PBS) for 15 min before incubation with mouse monoclonal anti-α-smooth muscle actin, at a dilution of 1:400, made up in blocking solution, for 2 h at room temperature. After extensive washing in PBS, fluorescein-linked anti-mouse Ig diluted 1:30 in blocking solution was applied for 2 h in the dark. The coverslips were washed in PBS and placed on microscope slides with Citifluor mounting medium, sealed with nail varnish and examined under a fluorescence microscope. These experiments showed that approximately 90% of the cells stained positively for the α-smooth muscle actin antibody. No staining was observed in the bladder smooth muscle cells with control ascites fluid and anti-α-actin did not stain Chinese Hamster Ovary (CHO) cells, which were used as negative control.

2.2. Measurement of $^{45}Ca^{2+}$ efflux

Measurement of $^{45}Ca^{2+}$ efflux was carried out as described in Smith et al. (1995). Briefly, bladder smooth muscle cells were plated onto 60-well Terasaki plates at approximately 1500 cells/well and grown overnight. After loading with 45 μCi/ml [^{45}Ca]calcium chloride for 4 h at 37 °C, the cells were washed for 17 min in assay buffer (10 mM HEPES in HBSS, pH 7.4). Agonists were applied for 1 min and antagonists were applied for the 4 min prior to, and during, the 1-min application of agonist. The amount of $^{45}Ca^{2+}$ in each wash was measured by liquid scintillation counting in a Wallac scintillation counter.

In some experiments, the method was adapted for 96-well plates; the smooth muscle cells were plated at 35,000 cells/well and grown overnight. The medium was removed from the cells and replaced with 50 μl of 50 μCi/ml [^{45}Ca]calcium chloride and incubated for 4 h at 37 °C. The cells were washed for 20 min with assay buffer using a Denley cell washer and then exposed to the agonist for 4 min. Where antagonists were used, they were added for 4 min before the addition of the agonist as well as during the application of agonist. The amount of $^{45}Ca^{2+}$ in each wash was determined by counting on a Packard Topcount.

The amount of $^{45}Ca^{2+}$ efflux for each wash was calculated as a fraction of the total $^{45}Ca^{2+}$ in the cells (determined by solubilising the cells with 0.2% sodium dodecyl sulphate). The rate of $^{45}Ca^{2+}$ efflux in response to an agonist was expressed as the fold increase over the basal efflux. Typical values for basal and agonist-evoked efflux in the Terasaki-based method were 0.045 ± 0.002 min^{-1} ($n = 24$), 0.114 ± 0.002 min^{-1} ($n = 24$) and 0.296 ± 0.045 min^{-1} ($n = 6$) for basal, des-Arg^9-bradykinin (1 μM) and bradykinin (100 nM)-evoked efflux, respectively. In the 96-well plate-based method, basal efflux was 0.016 ± 0.001 min^{-1} ($n = 6$) and agonist-evoked efflux was 0.030 ± 0.005 min^{-1} ($n = 7$) for des-Arg^9-bradykinin (1 μM) and 0.091 ± 0.005 ($n = 9$) for bradykinin (100 nM).

2.3. Calcium imaging

Bladder smooth muscle cells were plated onto poly-D-ornithine-coated 22-mm glass coverslips at approximately 17,000 cells per coverslip in growth medium and grown

overnight. A video imaging system (Imagemaster, Photon Technology International) was used for measurement of $[Ca^{2+}]_i$. The cells were loaded with 2 μM of the cell permeant acetoxymethyl ester of fura-2 (fura-2/AM) for 30 min at 37 °C, rinsed, transferred to a perfusion chamber at room temperature and perfused (flow rate of 1 ml/min) with buffer (HBSS containing 10 mM HEPES, pH 7.4) in the presence and absence of drugs. Fura-2 was excited alternately at 340 and 380 nm, and the emitted light monitored at 510 nm. Images were captured every 4 s and ratio images calculated using Imagemaster software.

2.4. Aequorin-based assays

To measure the activity of the human bradykinin B_2 receptor, CHO cells stably expressing the receptor and aequorin, a protein which emits light on interaction with Ca^{2+}, were used. The cells were grown in Minimum Essential Medium Alpha without ribonucleosides and deoxyribonucleosides, supplemented with 10% dialysed FCS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 700 μg/ml G418. For the assay, the cells were plated onto Packard Viewplates at 50,000 cells/well, grown overnight and then incubated with 20 μM coelenterazine H plus 30 μM glutathione, made up in the growth media, for 2–3 h at 37 °C. The loading solution was removed and replaced with assay buffer (10 mM HEPES in HBSS, pH 7.4) and the plate was placed in a Luminoskan luminometer. Following injection of the agonist, the luminescence was measured for 20 s. For comparison Cos-7 cells (grown in Dulbecco's Modified Eagle Medium plus 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% FCS) were transiently transfected with the rat bradykinin B_2 receptor and aequorin cDNA. Following transfection with 50 μg aequorin cDNA and 20 μg of rat bradykinin B_2 receptor cDNA using electroporation (300 V, 500 μF using a Bio-Rad Gene Pulser), the cells were plated at 12,500 cells/well in Packard Viewplates and grown for 2 days. The cells were loaded with coelenterazine H and the luminescence measured as described above for the human bradykinin B_2 receptor.

