Br. J. Pharmacol. (1992), **107**, 1129–1134 © Macmillan Press Ltd, 1992

Bradykinin-induced activation of nociceptors: receptor and mechanistic studies on the neonatal rat spinal cord-tail preparation *in vitro*

A. Dray, I.A. Patel, M.N. Perkins & A. Rueff

Sandoz Institute for Medical Research, 5 Gower Place, London WC1E 6BN

1 The effects of bradykinin on nociceptors have been characterized on a preparation of the neonatal rat spinal cord with functionally connected tail maintained *in vitro*. Administration of bradykinin to the tail activated capsaicin-sensitive peripheral fibres and evoked a concentration-dependent (EC_{50} = 130 nM) depolarization recorded from a spinal ventral root (L_3–L_5).

2 The response to bradykinin was unaffected by the peptidase inhibitors, bestatin (0.4 mM), thiorphan (1 μM), phosphoramidon (1 μM) and MERGETPA (10 μM) or by the presence of calcium blocking agents, cadmium (200 μM) and nifedipine (10 μM).

3 Inhibition of cyclo-oxygenase with indomethacin (1–5 μM), aspirin (1–10 μM) and paracetamol (10–50 μM) consistently attenuated responses to bradykinin.

4 The effect of bradykinin was mimicked by the phorbol ester PDBu, an activator of protein kinase C. The response to bradykinin was attenuated following desensitization to PDBu but desensitization to bradykinin did not induce a cross-desensitization to PDBu. The protein kinase C inhibitor staurosporine (10–500 nM) consistently attenuated the effects of PDBu and bradykinin.

5 Bradykinin responses were reversibly enhanced by dibutyryl cyclic AMP (100 μM). However dibutyryl cyclic GMP (0.5 mM) and nitroprusside (10 μM) produced prolonged block of responsiveness to bradykinin. Prolonged superfusion with pertussis toxin did not affect responses to bradykinin.

6 The B_1-receptor agonist des Arg^9-bradykinin (10–100 μM) was ineffective alone or after prolonged exposure of the tail to lipopolysaccharide (100 ng ml^{-1}) or epidermal growth factor (100 ng ml^{-1}) to induce B_1 receptors. The B_1-receptor antagonist, des Arg^9 Leu^8-bradykinin (10 μM) did not attenuate the response to bradykinin. A number of bradykinin B_2 antagonists selectively and reversibly attenuated the response to bradykinin. The rank order potency was Hoe 140 > LysLys [Hyp^3,$Thi^{5,8}$,D-Phe^7]-bradykinin > D-Arg[Hyp^3, $Thi^{5,8}$, D-Phe^7]-bradykinin = D-Arg[Hyp^2,$Thi^{5,8}$, D-Phe^7]-bradykinin.

7 These data show that bradykinin produces concentration-dependent activation of peripheral nociceptors in the neonatal rat tail. The responses were unaffected by calcium channel block and were partially dependent on the production of prostanoids. Bradykinin-evoked responses were consistent with the activation of protein kinase C-dependent mechanisms. Cyclic GMP-dependent mechanisms may be involved in bradykinin-receptor desensitization whereas cyclic-AMP dependent mechanisms increase fibre excitability and facilitate bradykinin-induced responses. The effects of bradykinin were mediated by a B_2 receptor.

Keywords: Bradykinin; peripheral nociceptors; bradykinin receptors; antagonists; second messengers

Introduction

Bradykinin, a pain producing and pro-inflammatory nonapeptide hormone is formed at sites of tissue trauma from proteolytic enzymes acting on kininogen precursors (Keele & Armstrong, 1964; Wilhelm, 1973; Erdos, 1979). The mechanisms of pain production involve the activation of a sub-population of polymodal nociceptors and the subsequent transmission of nociceptive signals to the spinal cord.

