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B_1 bradykinin receptors and sensory neurones

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1 The location of the B_1 bradykinin receptors involved in inflammatory hyperalgesia was investigated.

2 No specific binding of the B_1 bradykinin receptor ligand [^{3}H]-des-Arg^{10}-kallidin was detected in primary cultures of rat dorsal root ganglion neurones, even after treatment with interleukin-1β (100 iu ml^{-1}).

3 In dorsal root ganglion neurones, activation of B_2 bradykinin receptors stimulated polyphosphoinositidase C. In contrast, B_1 bradykinin receptor agonists (des-Arg^9-bradykinin up to 10 μM and des-Arg^{10}- kallidin up to 1 μM) failed to activate polyphosphoinositidase C, even in neurones that had been treated with interleukin-1β (100 iu ml^{-1}), prostaglandin E_2 (1 μM) or prostaglandin I_2 (1 μM).

4 Dorsal root ganglion neurones removed from rats (both neonatal and 14 days old) that had been pretreated with inflammatory mediators (Freund's complete adjuvant, or carrageenan) failed to respond to B_1 bradykinin receptor selective agonists (des-Arg^9-bradykinin up to 10 μM and des-Arg^{10}-kallidin up to 1 μM).

5 Bradykinin (25 nM to 300 nM) evoked ventral root responses when applied to peripheral receptive fields or central terminals of primary afferents in the neonatal rat spinal cord and tail preparation. In contrast, des-Arg^9-bradykinin (50 nM to 500 nM) failed to evoke ventral root depolarizations in either control rats or in animals that developed inflammation following ultraviolet irradiation of the tail skin.

6 The results of the present study imply that the B_1 bradykinin receptors that contribute to hypersensitivity in models of persistent inflammatory hyperalgesia are located on cells other than sensory neurones where they may be responsible for releasing mediators that sensitize or activate the nociceptors.

Keywords: Bradykinin; des-Arg^9-bradykinin; B_1 bradykinin receptors; sensory neurones; hyperalgesia; polyphosphoinositidase C; interleukin-1β

Introduction

Bradykinin and kallidin play an important part in mediating pain and inflammation (Proud & Kaplan, 1988; Dray & Perkins, 1993). They are broken down by proteolytic enzymes to form a variety of metabolites including the active fragments, des-Arg^9-bradykinin and des-Arg^{10}-kallidin (Regoli & Barabe, 1980; Regoli *et al.*, 1986). Two classes of bradykinin receptor have been cloned: B_2 bradykinin receptors (Hess *et al.*, 1992), which are activated selectively by bradykinin and kallidin (Regoli *et al.*, 1992), and B_1 bradykinin receptors (Menke *et al.*, 1994) which are activated selectively by des-Arg^9-bradykinin and des-Arg^{10}-kallidin (Regoli *et al.*, 1992).

B_2 bradykinin receptors have been localized to sensory neurones (Steranka *et al.*, 1988; Nagy *et al.*, 1993) and their activation leads to neuronal excitation (Dray *et al.*, 1992) and the generation of pain (see Dray & Perkins, 1993 for a review). Recent studies have indicated that B_1 bradykinin receptors may also have a role in generating and maintaining hyperalgesia in conditions that involve long-term inflammation (Farmer *et al.*, 1991; Perkins *et al.*, 1993; Perkins & Kelly, 1993; Davis & Perkins, 1994). For example, in two models: persistent thermal hyperalgesia induced by ultra-violet (u.v.) irradiation and mechanical hyperalgesia that accompanies the prolonged inflammation induced by injection of Freund's complete adjuvant (FCA) into the rat knee joint, systemic administration of the B_1 bradykinin receptor antagonist des-Arg^9,Leu^8-bradykinin can prevent and reverse hyperalgesia (Perkins *et al.*, 1993). In contrast, the B_2 bradykinin receptor antagonist D-Arg[Hyp^3,Thi^5,D-Tic^7,Oic^8]-bradykinin (Hoe140) (Hock *et al.*, 1991) is relatively inactive in these models. Furthermore, it has been shown that following either an inflammatory insult, or a local injection of interleukin-1 β (IL-1β), which upregulates B_1 bradykinin receptor expression in smooth muscle (Regoli *et al.*, 1978; Deblois *et al.*, 1988; 1989; 1991), the administration of B_1 bradykinin receptor agonists evokes hyperalgesia (Davis & Perkins, 1994), an effect not seen in non-inflamed tissue.

Current knowledge of the distribution of B_1 bradykinin receptors is poor and the cellular location of the B_1 bradykinin receptors that mediate the hyperalgesia observed in the models described above is not clear. Studies of acute nociceptor activation have shown no involvement of B_1 bradykinin receptors (Haley *et al.*, 1989; Dray *et al.*, 1992), although they could be synthesized on sensory neurones *de novo* following an inflammatory insult. Another possibility is that B_1 bradykinin receptors are induced on other cells, such as endothelial cells, fibroblasts or invading macrophages where they could be responsible for releasing mediators that sensitize or activate the nociceptors.

The aim of this study was to determine whether B_1 bradykinin receptors are present on rat sensory neurones. Two approaches have been used to address this question. Firstly, the presence of specific binding sites for the B_1 bradykinin receptor-selective radioligand [^{3}H]-des-Arg^{10}-kallidin on cultured dorsal root ganglion (DRG) neurones has been investigated along with the ability of B_1 bradykinin receptor agonists to cause second messenger activation in these cells. Secondly, the ability of B_1 bradykinin receptor agonists to activate primary afferents and spinal neurones was studied in a neonatal rat spinal cord and tail preparation (Dray *et al.*, 1990).