2.5. Data analysis

The data were calculated as the mean and S.E.M. of at least three independent experiments. Agonist and antagonist concentration–response curves were fitted using a Logistic fit in Microcal Origin. Statistical comparisons were made using two-tailed unpaired Student's test; results were considered significant if $P < 0.05$.

2.6. Materials

Bradykinin, des-Arg^9-bradykinin, [Leu^8]-des-Arg^9-bradykinin and kallidin were obtained from Bachem (UK). Des-Arg^{10}-kallidin, HOE140 (D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]-bradykinin) and des-Arg^{10}-HOE140 were obtained from Peninsula Laboratories Europe. T-kinin and Sar-[D-Phe^8]-des-Arg^9-bradykinin were obtained from Novabiochem. B9858 (Lys-Lys-[Hyp^3,Igl^5,D-Igl^7,Oic^8]-des-Arg^9-bradykinin; Stewart et al., 1996) was custom synthesised by Phoenix Pharmaceuticals, and des-Arg^{11}-T-kinin was custom synthesised by Peptide and Protein Research (University of Exeter, Exeter, UK). The bradykinin B_2 receptor antagonist FR173657 ((*E*)-3-(6-acetamido-3-pyridyl)-*N*-[*N*-(2,4-dichloro-3-[(2-methyl-8-quinolinyl)-oxymethyl]-phenyl]-*N*-methylaminacarbonylmethyl]-acrylamide; Asano et al., 1997) was kindly synthesised by Dr. David Xu at Novartis, Summit, USA. [^{45}Ca]calcium chloride (specific activity 0.185–1.85 MBq/mg calcium) was from Amersham Pharmacia Biotech. The collagenase was from Worthington Biochemical (Lakewood, NJ, USA), elastase was from Roche Diagnostics. The anti-α-actin was from Sigma-Aldrich and fluorescein-linked anti-mouse Ig was from Amesham Pharmacia Biotech. Citifluor mounting medium was from Citifluor (City University, London, UK). Microscint-40 was from Packard Bioscience. Fura-2/AM was from Molecular Probes. All tissue culture media and supplies were from Life Technologies, all other reagents were from Sigma-Aldrich or BDH.

3. Results

As both B_1 and B_2 bradykinin receptors have been reported to be linked to phosphoinositidase C and increased $[Ca^{2+}]_i$ in other smooth muscle cells (Levesque et al., 1993; Tropea et al., 1993; Mathis et al., 1996), changes in Ca^{2+} homeostasis were used a measure of receptor activation in the isolated bladder smooth muscle cells. Calcium imaging experiments showed that bladder smooth muscle cells responded to bradykinin B_1 and B_2 receptor agonists with a rise in $[Ca^{2+}]_i$ (Fig. 1). Out of 15 cells exposed to bradykinin (100 nM), all 15 responded and des-Arg^9-bradykinin (300 nM) evoked an increase in $[Ca^{2+}]_i$ in 14 out of 17 cells tested.

The cells responded to bradykinin stimulation with a rapid and transient increase in $[Ca^{2+}]_i$ (Fig. 1A), whereas the response to des-Arg^9-bradykinin was usually slower, more prolonged and was often oscillatory in nature (B,C). Individual cells were able to respond to both bradykinin and des-Arg^9-bradykinin (C,D). Out of 11 cells challenged with both agonists, all responded to bradykinin and 8 responded to des-Arg^9-bradykinin. This indicates that both receptors can be expressed in the same cell.

In order to characterise these responses more fully, the pharmacology was investigated using agonist-evoked $^{45}Ca^{2+}$ efflux. Bladder smooth muscle cells responded to both B_1 and B_2 bradykinin receptor agonists with an increase in $^{45}Ca^{2+}$ efflux (Fig. 2). The maximum responses evoked by the bradykinin B_2 receptor agonists bradykinin and kallidin (8.0 ± 1.6 ($n=6$)- and 8.4 ± 0.9 ($n=4$)-fold

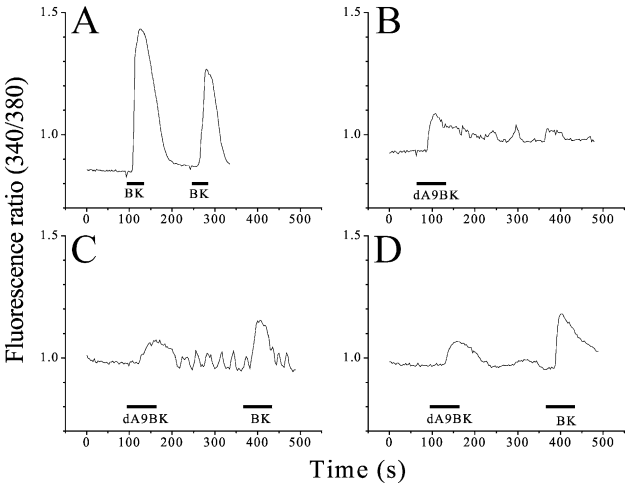

Fig. 1. Increases in $[Ca^{2+}]_i$ evoked by des-Arg^9-bradykinin and bradykinin in bladder smooth muscle cells. The data represent the ratio of the fluorescence emitted at 510 nm, following excitation at 340 and 380 nm, in individual bladder smooth muscle cells loaded with fura-2/AM. The cells were stimulated with 300 nM des-Arg^9-bradykinin (1 min) or 100 nM bradykinin (1 min). The bar indicates the presence of either des-Arg^9-bradykinin (dABK) or bradykinin (BK). Each trace represents the data from a single cell.