Studies of nociceptor activation by bradykinin have been made in sensory fibres *in vivo* (Beck & Handwerker, 1974; Mense & Schmidt, 1974; Franze & Mense, 1975) but because of the difficulties of studying fine sensory nerve terminals *in situ* these experiments have provided little quantitative information about the events and factors on which the effects of bradykinin depend. Mechanistic studies of bradykinin have been performed mainly on cultured sensory neurones or other hybrid cells. These have shown that bradykinin changes a number of membrane ion conductance mechanisms through interactions with several second messenger systems (Weinreich, 1986; Miller, 1987; Brown & Higashida, 1988; Dunn *et al.*, 1991) including phospholipase C to produce inositol 1,4,5, trisphosphate and diacyl glycerol (Thayer *et al.*, 1988; Burgess *et al.*, 1989b; Gammon *et al.*, 1989) and phospholipase A_2 to generate prostanoids, especially prostaglandins. These effects of bradykinin are likely to be coupled with specific receptors. At present two bradykinin receptors, B_1 and B_2 (Regoli & Barabe, 1980), have been convincingly demonstrated by the use of several generations of peptide antagonists (Vavrek & Stewart, 1985; Hock *et al.*, 1991).

To circumvent some of the disadvantages of *in vivo* studies of nociceptors we have used an *in vitro* preparation of the neonatal rat spinal cord/tail to characterize the receptors and second messengers involved in the activation of peripheral nociceptors by bradykinin. Some findings of this study have been reported previously (Dray *et al.*, 1988a,b).

Methods

The intact spinal cord and the functionally connected tail were taken from 1–2 day old rats following decapitation. The skin was carefully removed from the tail to expose cutaneous fibres and their endings to allow activation by bradykinin and to facilitate activation by capsaicin (Dray *et al.*, 1990a). Histology was not routinely performed but recent unpublished findings using immunological markers for developing neurones (GAP-43; Reynolds *et al.*, 1991) and for

specific neuronal cytoplasmic protein (PGP 9.5, Dalsgaard *et al.*, 1989) did not give any indication of damage to cutaneous fibres following skin removal. Bradykinin is not known to activate axons of nociceptors; it is more likely to stimulate nociceptors via the signal transducing elements localized on the terminations of intact primary afferent fibres. In addition the effects of other peripheral stimuli were robust and reproducible over many hours. This would be unlikely in the face of significant tissue damage.

The preparation was placed in a chamber and the spinal-cord and tail were separately superfused (2 ml min^{-1} on the cord and 4 ml min^{-1} on the tail) with a physiological salt solution (composition 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; at 24°C and gassed with 95% O_2/5% CO_2). Peripheral nociceptive fibres were activated by superfusion of the tail with bradykinin, capsaicin and by superfusate heated to 48–50°C (noxious heat). Each stimulus was applied for 10 s with an intervening period of 15 min between stimuli. Bradykinin applications were separated by at least 30–60 min to avoid tachyphylaxis.

The activation of peripheral fibres was assessed by measuring the depolarization produced in a spinal ventral root (L_3–L_5). The ventral root potential was recorded (d.c. with respect to the spinal cord which was earthed) using a low impedance glass pipette which was placed in an electrolyte-filled well containing the selected ventral root. The signals were amplified using conventional means (Neurolog System) and displayed simultaneously on an oscilloscope and on a rectilinear chart recorder.

In the following studies we examined whether bradykinin-induced activation of nociceptors was coupled to a G-protein or mediated via the activation of a second messenger system. In addition we have characterized the receptor involved in the bradykinin-induced activation of nociceptors and compared the potency of a number of bradykinin antagonists. Antagonist activity was expressed as the IC_{50} concentration, determined by measuring the responses to a submaximal concentration of bradykinin (usually the EC_{50}) in the presence of cumulative increases in the concentration of the antagonist. Three or more antagonist concentrations were used in the IC_{50} determination.