Methods

Culture of dorsal root ganglion cells

Unless otherwise specified, DRG were removed from all spinal levels of neonatal rats and neuronal cells cultured as described previously (Burgess *et al.*, 1989). Following dissociation, the

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cells were plated onto poly-ornithine coated petri dishes to facilitate separation of neuronal and non-neuronal cells. The non-neuronal cells attached firmly to the petri dishes and the neurones could be washed off. The neurones were then plated onto poly-ornithine and laminin coated coverslips (approximately 30,000 cells per coverslip) and grown in 50% Hams F-14 medium containing 14 mM $NaHCO_3$, 1 mM glutamine, 50 iu ml^{-1} penicillin, 50 $\mu g\ ml^{-1}$ streptomycin and 4% Ultroser G and 50% conditioned medium from C6 glioma cells (CM) with 300 ng ml^{-1} nerve growth factor (growth medium). Cytosine arabinoside (10 μM) was included in the growth medium for the first 3 to 4 days and reduced the proportion of non-neuronal cells to about 10%. The neurones were maintained in culture for a further 4 days before use. In some experiments neonatal or 14 day old animals were injected i.p. with 50 μl of either carrageenan (2%), FCA or saline and the DRG removed one day later and kept in culture for one or two days as indicated.

Measurement of [^{3}H]-inositol trisphosphate

The DRG neurones were grown in Minimum Essential Medium with Earle's salts, without L-methionine or glutamine (MEM), containing [^{3}H]-myo-inositol (50μ Ci ml^{-1}) for 20 h at 37°C. Measurement of [^{3}H]-inositol phosphate formation was performed as described by Burgess *et al.* (1989). Briefly, the cells were dipped into solutions containing the drugs made up in 25 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES) buffered Dulbecco's Modified Eagle Medium (DMEM) at 37°C. Reactions were stopped in 4.5% perchloric acid, containing 1 mg ml^{-1} phytic acid at 4°C. Following neutralization, the extracted ^{3}H-inositol phosphates were separated by anion-exchange chromatography on Dowex columns (formate form) as described by Berridge (1983). [^{3}H]-inositol trisphosphate ([^{3}H]-IP_3) was eluted from the columns with 0.8 M ammonium formate in 0.1 M formic acid and expressed as a percentage of cellular [^{3}H]-phosphoinositides, the majority of which (>90%) is [^{3}H]-phosphatidylinositol ([^{3}H]-PI) (Burgess *et al.*, 1989). The amount of radioactivity in the lipid fraction did not alter during the time-course of the experiments to be described. IL-1β and prostaglandins were added to the cultures at the times and concentrations indicated. For exposures of longer than 24 h, IL-1β was added daily. In experiments in which the DRG were removed from neonatal animals that had been pretreated (i.p.) with either 50 μl of FCA or 50 μl of 2% carrageenan, the neurones were plated in 50 μl growth medium, to which 200 μl of 50% MEM, 50% CM containing 300 ng ml^{-1} nerve growth factor and 80 μ Ci ml^{-1} [^{3}H]-myo-inositol was added 1 h later. The cells were cultured for a further 20 h. In experiments in which the DRG were removed from 14 day old animals the cells were grown for 1 day in growth medium in the presence or absence of IL-1β. The medium was then changed to MEM containing 300 ng ml^{-1} nerve growth factor plus 50 μ Ci ml^{-1} [^{3}H]-inositol with or without IL-1β and the cells incubated overnight.

Binding experiments

The DRG neurones were washed by dipping the coverslips in 20 ml of 25 mM HEPES-buffered DMEM (pH 7.5) at 37°C for 20 s, then incubated with either 1 nM [^{3}H]-bradykinin (specific activity 89 Ci $mmol^{-1}$) made up in 25 mM K_2HPO_4, pH 6.8, 240 mM sucrose, 0.2% bovine serum albumin (BSA), 1 μM captopril and 1 μM Plummers reagent (final volume 500 μl), or (1–5 nM) [^{3}H]-des-Arg^{10}-kallidin (specific activity 110 Ci $mmol^{-1}$) made up in 10 mM tris[hydroxymethyl]methyl-2-aminoethane sulphonic acid (TES) pH 7.4, 300 mM glucosamine, 0.1% BSA, 0.14 g l^{-1} bacitracin and 0.2 g l^{-1} phenanthroline (final volume 500 μl) in 24 well plates that had been pre-coated with poly-ornithine. Non-specific binding was determined in the presence of 1 μM bradykinin or 1 μM des-Arg^{10}-kallidin respectively. The cells were incubated in [^{3}H]-bradykinin for 90 min at 4°C and then washed once in assay buffer plus 0.2% BSA for 45 s followed by two 20 s washes in assay buffer without BSA. To measure [^{3}H]-des-Arg^{10}-kallidin binding the cells were incubated for 60 min at 4°C and then washed once for 45 s and twice for 20 s in 50 mM Tris[hydroxymethyl]aminomethane (Tris), 300 mM sucrose pH 7.4. The cells were then solubilized in 200 μl of 1 M NaOH and an aliquot (25 μl) removed for protein determination (Lowry *et al.*, 1951). The radioactivity in the remainder of the sample was determined by liquid scintillation counting. A number of experiments were also performed on WI38 human fibroblasts which express B_1 bradykinin receptors constitutively (Webb *et al.*, 1994). They were treated in the same way as the DRG neurones except that they were grown on petri dishes.