increases over basal, respectively) were significantly greater ($P<0.05$) than the maximum responses evoked by the bradykinin B_1 receptor agonists, 3.0 ± 0.2 ($n=10$)-fold increase over basal for des-Arg^9-bradykinin and 2.8 ± 0.5 ($n=5$)-fold increase over basal for des-Arg^{10}-kallidin. In addition, the bradykinin B_2 receptor agonists were more potent than the bradykinin B_1 receptor agonists. The EC_{50} values for bradykinin and kallidin were 2.7 ± 1.6 nM ($n=6$) and 3.2 ± 1.1 nM ($n=4$), respectively, and for des-Arg^9-bradykinin and des-Arg^{10}-kallidin, they were 37 ± 9 nM ($n=10$) and 39 ± 14 nM ($n=5$), respectively.

The pharmacology of the bradykinin receptors on bladder smooth muscle cells was characterised further by measuring the ability of HOE140 (a selective bradykinin B_2 receptor peptide antagonist), FR173657 (a non-peptide bradykinin B_2 receptor antagonist), des-Arg^{10}-HOE140 (a peptide bradykinin B_1 receptor antagonist), Lys-Lys-[Hyp^3,Igl^5,D-Igl^7,Oic^8]-des-Arg^9-bradykinin (B9858, a peptide bradykinin B_1 receptor antagonist) and [Leu^8]-des-Arg^9-bradykinin (a peptide bradykinin B_1 receptor antagonist) to inhibit the response to 3 nM bradykinin

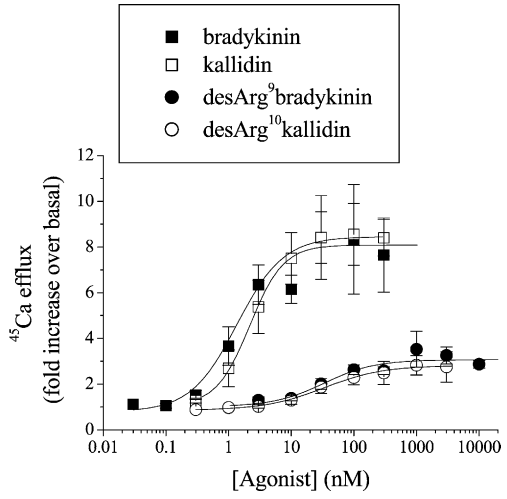

Fig. 2. Stimulation of $^{45}Ca^{2+}$ efflux in bladder smooth muscle cells by bradykinin, kallidin, des-Arg^9-bradykinin and des-Arg^{10}-kallidin. The data represent the increase in $^{45}Ca^{2+}$ efflux, expressed as fold increase over basal, evoked by the concentrations of the agonists shown and are the means and S.E.M. of 4–10 independent experiments. Des-Arg^9-bradykinin and des-Arg^{10}-kallidin were applied in the presence of 30 nM HOE140 to block activation of bradykinin B_2 receptors.

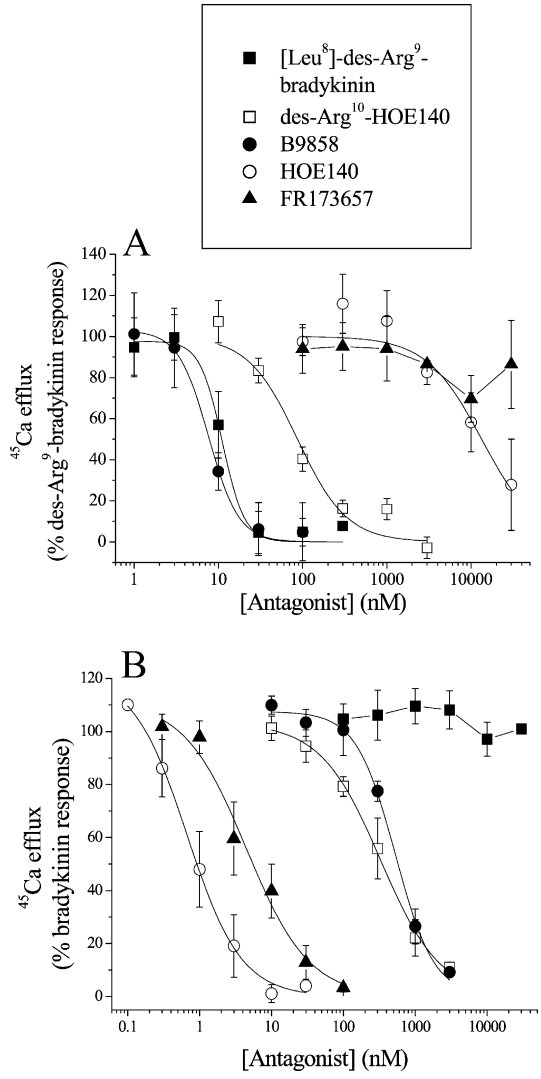

Fig. 3. Inhibition of des-Arg^9-bradykinin and bradykinin-evoked $^{45}Ca^{2+}$ efflux by a range of bradykinin receptor antagonists. The data represent the $^{45}Ca^{2+}$ efflux evoked by (A) 40 nM des-Arg^9-bradykinin or (B) 3 nM bradykinin in the presence of antagonists. The results are calculated as a percentage of the control response to 40 nM des-Arg^9-bradykinin or 3 nM bradykinin and represent the means and S.E.M. of three to seven independent experiments.