Drugs

The following substances were used; capsaicin (Sigma, 10 mM stock solution in dimethylsulpoxide (DMSO) made up to the desired concentration in physiological salt solution), bradykinin (Bachem, Nova), forskolin, sodium nitroprusside, sodium dibutyryl cyclic AMP, dibutyryl cyclic GMP, trifluoperazine, indomethacin, mepacrine, phenobarbital sodium, ruthenuim red (all from Sigma), nifedipine (Research Biochemicals Incorporated); R_p isomer of adenosine 3'-5'-cyclic monophosphathioate (R_p-cAMPS, BIOLOG); β-phorbol 12,13 dibutyrate, (Avanti Polar Lipids); staurosporine (Fluka), H7 [1-(5-isoquinolinesulphonyl)-2-methylpiperazine] (Seikagaku America Inc); sodium chromoglycate (gift from Pfizer); DL-2-mercaptomethyl-3-guanidoethylthiopropionic acid (MERGETPA, Calbiochem-Behring), phosphoramidon (Peninsula), des-Arg^9 bradykinin; Leu^8 (des Arg^9)-bradykinin; D-Arg [Hyp^2 $Thi^{5,8}$ D-Phe^7 bradykinin], D-Arg [Hyp^3 $Thi^{5,8,}$ D-Phe^7 bradykinin], Lys,Lys [Hyp^3 $Thi^{5,8}$ D-Phe^7 bradykinin] (where Thi = β (2-thienyl)-L-alanine; Hyp = L-4-hyroxyproline: Peninsula Labs); D-Arg[Hyp^3-Thi^5-D-Tic^7-Oic^8-bradykinin] (HOE 140, where Tic = 1,2,3,4 tetrahydroisoquinolin-2-yl-carbonyl, Oic = (3aS,7aS)-octahydroindol-2-yl-carbonyl: synthesized at the Sandoz Institute, London); pertussis toxin (Porton Products Ltd).

Results

A 10 s administration of bradykinin to the tail evoked a ventral root response. Application of bradykinin (1–5 μM) to the intact tail without prior removal of the superficial skin did not produce a response ($n = 5$). A ventral root response was also evoked by brief applications of capsaicin (0.2–1.0 μM) and noxious heat. In any given tissue the noxious heat stimulus produced the maximal response. Therefore for quantification of data, chemically induced responses were normalized, in each tissue, relative to the noxious heat response.

The amplitude of the ventral root depolarization produced by bradykinin was concentration-related (Figures 1 and 3). A concentration-response curve (EC_{50} = 130 nM, maximum concentration = 1 μM), shown in Figure 1, was determined from the response produced by the first application of bradykinin at any given concentration. This provided only one data point per experiment but avoided possible sensitivity changes due to tachyphylaxis upon repeated administration.

The onset of the effect of a 10 s application of bradykinin occurred within 20–60 s of the start of the superfusion and the duration of the effect ranged from 40–190 s. The onset of the effect was shorter and the duration longer with higher concentrations (e.g. 20 s latency and 190 s duration at 1 μM) but this relationship was not systematically studied. Under the present conditions, responses evoked by a submaximal concentration of bradykinin (0.3 μM) were not significantly influenced by enzymatic degradation since they were unchanged in the presence of the peptidase inhibitors, bestatin (0.4 mM, $n = 4$; control response = 65 ± 5%; test response = 71 ± 9%, $P > 0.05$), thiorphan (1 μM, $n = 4$; control response = 56 ± 8%; test response = 59 ± 5%, $P > 0.05$), phosphoramidon (1 μM, $n = 5$; control response = 60 ± 5%, test response = 63 ± 7%, $P > 0.05$) or MERGETPA (10 μM, $n = 5$, 61 ± 9%; test response = 64 ± 9%, $P > 0.05$), each superfused 15 min prior to and throughout the test with bradykinin. In addition bradykinin-evoked (0.35 μM) responses were unaffected in the presence of cadmium chloride (200 μM, $n = 4$; control response = 100 ± 10%; test response = 88 ± 17%, $P > 0.05$) or nifedipine (10 μM, $n = 5$; control response = 89 ± 11%; test response = 80 ± 8%, $P > 0.05$).

Second messenger studies

The effects of phorbol esters, non-hydrolysable protein kinase C activators (Castagna *et al.*, 1982), and their interactions with bradykinin were tested on peripheral nociceptors. Both phorbol 12, myristate-13,acetate (PMA, 1 μM, $n = 4$, response = 32 ± 9%) and β-phorbol 12,13dibutyrate (PDBu 1 μM, $n = 10$, response = 48 ± 13%) application to the tail

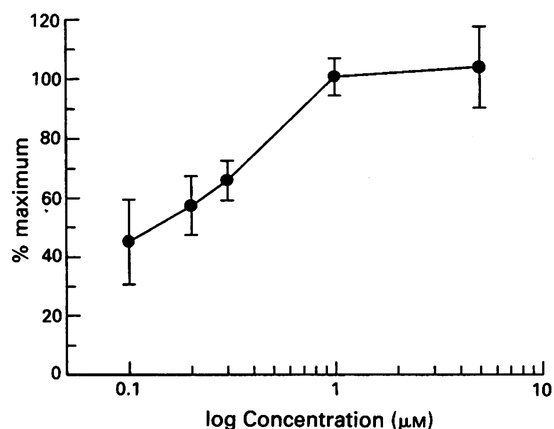

Figure 1 The concentration-effect curve to bradykinin. The responses to bradykinin were normalized with respect to the maximal tissue response evoked by noxious heat response and plotted as % maximal effect vs log concentration of bradykinin (nM). The EC_{50} concentration = 130 nM. Each point is the mean ± s.e.mean (vertical bars) of 4–5 determinations.