Neonatal spinal cord tail preparation

Sprague-Dawley rats (0–2 days old) were decapitated and the spinal cords exposed by laminectomy and dissected with the pelvic bone and the tail attached (Dray *et al.*, 1990). After removal of the tail skin, the preparation was placed in a recording chamber that allowed the spinal cord and tail to be superfused separately (2.5 ml min^{-1}) with an aerated (95% O_2 and 5% CO_2) physiological salt solution (composition in mM: NaCl 138.6, KCl 3.35, $CaCl_2$ 1.26, $MgCl_2$ 1.16, $NaHCO_3$ 21.0, $NaHPO_4$ 0.58, glucose 10.0) at 24°C. Agonists were superfused for 10 s to either the tail or cord with a 20 min interval between each application. Activation of spinal neurones was measured by recording ventral root (VR,L4) depolarization with an extracellular glass electrode. Signals were amplified (Grass DC amplifier, Model P16) and displayed on a Graphtec WR3107 chart-recorder. In a separate group of 1 day old animals ($n=3$), the base of the tail was irradiated by an u.v. source (maximum intensity of 365 nm, 69 mW cm^{-2}) for 90 s, 18 h prior to the preparation of the spinal cord and tail as described above.

Data analysis

Results were compared by use of paired or unpaired one-tailed Student's t test. Differences were considered to be significant if $P<0.05$.

Drugs and chemicals

D-Arg[Hyp^3,Thi^5,D-Tic^7,Oic^8]-bradykinin (Hoe140) was synthesized at the Sandoz Institute for Medical Research, London, by Dr A. Hallet. The following materials were obtained from the sources indicated: bradykinin, des-Arg^9-bradykinin, des-Arg^9,Leu^8-bradykinin, des-Arg^{10}-kallidin (Bachem A.G., Switzerland); penicillin, streptomycin, foetal calf serum (Myoclone Plus), Ultroser G, glutamine, DMEM and MEM (Gibco BRL, Life Technologies Ltd., Paisley, U.K.); nerve growth factor (Promega, Southampton, Hants U.K.); myo-2-[^{3}H]-inositol (17.1 Ci $mmol^{-1}$) (Amersham International, Bucks U.K.), [^{3}H]-bradykinin (89 Ci $mmol^{-1}$) (Zeneca, Cambridge Research Biochemicals, Northwich U.K.); [^{3}H]-des-Arg^{10}-kallidin (110 Ci $mmol^{-1}$) (Du Pont NEN, Stevenage U.K.); human recombinant IL-1β (The National Institute for Biological Standards and Control, U.K.); bacitracin, prostaglandin E_2, captopril, cytosine arabinoside, 1,10-phenanthroline, FCA (Sigma Chemical Co. Poole, U.K.); Plummer's inhibitor (Calbiochem, Nottingham U.K.). All other chemicals and reagents were obtained from BDH (Dagenham, U.K.).

Results

Binding studies with [^{3}H]-des-Arg^{10}-kallidin

In order to determine whether there were any specific binding sites for B_1 bradykinin receptor agonists on cultured DRG neurones, the cells were incubated with the B_1 bradykinin receptor-selective radioligand [^{3}H]-des-Arg^{10}-kallidin (see Menke

et al., 1994; Galizzi *et al.*, 1994) using des-Arg^{10}-kallidin (1 μM) to determine non-specific binding. No specific binding of [^{3}H]-des-Arg^{10}-kallidin (2 nM) was detected in the DRG neurones, even if the cells had been incubated with IL-1β (100 iu ml^{-1}) for 2 h (Table 1). Longer incubations with IL-1β (up to 4 h) were also ineffective (data not shown). In some experiments, the concentration of [^{3}H]-des-Arg^{10}-kallidin was increased to 5 nM, but no specific B_1 bradykinin receptor binding was detected at this higher concentration of radioligand (data not shown). In parallel experiments specific binding of the B_2 bradykinin receptor-selective radioligand [^{3}H]-bradykinin was detected in the cultured DRG neurones (Table 1). WI38 fibroblasts, which express B_1 bradykinin receptors constitutively (Webb *et al.*, 1994) were used as a positive control and, under conditions similar to those used for the DRG neurones, it was possible to detect specific binding of [^{3}H]-des-Arg^{10}-kallidin (1 nM) to these fibroblasts (Table 1). The specific binding in the WI38 cells increased ($P=0.018$, paired Student's t test) from 3510±993 d.p.m. mg^{-1} of protein ($n=4$) to 8523±2063 d.p.m. mg^{-1} of protein ($n=4$) following a 2 h incubation with 100 iu ml^{-1} IL-1β. This increase in the specific binding in the fibroblasts peaked between 2 h and 4 h and remained significantly above the basal value for up to 10 h (data not shown).

Effect of B_1 bradykinin receptor agonists on polyphosphoinositidase C activity in primary cultures of DRG neurones

Bradykinin activates polyphosphoinositidase C (PIC) in DRG neurones (Thayer *et al.*, 1988; Burgess *et al.*, 1989). Figure 1 shows that the increase in [^{3}H]-IP$_3$ induced by bradykinin (30 nM) could be blocked by 100 nM Hoe140, but not by the B_1 bradykinin receptor-selective antagonist des-Arg^9,Leu^8-bradykinin (10 μM).