Table 1
Inhibition of bradykinin and des-Arg^9-bradykinin-induced $^{45}Ca^{2+}$ efflux in bladder smooth muscle cells

Compound	IC_{50} (nM) vs. bradykinin	IC_{50} (nM) vs. des-Arg^9-bradykinin
HOE140	1.2 ± 0.6	16316 ± 7422
FR173657	7.0 ± 3.0	>30000
Des-Arg^{10}-HOE140	432 ± 180	59 ± 10
B9858	536 ± 81	8.1 ± 2.3
[Leu^8]-des-Arg^9-bradykinin	>30000	10 ± 2

The data represent the concentration required to inhibit the bradykinin (3 nM) or des-Arg^9-bradykinin (40 nM)-induced $^{45}Ca^{2+}$ efflux by 50% and are the means and S.E.M. of three to seven independent experiments.

and 40 nM des-Arg^9-bradykinin, concentrations close to their respective EC_{50} values. The rank order of potency against 3 nM bradykinin was HOE140>FR173657>des-Arg^{10}-HOE140 = B9858 ≫ [Leu^8]-des-Arg^9-bradykinin, which is consistent with the known pharmacology of the bradykinin B_2 receptor (see Fig. 3). In contrast, the rank order of potency against 40 nM des-Arg^9-bradykinin was B9858=[Leu^8]-des-Arg^9-bradykinin>des-Arg^{10}-HOE140> HOE140>FR173657. The IC_{50} values for the antagonists tested are given in Table 1. These profiles are consistent with the bladder smooth muscle cells expressing both B_1 and B_2 bradykinin receptors.

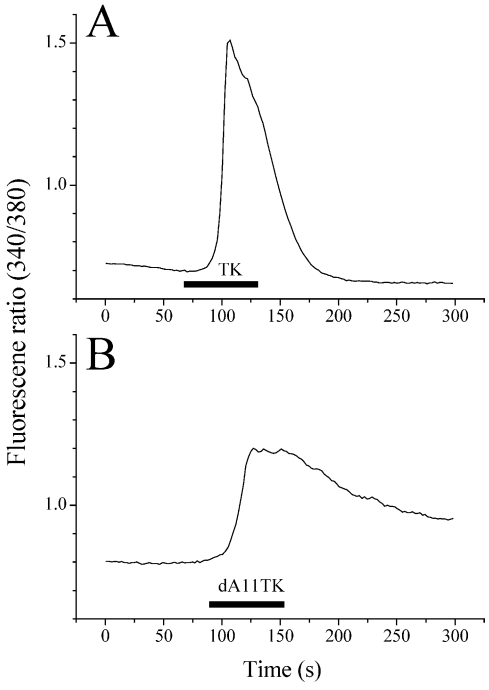

Fig. 4. Increases in $[Ca^{2+}]_i$ evoked by T-kinin and des-Arg^{11}-T-kinin in bladder smooth muscle cells. The data represent the ratio of the fluorescence emitted at 510 nm, following excitation at 340 and 380 nm, in individual bladder smooth muscle cells loaded with fura-2/AM. The cells were stimulated with 30 nM T-kinin or 300 nM des-Arg^{11}-T-kinin for 1 min. The bar indicates the presence of either T-kinin (TK) or des-Arg^{11}-T-kinin (dA11TK). Each trace represents the data from a single cell.

Having demonstrated that both B_1 and B_2 bradykinin receptors were expressed on bladder smooth muscle cells, the ability of T-kinin ligands to evoke changes in $[Ca^{2+}]_i$ was investigated using calcium imaging. These experiments