evoked ventral root responses due to activation of capsaicin-sensitive afferents (Dray *et al.*, 1988a). In further studies, 1 μM PDBu was used, as responses with this agent were obtained more consistently and were comparable to those produced by bradykinin (0.3 μM).

The onset of responses evoked by PDBu was delayed like those of bradykinin. Frequently repeated (every 10–20 min) or prolonged (2 min) administration of 1 μM PDBu produced a tachyphylaxis at which time the response to bradykinin was also attenuated ($n = 5$, control bradykinin response = 83 ± 10%; post PDBu = 12 ± 9%, $P < 0.01$). On the other hand prolonged administration of bradykinin (10–20 μM, $n = 5$), to induce desensitization to bradykinin did not induce a cross desensitization to PDBu (control PDBu = 60 ± 15%; post bradykinin = 54 ± 12%, $P > 0.05$). The degree of tachyphylaxis to repeated brief administrations of PDBu could be minimized by restricting drug administration to once every 60 min.

Prolonged application of the protein kinase C inhibitor, H7 (Hidaka & Hagiwara, 1987) (100 μM, $n = 3$) did not affect the response to PDBu (control = 40 ± 12%; post H7 = 45 ± 10%, $P > 0.05$) or bradykinin (control = 72 ± 11%; post H7 = 66 ± 14%). However another protein kinase C inhibitor, staurosporine (Tamaoki *et al.*, 1986) consistently (10–500 nM, $n = 8$) attenuated the responses to PDBu (control = 46 ± 10%; post staurosporine = 22 ± 12%, $P < 0.01$) and bradykinin (control = 69 ± 9%; post staurosporine = 14 ± 9%, $P < 0.01$) without affecting responses to other stimulants such as capsaicin (control = 61 ± 8%; post staurosporine = 54 ± 8%, $P > 0.05$) and noxious heat. Prolonged (20–30 min) superfusion of the tail with phenobarbitone (0.1–1.0 mM, $n = 4$), previously suggested to be an inhibitor of protein kinase C (Chaouhan & Brockerhoff, 1987), did not attenuate the responses to bradykinin (control = 72 ± 14%; post phenobarbitone = 67 ± 12%, $P > 0.05$). At higher concentrations (3–10 mM, $n = 3$) phenobarbitone depressed responsiveness to all sensory stimuli.

Previous studies have shown that adenosine 3′:5′-cyclic monophosphate (cyclic AMP) increased membrane excitability by blocking hyperpolarizing potassium conductance (Weinreich & Wonderlin, 1987; Grega & Macdonald, 1987). In our experiments the addition of dibutyryl cyclic AMP (30 s, 100 μM, $n = 13$) or stimulation of adenylate cyclase by the addition of forskolin (30 s, 10 μM, $n = 5$) did not evoke a response. However in 8 of 13 preparations the effects of bradykinin (control = 32 ± 12%; with dibutyryl cyclic AMP (db cyclic AMP) = 122 ± 19%, $P < 0.01$) and capsaicin (control = 74 ± 5%; with db cyclic AMP = 123 ± 12%, $P < 0.01$) were significantly enhanced (Figure 2) by db cyclic AMP but were not significantly affected in the 4 other experiments. The effects of db cyclic AMP were reversed following 10–20 min of washing (Figure 2). The effect of bradykinin was not significantly changed in the presence of forskolin (10 μM, $n = 5$) or by 10 μM, R_p-cAMPS ($n = 4$), a cyclic AMP-dependent kinase inhibitor (Botelho *et al.*, 1988).