The neurones did not respond to 10 μM des-Arg^9-bradykinin (Figure 1) and the more potent B_1 bradykinin receptor agonist, des-Arg^{10}-kallidin, applied at 1 μM, also failed to evoke a rise in [^{3}H]-IP$_3$ (data not shown). Incubation of the neurones with IL-1β (100 iu ml^{-1}) for either 2 h or 20 h did not induce B_1 bradykinin receptor responsiveness, nor did it alter the response to bradykinin (Figure 2). A 7 day treatment with IL-1β was also ineffective (data not shown).

The possibility that prostaglandins might regulate the sensitivity of DRG neurones to B_1 bradykinin receptor agonists was investigated. Pretreatment of the cultures with prostaglandin E_2 (PGE_2) (1 μM) for periods from 30 min up to 20 h did not cause the neurones to respond to des-Arg^9-bradykinin (basal [^{3}H]-IP$_3$, 0.17±0.065% [^{3}H]-PI ($n=3$); after a 20 s exposure to 1 μM des-Arg^9-bradykinin, 0.14±0.04% [^{3}H]-PI ($n=3$); after a 20 s exposure to 1 μM des-Arg^9-bradykinin following a 30 min treatment with PGE_2, 0.19±0.06% [^{3}H]-PI ($n=3$), after a 20 s exposure to 1 μM des-Arg^9-bradykinin following a 20 h treatment with PGE_2 0.18±0.06% [^{3}H]-PI ($n=3$)). Similar results were obtained when neurones were pretreated for either 30 min or 20 h with 1 μM PGI_2 (data not shown).

Effect of B_1 bradykinin receptor agonists on polyphosphoinositidase C in DRG neurones from animals treated with inflammatory mediators

The possibility that B_1 bradykinin receptors could be induced on sensory neurones innervating inflamed tissue was investigated by pretreating neonatal rats with 50 μl of either FCA or 2% carrageenan (i.p.). In these experiments, the neurones were challenged with agonists within 22 h of removal of the DRG. Des-Arg^9-bradykinin did not activate PIC in the neurones from animals treated with either FCA or carrageenan, both of which have been shown to induce hyperalgesia in response to B_1 agonists (Perkins *et al.*, 1993). The level of [^{3}H]-IP$_3$ in neurones from naïve animals after a 20 s exposure to 10 μM des-Arg^9-bradykinin was 107.8±5.2% of the basal value ($n=3$); in neurones from FCA-treated animals after a 20 s exposure to 10 μM des-Arg^9-bradykinin it was 107.5± 33.2% of the basal value ($n=3$); in neurones from carrageenan-treated animals after a 20 s exposure to 10 μM des-Arg^9-bradykinin it was 122.9±27.6% of the basal value. Des-Arg^{10}-kallidin,

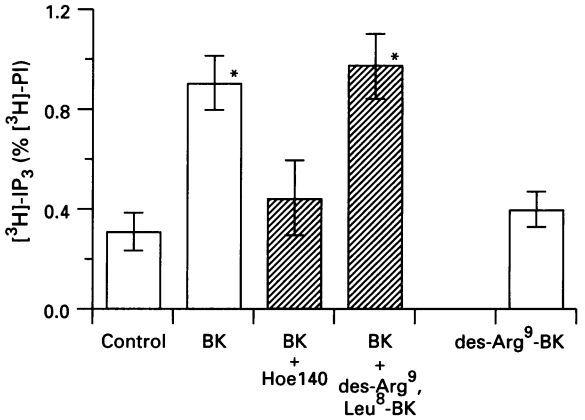

Figure 1 The B_1 bradykinin receptor agonist des-Arg^9- bradykinin failed to increase [^{3}H]-IP$_3$ formation in DRG neurones. Neonatal rat DRG neurones were challenged with either 30 nM bradykinin (BK) or 10 μM des-Arg^9- bradykinin (des-Arg^9-BK) for 20 s. The antagonists, Hoe140 (100 nM) and des-Arg^9,Leu^8-bradykinin (10 μM) were applied for 2 min before and during application of bradykinin. The level of [^{3}H]-IP$_3$ was expressed as a percentage of the [^{3}H]-PI in the sample and the data represent the mean±s.e.mean of 3 independent experiments. An asterisk indicates that the value is significantly above the basal value ($P<0.05$).

Table 1 Binding of [^{3}H]-des-Arg^{10}-kallidin and [^{3}H]-bradykinin to primary cultures of rat DRG neurones

	[^{3}H]-des-Arg^{10}-kallidin		*[^{3}H]-bradykinin*	
	Total binding	*Non-specific binding*	*Total binding*	*Non-specific binding*
DRG neurones (control)	2585±627	2384±807	21180±5178	2593±312
DRG neurones (IL-1β)	3126±1391	2450±876	–	–
WI38 fibroblasts (control)	9487±4814	6177±3381	–	–
WI38 fibroblasts (IL-1β)	15192±4335*	6668±2880	–	–

DRG neurones and WI38 fibroblasts were incubated in the presence or absence of interleukin-1β (IL-1β; 100 iu ml^{-1}) for 2 h. The cells were then incubated with either 2 nM [^{3}H]-des-Arg^{10}-kallidin (DRG) or 1 nM [^{3}H]-des-Arg^{10}-kallidin (WI38) or 1 nM [^{3}H]-bradykinin. Non-specific binding was defined in the presence of 1 μM des-Arg^{10}-kallidin or bradykinin, respectively. The data shown are in d.p.m. mg^{-1} of protein and are the mean±s.e.mean of at least 3 independent experiments each performed in triplicate. (–) Not determined. *Indicates that the total binding in the IL-1β -treated fibroblasts was significantly greater ($P<0.05$) than in the control fibroblasts.