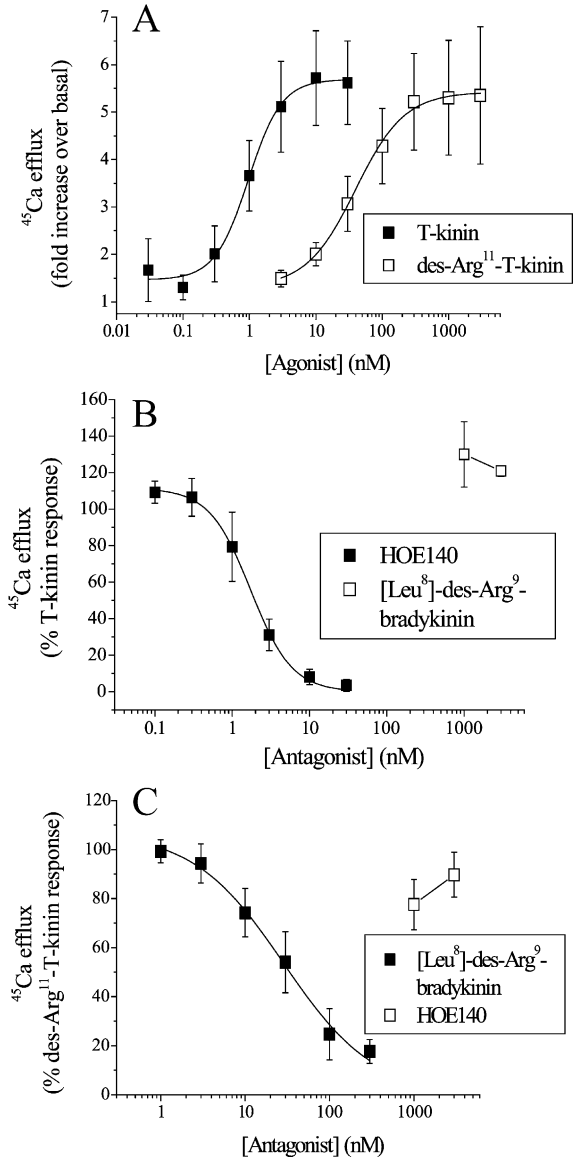

Fig. 5. Pharmacology of T-kinin derived ligands in bladder smooth muscle cells. (A) Stimulation of $^{45}Ca^{2+}$ efflux in bladder smooth muscle cells by T-kinin and des-Arg^{11}-T-kinin. The data represent the increase in $^{45}Ca^{2+}$ efflux, expressed as fold increase above basal, evoked by the concentrations shown of T-kinin and des-Arg^{11}-T-kinin, in the presence of 30 nM HOE140 to block activation of bradykinin B_2 receptors, and are the means and S.E.M. of four independent experiments. (B) Inhibition of T-kinin-evoked $^{45}Ca^{2+}$ efflux by HOE140 and [Leu^8]-des-Arg^9-bradykinin. The data represent the $^{45}Ca^{2+}$ efflux evoked by 1 nM T-kinin in the presence of the antagonists as a percentage of the control response to 1 nM T-kinin and are the means and S.E.M. of three independent experiments. (C) Inhibition of des-Arg^{11}-T-kinin-evoked $^{45}Ca^{2+}$ efflux by HOE140 and [Leu^8]-des-Arg^9-bradykinin. The data represent the $^{45}Ca^{2+}$ efflux evoked by 40 nM des-Arg^{11}-T-kinin in the presence of the antagonists as a percentage of the control response to 40 nM des-Arg^{11}-T-kinin and are the means and S.E.M. of five independent experiments.

showed that both T-kinin and des-Arg^{11}-T-kinin could evoke an increase in $[Ca^{2+}]_i$ in bladder smooth muscle cells (Fig. 4). Out of 19 cells tested, 10 responded to T-kinin and 7 out of 21 cells responded to des-Arg^{11}-T-kinin. The pattern of responses seen with T-kinin and des-Arg^{11}-T-kinin was similar to that obtained with bradykinin and des-Arg^{9}-bradykinin, with T-kinin evoking a rapid and transient increase in $[Ca^{2+}]_i$, (Fig. 4a) and des-Arg^{11}-T-kinin evoking a more prolonged response (Fig. 4b); however, no oscillatory responses to des-Arg^{11}-T-kinin were observed.

The pharmacology of the T-kinin peptides was characterised further using $^{45}Ca^{2+}$ efflux (Fig. 5A). T-kinin caused an increase in $^{45}Ca^{2+}$ efflux with an EC_{50} value of 1.1 ± 0.3 nM (n = 4) (Fig. 5A) and the maximum response (5.7 ± 0.9-fold increase over basal) was not significantly different (P>0.1) from the maximum response to bradykinin. Des-Arg^{11}-T-kinin also evoked $^{45}Ca^{2+}$ efflux in bladder smooth muscle cells with an EC_{50} value of 40 ± 8 nM (n = 4). The response to des-Arg^{11}-T-kinin (5.4 ± 1.0-fold increase over the basal level) was significantly greater (P < 0.05) than the response to either des-Arg^{9}-bradykinin or des-Arg^{10}-kallidin and in fact was not significantly (P>0.05) different from the responses evoked by the bradykinin B_2 receptor agonists bradykinin, kallidin and T-kinin itself.

The pharmacology of the responses evoked by T-kinin and des-Arg^{11}-T-kinin was investigated using a selection of the bradykinin B_1 and B_2 receptor antagonists used previously. The $^{45}Ca^{2+}$ efflux induced by 1 nM T-kinin was inhibited by HOE140 with an IC_{50} value of 1.7 ± 0.5 nM (n = 3) but was unaffected by high concentrations of [Leu^{8}]-des-Arg^{9}-bradykinin (up to 3 μM) (Fig. 5B). The response to 40 nM des-Arg^{11}-T-kinin was inhibited by [Leu^{8}]-des-Arg^{9}-bradykinin with an IC_{50} value of 38 ± 13 nM (n = 4) but not by high concentrations of HOE140 (up to 3 μM) (Fig. 5C). These data confirm that in bladder smooth muscle cells, T-kinin is a selective activator of the bradykinin B_2 receptor whereas des-Arg^{11}-T-kinin selectively activates bradykinin B_1 receptors.