Bradykinin has previously been shown to increase guanosine 3′:5′-cyclic monophosphate (cyclic GMP) production in sensory neurones (Burgess *et al.*, 1989a). In our experiments neither db cyclic GMP (0.5 mM, $n = 5$) nor nitroprusside (2–50 μM, $n = 7$) evoked a measurable response. In the presence of these agents the response to bradykinin was selectively attenuated or abolished ($n = 5$, db cyclic GMP, 0.5 mM; control response = 78 ± 11%; test response = 18 ± 9%, $P < 0.01$: sodium nitroprusside, 2–10 μM, $n = 6$, control response = 80 ± 14%; test response = 18 ± 10%, $P < 0.01$). In contrast to the short lived effect of cyclic AMP, that produced by cyclic GMP or nitroprusside was prolonged and was incompletely reversed even by 1–2 h after continuous washing of the tissue. In this respect the loss of bradykinin sensitivity was similar to that seen during bradykinin-induced tachyphylaxis.

Prolonged superfusion of the tail (20–30 min) with the calmodulin-kinase inhibitor trifluoperazine (30 μM, $n = 4$) or

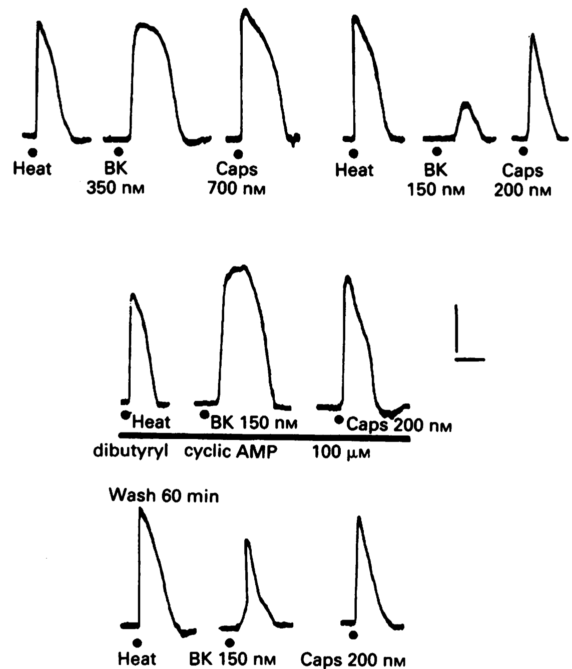

Figure 2 Enhancement of the bradykinin-evoked response during the administration of dibutyryl cyclic AMP. The top row of traces show responses to heat, bradykinin (BK) (350 nM and 150 nM respectively) and capsaicin (Caps, 700 and 200 nM). Middle traces: the responses to bradykinin (150 nM) and capsaicin (200 nM) were enhanced in the presence of dibutyryl cyclic AMP (100 μM). Bottom traces: the effect of cyclic AMP was reversed 60 min after the end of the cyclic AMP superfusion. The calibration bars are 0.2 mV and 60 s.

the phospholipase A_2 inhibitor, mepacrine (10–30 μM, $n = 4$) did not significantly change the responses to bradykinin, capsaicin or noxious heat. In addition similar applications of sodium chromoglycate (1–100 μM, $n = 4$), reported to depress C-fibre excitability in the lung (Dixon *et al.*, 1980) and bradykinin-induced bronchoconstriction (Fuller *et al.*, 1987), did not significantly affect responses to bradykinin. Finally, ruthenium red (100–500 nM, $n = 6$), which selectively inhibited C-fibre activation by capsaicin (Dray *et al.*, 1990b) did not alter responses to bradykinin.

Indomethacin (1.5 μM) reduced, but did not abolish, the response to bradykinin (300 nM) in 7 of 9 preparations (control responses = 42 ± 15%; with indomethacin = 17 ± 11%, $P < 0.01$, $n = 7$). Higher concentrations of indomethacin (10–50 μM, $n = 5$) produced an additional non-selective depression of responses to capsaicin (43 ± 2% of control) and noxious heat (56 ± 9% of control). Aspirin (1–10 μM, $n = 6$, control response = 65 ± 10%; with aspirin = 21 ± 8%, $P < 0.01$) or paracetamol (10–50 μM, $n = 6$, control response = 57 ± 12%; with paracetamol = 30 ± 7%, $P < 0.01$) also selectively reduced the responses to bradykinin (300 nM).

Superfusion of the tail for 4–6 h with recirculated and reoxygenated pertussis toxin (200 ng–1.0 μg ml^{-1}) (Dolphin, 1987) to inactivate G_i and G_o did not affect the responses to 300 nM bradykinin ($n = 3$, control = 59 ± 15%; test = 66 ± 11%, $P > 0.05$).