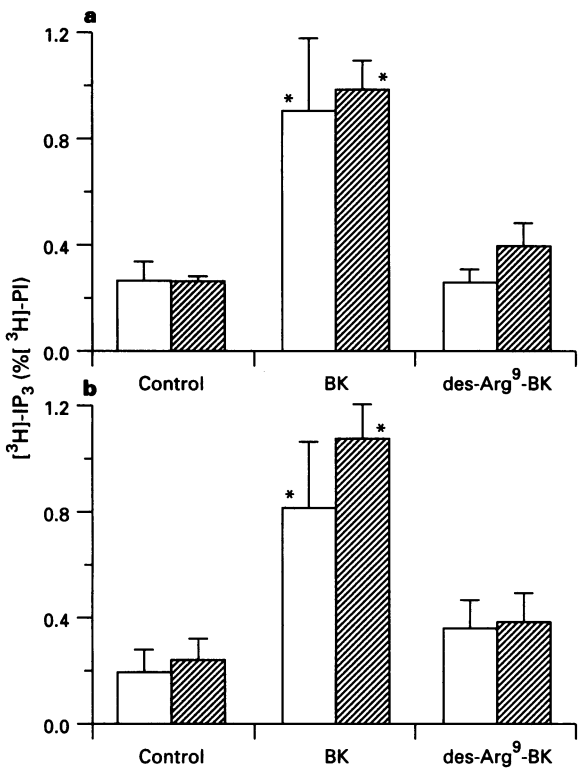

Figure 2 Treatment with IL-1β did not induce responsiveness to des-Arg^9-bradykinin in cultured DRG neurones. The neurones were incubated in the absence (open columns) or presence (hatched columns) of IL-1β (100 iu ml^{-1}) for 2 h (a) or 20 h (b), then challenged with either 30 nM bradykinin (BK) or 10 μM des-Arg^9-bradykinin (des-Arg^9-BK) for 20 s. The data represent the mean ± s.e.mean of 3 independent experiments. An asterisk indicates that the value is significantly above the basal value ($P<0.05$).

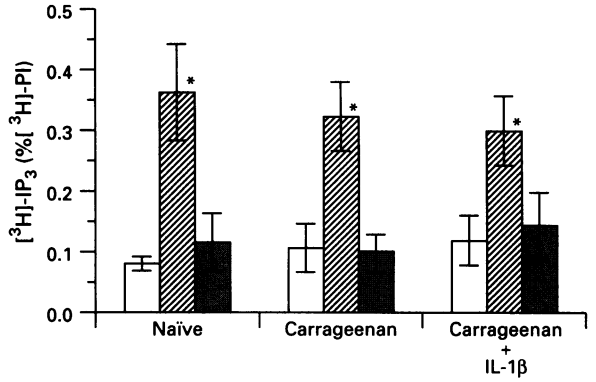

Figure 3 Pretreatment of 14 day old rats with carrageenan did not result in des-Arg^9-bradykinin-mediated [^{3}H]-IP$_3$ formation in DRG neurones. Fourteen day old rats were given an i.p. injection of carrageenan and 24 h later thoracic ganglia 11 and 12 and lumbar ganglia 1, 2 and 3 were removed. The DRG neurones were cultured in the presence or absence of IL-1β 100 iu ml^{-1} and then challenged with 100 nM bradykinin (hatched columns) or 1 μM des-Arg^9-bradykinin (stippled columns) for 20 s. The open columns represent the basal value. The data represent the mean ± s.e.mean of 3 independent experiments. An asterisk indicates that the value is significantly above the basal value ($P<0.05$).

applied up to 1 μM, also failed to evoke a response in the cells from the treated animals (data not shown). There was no significant difference ($P>0.05$) in the magnitude of the response to bradykinin in DRG neurones obtained from naïve animals and neurones from animals treated with either FCA or carrageenan ([^{3}H]-IP$_3$ in neurones from naïve animals after a 20 s exposure to 30 nM bradykinin, 199 ± 14.8% of the basal value ($n=3$); in neurones from FCA-treated animals after a 20 s exposure to 30 nM bradykinin, 253 ± 44.2% of the basal value ($n=3$); in neurones from carrageenan-treated animals after a 20 s exposure to 30 nM bradykinin, 291 ± 55.1% ($n=3$) of the basal value).

It has been suggested that C fibre-mediated inflammatory reactions do not occur in rats until they are 11 days old (Fitzgerald & Gibson, 1984). For this reason, some experiments were performed on 14 day old rats. The animals were injected (i.p.) with 50 μl of 2% carrageenan and, in order to increase the possibility of detecting changes, only those ganglia supplying the abdominal viscera were removed (i.e. thoracic ganglia 11 and 12 and lumbar ganglia 1, 2 and 3). Some of the neurones from these ganglia were maintained in the presence of IL-1β (100 iu ml^{-1}) until they were tested, 48 h after removal from the animals. Des-Arg^9-bradykinin (1 μM) did not increase [^{3}H]-IP$_3$ in either naïve or carrageenan-treated animals (Figure 3). Furthermore, the inclusion of IL-1β (100 iu ml^{-1}) in the culture medium did not alter the lack of responsiveness to the B_1 bradykinin receptor agonist (Figure 3).