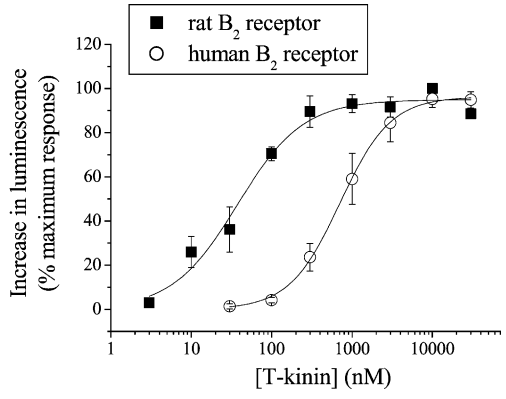

Fig. 6. Increases in $[Ca^{2+}]_i$ evoked by T-kinin in at the human and rat bradykinin B_2 receptors. The data represent the increase in luminescence evoked by the concentrations shown of T-kinin in CHO cells expressing the human bradykinin B_2 receptor plus aequorin or Cos-7 cells expressing the rat bradykinin B_2 receptor plus aequorin. The data are expressed as a percentage of the maximum response obtained and are the mean and S.E.M. of three (human) or four (rat) independent experiments.

We have demonstrated previously that des-Arg^{11}-T-kinin is more potent at the rat than the human bradykinin B_1 receptor using radioligand binding (Jones et al., 1999). To investigate whether T-kinin was more selective for the rat bradykinin B_2 receptor than the human, its ability to increase $[Ca^{2+}]_i$ in cells transfected with either the rat or human bradykinin B_2 receptor and the Ca^{2+}-sensitive protein aequorin was measured (Fig. 6). In Cos-7 cells transfected with the rat bradykinin B_2 receptor plus aequorin, the EC_{50} value for T-kinin-induced luminescence was 42 ± 10 nM (n = 4). In contrast, in CHO cells expressing the human bradykinin B_2 receptor and aequorin, the EC_{50} value was 741 ± 81 nM (n = 3), indicating a preference of this kinin for the rat bradykinin B_2 receptor.

4. Discussion

The T-kinin system is a poorly characterised part of the kinin system in the rat. T-kinin is produced from T-kininogen by the action of the T-kininogenase enzyme. Unlike high and low molecular weight kininogens, which are the precursors of bradykinin and kallidin, the levels of T-kininogen are increased in inflammatory conditions. For example, it has been shown that T-kininogen was the most abundant kininogen in rat plasma following induction of adjuvant arthritis (Barlas et al., 1985a) and the expression of T-kininogen was upregulated by inflammatory mediators such as cytokines (Takano et al., 1995; Yayama et al., 2000). Like all kinins, T-kinin is subject to degradation by a variety of protease enzymes. The two major pathways of kinin degradation are via angiotensin-converting enzyme (ACE), which hydrolyses the Pro−Phe bond to generate inactive fragments, and via carboxypeptidase, which generates selective bradykinin B_1 receptor agonists. In vitro studies showed that T-kinin is susceptible to carboxypeptidase but resistant to ACE (Passaglio and Vieira, 1996). The major product of T-kinin degradation by carboxypeptidase would be des-Arg^{11}-T-kinin. In addition, degradation of T-kinin by aminopeptidases to bradykinin may also occur (Vieira et al., 1994).

In view of the potential importance of the T-kinins as activators of the rat bradykinin receptors, we decided to investigate their pharmacology. As rat bladder has been reported to express both B_1 and B_2 bradykinin receptors (Lecci et al., 1995; Belichard et al., 1999), the possibility that bladder smooth muscle cells would prove to be a useful cellular model for bradykinin receptors was explored.

Before studying the responses to T-kinin and des-Arg^{11}-T-kinin, it was necessary to confirm that cultured bladder smooth muscle cells expressed both B_1 and B_2 bradykinin receptors and that the pharmacological profile of well-studied bradykinin receptor agonists was consistent with

that previously reported for rat bradykinin receptors. Calcium imaging experiments demonstrated that bradykinin and des-Arg^9-bradykinin evoked increases in $[Ca^{2+}]_i$ in bladder smooth muscle cells. The results with bradykinin and des-Arg^9-bradykinin showed that a high proportion of the cells responded to each agonist (100% and 82%, respectively) and that B_1 and B_2 bradykinin receptors could be, but were not always, expressed on the same cell. Cells were either responsive to bradykinin alone, or bradykinin and des-Arg^9-bradykinin. None were found to respond to des-Arg^9-bradykinin alone. The responses to des-Arg^9-bradykinin were generally longer in duration than the bradykinin-evoked responses and sometimes oscillatory in nature. This pattern is similar to that reported by Mathis et al. (1996) for rabbit mesenteric artery smooth muscle cells and Smith et al. (1995) for bovine aortic endothelial cells.

The pharmacology of the responses to the bradykinin B_1 receptor agonists des-Arg^9-bradykinin and des-Arg^{10}-kallidin and the bradykinin B_2 receptor agonists bradykinin and kallidin in bladder smooth muscle cells was comparable to that reported for these agonists in other rat cells and tissues. For example, the EC_{50} values for bradykinin (2.9 nM)- and kallidin (3.2 nM)-evoked $^{45}Ca^{2+}$ efflux were similar to the values reported for bradykinin-evoked Ins(1,4,5)P_3 formation in arterial smooth muscle cells (5 nM) (Dixon et al., 1994), and cultured dorsal root ganglion cells (7.6 nM) (Harvey and Burgess, 1996). The EC_{50} values for the bradykinin B_1 receptor agonists des-Arg^9-bradykinin (37 nM) and des-Arg^{10}-kallidin (39 nM) were similar to those recently reported in rat aortic smooth muscle cells, 24 and 16 nM, respectively (Schaeffer et al., 2001). They are also within the same range as the values reported for des-Arg^9-bradykinin-evoked contractions in rat smooth muscle preparations. For example, the pD_2 value for des-Arg^9-bradykinin-induced contraction in the ileum was 8.3 (Meini et al., 1996), in portal vein rings, the EC_{50} value was 46 nM (Campos and Calixto, 1994) and in the bladder, the EC_{50} value was 58 nM (Meini et al., 1998).