Pharmacology of bradykinin on peripheral fibres

Brief (10 s) or prolonged (5 min) application of the B_1 receptor agonist des-Arg^9-bradykinin (10–100 μM, $n = 5$) did not evoke a response. Also prolonged superfusion (1–4 h) with lipopolysaccharide (100 ng ml^{-1}, $n = 4$) and epidermal growth factor (100 ng ml^{-1}, $n = 3$), both shown to induce responses to B_1 receptor agonists *in vitro* (Bouthillier *et al.*, 1987) did not induce any measurable response to a subse

quent administration of des-Arg[9]-bradykinin (10 μM). Prolonged superfusion of the tail with the B_1-antagonist, des-Arg[9]Leu[8]-bradykinin (10 μM, $n = 5$) did not change responsiveness to bradykinin (control = 77 ± 10%; test = 84 ± 12%, $P > 0.05$).

The response to bradykinin was reduced, in a concentration-dependent manner, by a number of competitive peptide antagonists. Antagonism was quantified by determining the concentration of the antagonist (IC_{50}), incremented in a cumulative manner, required to reduce the response produced by a submaximal 2x-dose of bradykinin to that produced by a single x-dose (Figure 3). The relative potency of a number of bradykinin antagonists, determined in this manner was Hoe 140 = 1.5 ± 0.4 nM, $n = 5$; LysLys[Hyp^3,Thi^{5,8} D-Phe^7-bradykinin] = 36 ± 14 nM, $n = 5$; D-Arg[Hyp^3,Thi^{5,8}, D-Phe^7-bradykinin] = 44 ± 23 nM, $n = 6$; D-Arg[Hyp^2, Thi^{5,8}, D-Phe^7-bradykinin], = 69 ± 21 nM, ($n = 5$ for each determination). The effect of each antagonist was reversed within a 60 min wash period though more prolonged washing (120–180 min) was required to show partial reversal of the Hoe 140-induced antagonism. This suggested that Hoe 140 may have been more tightly bound to the tissue. The reversibility of the antagonism with each compound readily distinguished this effect from desensitization. None of these substances exhibited any agonistic activity when administered up to 10 μM ($n = 3$ for each substance).

Discussion

By using an *in vitro* preparation we have been able to study in greater detail the receptor-mediated interactions of bradykinin with peripheral nociceptors. In the neonatal rat, cutaneous nociceptors have similar sensitivity to physiological stimuli to those in adults (Fitzgerald, 1987).

The peripheral nerve elements activated by bradykinin were likely to be C and Aδ nociceptive fibres because previous studies have shown that bradykinin affected C-cells but not larger A-cells (Burgess *et al.*, 1989b) and that bradykinin activated a subpopulation of capsaicin-sensitive C and Aδ nociceptors (Lang *et al.*, 1990). In addition the effects

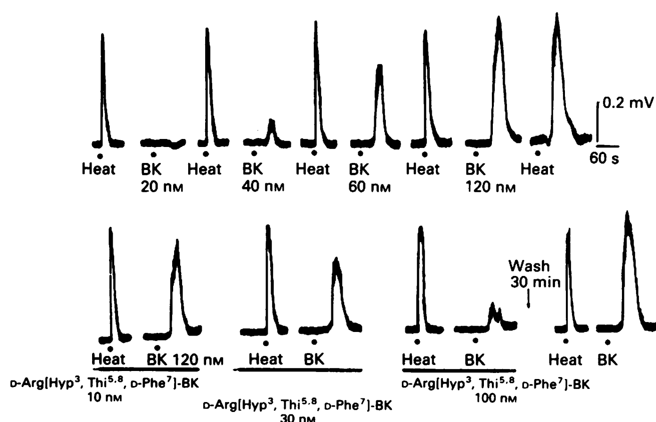

Figure 3 Concentration-related antagonism of bradykinin by D-Arg[Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin. The top traces show response to brief administration of noxious heat and concentration related responses to bradykinin (20, 40, 60 and 120 nM). The response to 120 nM bradykinin was chosen as the test concentration of bradykinin and in the bottom traces the antagonistic effect of D-Arg [Hyp^3,Thi^{5,8}D-Phe^7]-bradykinin was determined. The antagonist was administered 15 min before and throughout the repeated tests with bradykinin. The concentration of the antagonist (10, 30, 100 nM) was cumulatively increased until the response to bradykinin was reduced to or beyond that produced by the 60 nM control concentration. The IC_{50} was subsequently determined. The effect of the antagonist was readily reversed and therefore distinguishable from any possible bradykinin-induced tachyphylaxis. The calibration bars are 0.2 mV and 60 s.