Effect of B_1 bradykinin receptor agonists in an in vitro *spinal cord and tail preparation*

In spinal cords removed from one day old rats, superfusion of the attached tail with either bradykinin (300 nM) or capsaicin (700 nM) evoked a maximal ventral root depolarization. In contrast, application of 500 nM des-Arg^9-bradykinin to the tail had no effect (see Figure 4a and Table 2). Higher concentrations of des-Arg^9-bradykinin (up to 2 μM, data not shown) applied to the tail were also ineffective. The spinal terminals of primary afferents are more sensitive to alogenic substances than the terminals in the tail (Dray *et al.*, 1988) and lower concentrations of bradykinin (25 nM) and capsaicin (100 nM) produced ventral root depolarizations when applied directly to the spinal cord. However, direct application of 50 nM des-Arg^9-bradykinin (or 100 nM data not shown) to the spinal cord did not evoke ventral root depolarizations (see Figure 4a and Table 2). The more potent B_1 bradykinin receptor agonist des-Arg^{10}-kallidin was also ineffective when applied either to the tail at 1 μM or to the cord at 100 nM (data not shown).

When spinal cords were removed from animals that had received u.v. irradiation to the base of the tail 18 h before ventral root recording, there was intensive spontaneous activity in the ventral root which is recognized as a sign of spinal hyperexcitability. The frequency of spontaneous responses increased ($P<0.005$) from 0.6 ± 0.47 spontaneous ventral root discharges min^{-1} ($n=4$) in control animals to 5.3 ± 0.75 spontaneous ventral root discharges min^{-1} ($n=3$) in the animals that received u.v. irradiation. Even in the u.v.-treated preparations, des-Arg^9-bradykinin failed to evoke a ventral root response when applied to primary afferent terminals in either the tail or cord (Figure 4b and Table 2). There was no significant difference in the responses to either bradykinin or capsaicin in naïve or u.v.-treated animals.

Discussion

The results of the present study imply that the B_1 bradykinin receptors that contribute to persistent inflammatory hyperalgesia in animal models (Perkins *et al.*, 1993) are not located on nociceptive neurones.

Experiments in cultured DRG neurones

Attempts to induce responses to B_1 bradykinin receptor agonists in vitro No specific binding was detected in primary cultures of DRG neurones with the recently developed B_1 bradykinin

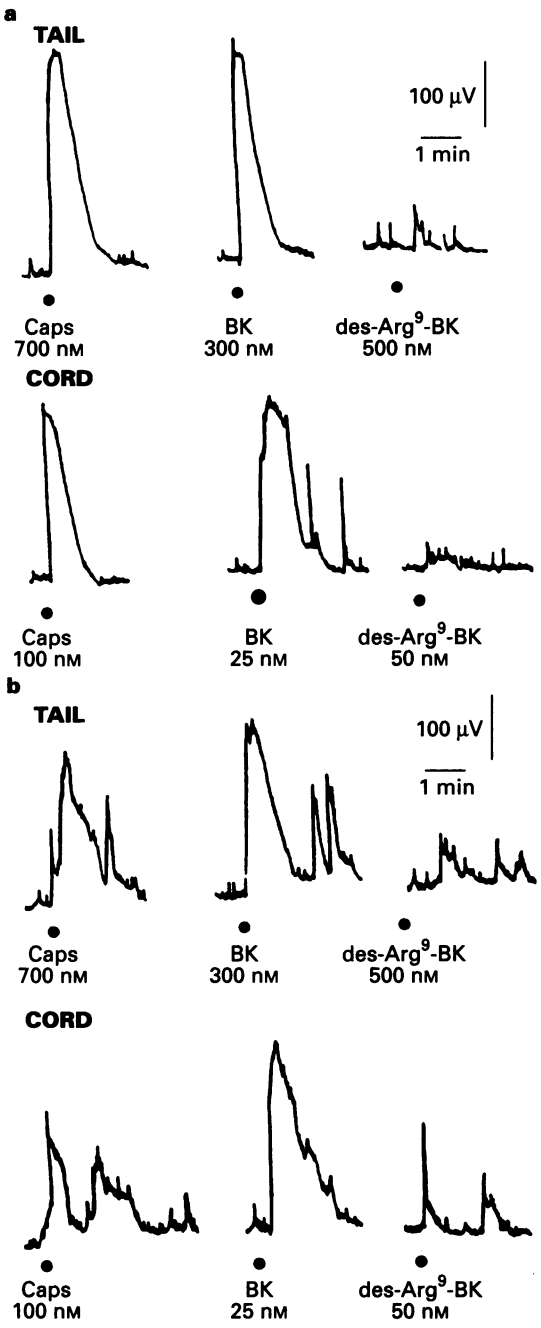

Figure 4 Effects of des-Arg^9-bradykinin, bradykinin and capsaicin on the spinal excitability of neonatal rats in naïve and u.v.-irradiated animals. (a) Effects of capsaicin (Caps), bradykinin (BK) and des-Arg^9-bradykinin (des-Arg^9-BK) on the spinal excitability of untreated neonatal rats. Ventral root responses were evoked by a brief application (10 s) of capsaicin, bradykinin and des-Arg^9-bradykinin to the tail (Tail) or to the spinal cord (Cord). Both capsaicin and bradykinin evoked ventral root depolarizations when applied either to the tail or the spinal cord, while des-Arg^9-bradykinin was ineffective. (b) Peripheral skin inflammation evoked by u.v.-irradiation of the tail base failed to induce responses to des-Arg^9-bradykinin in the ventral root. The agonists were applied for 10 s by superfusion to the tail or to the spinal cord.