Having demonstrated that bladder smooth muscle cells expressed both B_1 and B_2 bradykinin receptors, the responses evoked by T-kinin and des-Arg^{11}-T-kinin were investigated. Both T-kinin and des-Arg^{11}-T-kinin were capable of increasing $[Ca^{2+}]_i$ and the pattern of responses was similar to that for bradykinin and des-Arg^9-bradykinin, with the bradykinin B_1 receptor agonist giving a more prolonged response than the bradykinin B_2 receptor agonist. Although a lower proportion of the cells appeared to respond to T-kinin and des-Arg^{11}-T-kinin (53% and 33%, respectively) than to bradykinin and des-Arg^9-bradykinin, it would be necessary to study a greater number of cells to determine whether this was a statistically significant difference.

The potency of T-kinin (EC_{50} value of 1.1 nM) was very similar to that of bradykinin (2.9 nM) and kallidin (3.2 nM), and the response was inhibited by HOE140 and not by [Leu^8]-des-Arg^9-bradykinin. This is a similar profile of activity to that reported previously for T-kinin (Champion et al., 1997). As T-kinin is unique to the rat, it might be expected to have greater potency at the rat bradykinin B_2 receptor compared to the human bradykinin B_2 receptor. In fact, it was approximately 20-fold more potent in Cos-7 cells transfected with the rat bradykinin B_2 receptor than in CHO cells transfected with the human bradykinin B_2 receptor.

Des-Arg^{11}-T-kinin evoked $^{45}Ca^{2+}$ efflux with an EC_{50} value that was very close to that found for the other bradykinin B_1 receptor agonists in the rat bladder smooth muscle cells. The response to des-Arg^{11}-T-kinin was inhibited by [Leu^8]-des-Arg^9-bradykinin but not by HOE140, confirming that it was activating bradykinin B_1 receptors. We demonstrated previously the rat selectivity of this peptide by showing that it is a more potent displacer of [^{3}H]des-Arg^{10}-kallidin binding at the rat bradykinin B_1 receptor than the human (Jones et al., 1999). In contrast to bradykinin and kallidin, which evoked greater responses than their bradykinin B_1 receptor-selective derivatives, there was no difference in the maximum responses evoked by T-kinin and des-Arg^{11}-T-kinin. The higher efficacy of des-Arg^{11}-T-kinin was not due to activation of the bradykinin B_2 receptor as the experiments were carried out in the presence of HOE140 to block activation of the bradykinin B_2 receptor. This suggests that des-Arg^{11}-T-kinin is a full agonist at the rat bradykinin B_1 receptor, whereas des-Arg^9-bradykinin and des-Arg^{10}-kallidin may be classified as partial agonists. This is surprising for des-Arg^9-bradykinin, which is formed in the rat, but perhaps not for des-Arg^{10}-kallidin as it is not an endogenous ligand in the rat.

In summary, we have demonstrated that cultured rat bladder smooth muscle cells express functionally active B_1 and B_2 bradykinin receptors and it will be possible to compare the expression and regulation of both subtypes of bradykinin receptor in the rat bladder cells. In addition, using this cellular model, the putative breakdown product of T-kinin, des-Arg^{11}-T-kinin, has been shown to be a potent and selective agonist at the rat bradykinin B_1 receptor. As the expression of T-kininogen, the precursor of T-kinin and des-Arg^{11}-T-kinin, is increased under inflammatory conditions in the rat, this suggests that des-Arg^{11}-T-kinin may have an important role as a mediator of inflammation in the rat.

References

Asano, M., Inamura, N., Hatori, C., Sawai, H., Fujiwara, T., Katayama, K., Kayakiri, H., Satoh, S., Abe, Y., Inoue, T., Sawada, Y., Nakahara, K., Oku, T., Okuhara, M., 1997. The identification of an orally active, non-peptide bradykinin B_2 receptor antagonist, FR173657. Br. J. Pharmacol. 120, 617–624.

Barlas, A., Okamoto, H., Greenbaum, L.M., 1985a. T-kininogen—the major plasma kininogen in rat adjuvant arthritis. Biochem. Biophys. Res. Commun. 129, 280–286.

Barlas, A., Sugio, K., Greenbaum, L.M., 1985b. Release of T-kinin and bradykinin in carrageenan-induced inflammation in the rat. FEBS Lett. 190, 268–270.

Belichard, P., Luccarini, J.M., Defrêne, E., Faye, P., Franck, R.M., Duclos, H., Paquet, J.L., Pruneau, D., 1999. Pharmacological and molecular evidence for kinin B_1 receptor expression in urinary bladder of cyclophosphamide-treated rats. Br. J. Pharmacol. 128, 213–219.