of bradykinin could be abolished by pretreatment with capsaicin (Dray *et al.*, 1989) which has a selective action on these classes of fibres (Buck & Burks, 1986). Finally the effect of bradykinin or other peptide analogues (see later) were unlikely to be limited by the actions of endogenous degrading enzymes since the responses to bradykinin were unchanged in the presence of a variety of kinase inhibitors. The responses evoked by bradykinin were concentration-dependent with an EC_{50} = 130 nM, somewhat higher than that found for activation of sensory neurones in culture (Burgess *et al.*, 1989b; Thayer *et al.*, 1988).

Activation of peripheral fibres by bradykinin was unlikely to be due to an increase in permeability to extracellular calcium ions since bradykinin-evoked responses were not significantly changed by the calcium channel blockers, cadmium and nifedipine. Similar findings were reported with cultured sensory neurones where bradykinin-induced membrane currents were unaffected by calcium-free solution or by the presence of calcium channel blocking drugs. Moreover any intracellular calcium flux was considered to be secondary to membrane depolarization and the activation of voltage-sensitive calcium channels (Burgess *et al.*, 1989b). Indeed the depolarization of sensory neurones has been suggested to depend mainly on an increase membrane permeability to sodium ions (Burgess *et al.*, 1989b).

The effect of bradykinin appeared to be mediated in part via protein kinase C. Thus bradykinin and phorbol esters activated peripheral fibres. Responses to both agents were sensitive to the protein kinase C inhibitor, staurosporine (Tamaoki *et al.*, 1986) and bradykinin responses were attenuated or abolished after PDBu-induced tachyphylaxis. These observations confirm similar interactions in sensory neurones in culture (Burgess *et al.*, 1989b). On the other hand PDBu was still active following bradykinin-induced desensitization, suggesting that the mechanisms of desensitization to bradykinin and PDBu were different.

The response to bradykinin was also consistently and selectively attenuated, but not abolished, by a number of cyclo-oxygenase inhibitors including indomethacin, aspirin and paracetamol. These data indicate that bradykinin stimulated the production of prostanoids, as occurs in many tissues (Griesbacher & Lembeck, 1987; Conklin *et al.*, 1988). However, bradykinin was unlikely to have stimulated phospholipase A_2 (PLA_2) activity to any significant extent as the PLA_2 inhibitor, mepacrine, had litte effect on bradykinin-induced responses. Also it is unlikely that endogenously produced prostanoids directly activated nociceptors since in our preparation, exogenously administered prostaglandins only sensitized nociceptors to other direct activators, including bradykinin.

It is unclear how bradykinin-induced protein kinase C activation contributed to an increased excitability of nociceptors. On the one hand bradykinin or phorbol esters, which stimulate protein kinase C, depolarize C-fibres (Rang & Ritchie, 1988) and increase membrane conductance, to sodium ions, in sensory neurones (Baccaglini & Hogan, 1983; McGhee & Oxford, 1989; Burgess *et al.*, 1989b). Other studies however indicate that bradykinin and phorbol esters inhibit membrane calcium conductance (Rane *et al.*, 1989; Boland *et al.*, 1991). In visceral sensory neurones, a reduced calcium permeability (Gross & Macdonald, 1989) may account for the inhibition of calcium-dependent potassium conductance, the reduction of membrane spike after-hyperpolarization and the consequent increase in cell excitability (Weinreich, 1986). Bradykinin induced prostaglandin production may also increase sensory neurone excitability by inhibiting potassium permeability (Weinreich, 1986; Weinreich & Wonderlin, 1987). Clearly the actions of bradykinin on membrane excitability of sensory neurones are complex and depend on a multitude of factors including the prevailing level of membrane polarization.