receptor-selective radioligand, [^{3}H]-des-Arg^{10}-kallidin (see Schneck *et al.*, 1994; Menke *et al.*, 1994). Regulation of B_1 bradykinin receptor expression has been studied mostly in isolated smooth muscle preparations and primary cultures of smooth muscle cells, where it has been demonstrated that cytokines, including IL-1β, can upregulate receptor expression (e.g. DeBlois *et al.*, 1991; Bathon *et al.*, 1992; Levesque *et al.*, 1993; Galizzi *et al.*, 1994; Seabrook *et al.*, 1995). In the present study pretreatment of primary cultures of DRG neurones with IL-1β, (using incubation times ranging from 2 h up to 20 h) failed to induce specific [^{3}H]-des-Arg^{10}-kallidin binding, although specific B_1 bradykinin receptor binding in WI38 cells, which express B_1 bradykinin receptors constitutively, was upregulated by the same concentration of IL-1β with similar incubation times. In cultured cells, induction of B_1 receptors can be observed from 2 h up to 24 h after IL-1β treatment (Levesque *et al.*, 1993; Galizzi *et al.*, 1994) and *in vivo* treatment with IL-1β can induce B_1 bradykinin receptor-mediated hyperalgesia that is maintained for up to 48 h (e.g. Davis *et al.*, 1994).

In most cells, including sensory neurones (Thayer *et al.*, 1988; Burgess *et al.*, 1989), B_2 bradykinin receptors activate PIC. In DRG neurones this leads to activation of a protein kinase C-sensitive inward current, termed I_{BK}, that is carried mainly by sodium ions and is likely to be at least partly responsible for depolarizing and activating the neurones in response to bradykinin. B_1 bradykinin receptors have been shown to couple to PIC in a variety of cell types (Tropea *et al.*, 1993; Bascands *et al.*, 1993; Smith *et al.*, 1995). However, neither des-Arg^9-bradykinin, nor the more potent des-Arg^{10}-kallidin, were able to activate PIC in cultured DRG neurones, even in cells that had been treated with IL-1β.

Numerous studies using both behavioural measurements and extracellular recordings of sensory neurone activity have demonstrated that prostaglandins, including PGE_2 and PGI_2, increase sensitivity to noxious stimuli (e.g. Ferreira *et al.*, 1978; Martin *et al.*, 1987; Mizumura *et al.*, 1991; Rueff & Dray, 1993; Nicol & Cui, 1994). In the present study, neither of these prostaglandins induced sensitivity to B_1 bradykinin receptor agonists in the DRG neurones.

Attempts to induce responses to B_1 bradykinin receptor agonists in vivo No responses to B_1 bradykinin receptor agonists were obtained in DRG neurones removed from either neonatal or 14 day old rats that had been pretreated with inflammatory mediators even if the neurones were cultured with IL-1β. Again, this is in contrast to the observed induction of B_1 bradykinin receptor responsiveness in smooth muscle tissues following experimental inflammation (Marceau *et al.*, 1980; Kachur *et al.*, 1986; Farmer *et al.*, 1991), or the development of B_1 bradykinin receptor-mediated hyperalgesia following injections of FCA or IL-1β (Perkins *et al.*, 1993; Davis & Perkins, 1994). The hyperalgesia experiments described in the above references were done in young animals (4 week old rats), suggesting that B_1 bradykinin receptors can be induced at an early age. However, although there is good evidence from experiments with protocols similar to those used in the present study that inflammation, neurogenic oedema and hyperalgesia can occur in even younger animals (from postnatal day 11 onwards) (Fitzgerald & Gibson, 1984; Gonzales *et al.*, 1991; Thompson *et al.*, 1996), no direct evidence has, as yet, been obtained to indicate that B_1 bradykinin receptors can be induced in animals in this age range. This is being investigated currently.

In models of inflammatory hyperalgesia B_1 bradykinin receptor-mediated hyperalgesia can be observed up to 2 to 3 days after administration of either chemical irritants such as FCA or carrageenan or IL-1β. In the present experiments, although the time that the DRG neurones were kept in culture was kept to a minimum (22–48 h), it is possible that changes resulting from the inflammation might have been lost in culture. However, B_1 bradykinin receptors are maintained for up to 6 weeks in primary cultures of muscle cells (Schneck *et al.*, 1994).

Both the binding and second messenger experiments described above are population measurements and thus not ideal for detecting receptors that are expressed on a small subset of a cell population. The possibility that there is a small sub-population of B_1 bradykinin receptor-expressing DRG neurones is currently being explored by use of *in situ* hybridization.

Table 2 Effects of B_1 and B_2 bradykinin receptor agonists, and capsaicin on primary afferent and spinal neurones in naïve and u.v.-irradiated animals

	Capsaicin (μV)	n	*Bradykinin* (μV)	n	*des-Arg⁹-bradykinin* (μV)	n
Tail (control)	420±30	11	400±24	11	0	8
Tail (u.v.)	370±19	3	390±63	3	0	3
Cord (control)	410±60	4	380±63	4	0	4
Cord (u.v.)	410	2	300	2	0	2

Ventral root responses evoked by capsaicin, bradykinin and des-Arg⁹-bradykinin in control rats and in rats in which skin inflammation was induced in the tail by u.v. irradiation. Drugs were applied either to the tail (700 nM capsaicin, 300 nM bradykinin and 500 nM des-Arg⁹-bradykinin) or to the spinal cord (100 nM capsaicin, 25 nM bradykinin and 50 nM des-Arg⁹-bradykinin). Bradykinin and capsaicin evoked ventral root responses with similar amplitudes in control and u.v.-treated animals and des-Arg⁹-bradykinin failed to induce ventral root depolarization in either group. Results are expressed as means±s.e.mean of the number of observations (where *n* is greater than 2) shown.