Bhoola, K.D., Figuerao, C.D., Worthy, K., 1992. Bioregulation of kinins, kallikreins, kininogens and kininases. Pharmacol. Rev. 44, 1–79.

Campos, A.H., Calixto, J.B., 1994. Mechanisms involved in the contractile responses of kinins in rat portal vein rings: mediation by B_1 and B_2 receptors. J. Pharmacol. Exp. Ther. 268, 902–909.

Chamley-Campbell, J., Campbell, G.R., Ross, R., 1979. The smooth muscle cells in culture. Physiol. Rev. 59, 1–61.

Champion, H.C., Santiago, J.A., Czapla, M.A., Bivalacqua, T.J., Ilgenfritz, C., Kadowitz, P.J., 1997. Comparison of responses to T-kinin and bradykinin in the mesenteric vascular bed of the cat. Peptides 18, 1357–1364.

Dixon, B.S., Sharma, R.V., Dickerson, T., Fortune, J., 1994. Bradykinin and angiotensin II: activation of protein kinase C in arterial smooth muscle. Am. J. Physiol. 266, C1406–C1420.

Harvey, J.S., Burgess, G.M., 1996. Cyclic GMP regulates activation of phosphoinositidase C by bradykinin in sensory neurons. Biochem. J. 316, 539–544.

Jones, C., Phillips, E., Davis, C., Arbuckle, J., Yaqoob, M., Burgess, G.M., Docherty, R.J., Webb, M., Bevan, S., McIntyre, P., 1999. Molecular characterisation of cloned bradykinin B_1 receptors from rat and human. Eur. J. Pharmacol. 374, 423–433.

Lecci, A., Giuliani, S., Meini, S., Maggi, C.A., 1995. Pharmacological analysis of the local and reflex responses to bradykinin on rat urinary bladder motility in vivo. Br. J. Pharmacol. 114, 708–714.

Levesque, L., Drapeau, G., Grose, J.H., Rioux, F., Marceau, F., 1993. Vascular mode of action of kinin B_1 receptors and development of a cellular model for the investigation of these receptors. Br. J. Pharmacol. 109, 1254–1262.

Mathis, S.A., Criscimagna, N.L., Leeb-Lundberg, L.M.F., 1996. B_1 and B_2 kinin receptors mediate distinct patterns of intracellular Ca^{2+} signalling in single cultured vascular smooth muscle cells. Mol. Pharmacol. 50, 128–139.

Meini, S., Lecci, A., Maggi, C.A., 1996. The longitudinal muscle of rat ileum as a sensitive monoreceptor assay for bradykinin B_1 receptors. Br. J. Pharmacol. 117, 1619–1624.

Meini, S., Lecci, A., Cucchi, P., Catalioto, R.-M., Criscuoli, M., Maggi, C.A., 1998. Inflammation modifies the role of cyclooxygenases in the contractile responses of the rat detrusor smooth muscle to kinin agonists. J. Pharmacol. Exp. Ther. 287, 137–143.

Passaglio, K.T., Vieira, M.A.R., 1996. T-kinin is resistant to hydrolysis by angiotensin I-converting enzyme. Immunopharmacology 32, 166–168.

Schaeffer, P., Laplace, M.-C., Savi, P., Prabonnaud, V., Salel, V., Herbert, J.-M., 2001. Detection of bradykinin B_1 receptors in rat aortic smooth muscle cells. Biochem. Pharmacol. 61, 291–298.

Smith, J.A.M., Webb, C., Holford, J., Burgess, G.M., 1995. Signal transduction pathways for B_1 and B_2 receptors in bovine pulmonary artery endothelial cells. Mol. Pharmacol. 47, 525–534.

Stewart, J.M., Gera, L., Hanson, W., Zuzack, J.S., Burkard, M., McCullough, R., Whalley, E.T., 1996. A new generation of bradykinin antagonists. Immunopharmacology 33, 51–60.

Takano, M., Yokoyama, K., Yayama, K., Okamoto, H., 1995. Rat fibroblasts synthesise T-kininogen in response to cyclic-AMP, prostaglandin E_2 and cytokines. Biochim. Biophys. Acta 1268, 107–114.

Tropea, M.M., Gummelt, D., Herzig, M.S., Leeb-Lundberg, L.M.F., 1993. B_1 and B_2 kinin receptors on cultured rabbit superior mesenteric artery smooth muscle cells: receptor-specific stimulation of inositol phosphate formation and arachidonic acid release by des-Arg^9-bradykinin and bradykinin. J. Pharmacol. Exp. Ther. 264, 930–937.

Vieira, M.A.R., Moreira, M.F., Maack, T., Guimaraes, J.A., 1994. Conversion of T-kinin to bradykinin by rat kidney. Biochem. Pharmacol. 47, 1693–1699.

Yamawaki, I., Tamaoki, J., Takeda, Y., Chiyotani, A., Sakai, N., Kameyama, S., Konno, K., 1995. Effect of T-kinin on microvascular permeability and its modulation by peptidases in rat airways. J. Appl. Physiol. 79, 1129–1133.

Yayama, K., Nagaoka, M., Takano, M., Okamoto, H., 2000. Expression of kininogen, kallikrein and kinin receptor genes by rat cardiomyocytes. Biochim. Biophys. Acta 1495, 69–77.