Another possibility that must be considered, is that B_1 bradykinin receptors in DRG neurones are not coupled to PIC. For this reason, the ability of B_1 bradykinin receptor agonists to induce ventral root depolarizations in a sensory neurone preparation was also examined.

Experiments in the spinal cord and tail preparation

Consistent with the observations in cultured DRG neurones, des-Arg⁹-bradykinin failed to induce ventral root depolarizations when applied directly to primary afferent terminals in the rat tail or spinal cord. U.v irradiation has previously been shown to induce B_1 bradykinin receptor-mediated hyperalgesia (Perkins *et al.*, 1993) and in this study, u.v. irradiation applied to the base of the tail induced inflammation that resulted in a marked increase in the spontaneous activity in the spinal cord. However, even in animals that had been exposed to u.v. irradiation, B_1 bradykinin receptor agonists did not induce ventral root depolarizations when applied to the tail or spinal cord. In a previous study in the isolated spinal cord and tail preparation, Dray *et al.* (1992) were unable to evoke afferent responses to des-Arg⁹-bradykinin following perfusion of the tail with either lipopolysaccharide or epidermal growth factor, agents which have also been shown to induce B_1 bradykinin receptor responses in smooth muscle preparations *in vitro* (Deblois *et al.*, 1989). It is possible that des-Arg⁹-bradykinin might potentiate the responses to other noxious stimuli such as bradykinin, capsaicin or heat, in animals in which inflammation has been induced and this is currently under investigation.

Possible mechanisms of B_1 bradykinin receptor-mediated hyperalgesia

As B_1 bradykinin receptors do not appear to be expressed on sensory neurones, it is possible that the receptors involved in inflammatory hyperalgesia are located on sympathetic neurones. Bradykinin receptors have been shown to have hyperalgesic effects via the sympathetic nervous system (Levine *et al.*, 1986; Lee *et al.*, 1991) and a recent study in the superior cervical ganglion showed that, although the predominant form of bradykinin receptor was the B_2 bradykinin receptor subtype, there was evidence for B_1 bradykinin receptor-mediated depolarization after treatment with a combination of IL-1β and captopril (Seabrook *et al.*, 1995). This is in contrast, however, to a study by Babbedge *et al.* (1995), also in the superior cervical ganglion, who were unable to demonstrate any evidence for B_1 bradykinin receptor-mediated depolarization even after prior administration of bacterial lipopolysaccharide.

Another possibility is that the B_1 bradykinin receptors are located on other resident or infiltrating cells such as macrophages, fibroblasts or endothelial cells, where they are responsible for releasing modulators that act directly on the nociceptors. For example, des-Arg⁹-bradykinin has been shown to release inflammatory mediators such as IL-1β from macrophages (Tiffany & Burch, 1989) and prostaglandins from fibroblasts (Lerner & Modeer, 1991; Bathon *et al.*, 1992) and endothelial cells (Cahill *et al.*, 1988). As well as upregulating B_1 bradykinin receptor expression in a number of cell types (e.g. Levesque *et al.*, 1993; Galizzi *et al.*, 1994), IL-1β also potentiates B_1 bradykinin receptor-mediated prostanoid release from fibroblasts and vascular smooth muscle cells (Lerner & Modeer, 1991; Galizzi *et al.*, 1994) and increases prostaglandin production directly (Angel *et al.*, 1994) and indirectly by inducing increased levels of cyclo-oxygenase-2 (Habib *et al.*, 1993). Prostaglandins formed in response to both des-Arg⁹-bradykinin and IL-1β could be at least partly responsible for B_1 bradykinin receptor-induced hyperalgesia by sensitizing and exciting sensory neurones (Ferreira *et al.*, 1978; Martin *et al.*, 1987; Mizumura *et al.*, 1991; Nicol & Cui, 1994). In support of this suggestion, indomethacin has been shown to reduce the hyperalgesic effects of des-Arg⁹-bradykinin in IL-1β-treated animals (Davis & Perkins, 1994). Another mechanism by which des-Arg⁹-bradykinin might activate sensory neurones is via nitric oxide, which has been implicated in the activation of sensory fibres (Corrado & Ballejo, 1992; Haley *et al.*, 1992) and has been shown to cause pain when injected into human skin (Holthusen & Arndt, 1994). Des-Arg⁹-bradykinin increases $[Ca^{2+}]_i$ in endothelial cells (Morgan-Boyd *et al.*, 1987; Smith *et al.*, 1995) which results in the release of nitric oxide and IL-1β has also been shown to increase nitric oxide formation in endothelial cells (Szabo *et al.*, 1993).

Finally, in recent experiments, we have been unable to detect specific binding of [^{125}I]-IL-1β in DRG neurones (data not shown). This was in marked contrast to results obtained in human fibroblasts in which clear specific binding of [^{125}I]-IL-1β was observed. This provides an explanation for why IL-1β failed to induce B_1 bradykinin receptor binding or sensitivity to B_1 bradykinin receptor agonists in DRG neurones, whereas it did upregulate B_1 bradykinin receptor binding in WI38 cells. As IL-1β can induce hyperalgesic responses to B_1 bradykinin receptor agonists *in vivo* (Perkin *et al.*, 1993; Davis *et al.*, 1994), this finding supports the hypothesis that the B_1 bradykinin receptors involved in the hyperalgesic response are not located on sensory neurones. The nature of the cells and mediators involved in B_1 receptor-mediated hyperalgesia are under investigation.

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