Bradykinin-induced activation did not appear to involve cyclic AMP or cyclic GMP-dependent mechanisms as neither

the addition of nucleotide analogues nor stimulators of cyclic nucleotide formation (forskolin and nitroprusside) mimicked the effects of bradykinin. Moreover the effect of bradykinin was unchanged by the cyclic AMP-kinase inhibitor R_p-cAMPS (Botelho *et al.*, 1988). However cyclic AMP produced a short lasting increase in the responsiveness of nociceptors to bradykinin and capsaicin. This may have been due to a generalized increase in excitability of nociceptors, in keeping with findings *in vivo* which have suggested the participation of cyclic AMP mechanisms in peripheral hyperalgesia (Taiwo & Levine, 1991). On the other hand cyclic GMP consistently attenuated the response to bradykinin for a prolonged period. The reason for this is unclear at present but may have involved desensitization of bradykinin receptors due to receptor phosphorylation by a cyclic GMP-dependent kinase or the inhibition of bradykinin-induced second messenger production e.g. IP_3 and DAG, important for mediating the bradykinin response (Burgess & McNeill, 1989). In addition we were unable to show that the effect of bradykinin was coupled with a pertussis toxin-sensitive G-protein. Indeed evidence for the involvement of a G-protein in the effects of bradykinin on sensory neurones has been controversial. Neither Burgess *et al.* (1989b) nor McGehee & Oxford (1989) found altered sensitivity following pertussis toxin treatment while McGuirk *et al.* (1989) measured an increase in sensory neurone activity following treatment with a non-hydrolysable GTP-analogue, though it was unclear that this was related to a bradykinin mechanism.

Studies with the bradykinin analogues showed that the effects of bradykinin were mediated by a B_2 receptor. Thus no activity was observed with either the B_1 receptor agonist or antagonist. Moreover, we were unable to show the presence of B_1 receptors by incubating tissues with lipopolysaccharide or epidermal growth factor. These substances have been shown to enhance B_1-receptor expression in a number of smooth muscle preparations *in vitro* (Bouthillier *et al.*, 1987). On the other hand several peptide antagonists of bradykinin B_2 receptors produced a concentration-related attenuation of bradykinin-responses. The rank order potency of these substances was Hoe 140 > LysLys [Hyp^3,$Thi^{5,8}$D-Phe^7]-bradykinin > D-Arg^0[Hyp^3,$Thi^{5,8}$D-Phe^7]-bradykinin = D-Arg^0 [Hyp^2,$Thi^{5,8}$D-Phe^7]-bradykinin. In keeping with their *in vitro* activity, several of these agents have been shown to be anti-inflammatory and antinociceptive in a number of *in vivo* studies (Hargreaves *et al.*, 1988; Steranka *et al.*, 1988; 1989; Costello & Hargreaves, 1989; Burch & deHaas, 1990; Haley *et al.*, 1989). This also supports the likely involvement of endogenous bradykinin in inflammatory hyperalgesia.

In summary, we have shown that bradykinin produces a prolonged and concentration-related activation of peripheral nociceptors of the neonatal rat. The activation of nociceptors occurred independently of extracellular calcium but involved a number of cellular second messenger systems including cyclo-oxygenase products and protein kinase C. Finally a number of selective peptide antagonists of bradykinin suggested that these mechanisms are coupled to a bradykinin B_2 receptor.

References

BACCAGLINI, P.I. & HOGAN, P.G. (1983). Some rat sensory neurons in culture express characteristics of differentiated pain sensory cells. *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 594–598.

BECK, P.W. & HANDWERKER, H.O. (1974). Bradykinin and serotonin effects on various types of cutaneous nerve fibres. *Pflügers Arch.*, **347**, 209–222.

BOLAND, L.M., ALLEN, A.C. & DINGLEDINE, R. (1991). Inhibition by bradykinin of voltage-activated barium current in a rat dorsal root ganglion cell line: role of protein kinase C. *J. Neurosci.*, **11**, 1140–1149.

BOTELHO, L.H.P., ROTHERMAN, J.D., COOMBS, R.V. & JOSTORFF, B. (1988). cAMP analog antagonist of cAMP action. *Methods Enzymol.*, **159**, 159–172.

BOUTHILLIER, J., DE BOIS, D. & MARCEAU, F. (1987). Studies on the induction of pharmacological responses to des-Arg^9-bradykinin *in vitro* and *in vivo*. *Br. J. Pharmacol.*, **92**, 257–264.

BROWN, D.A. & HIGASHIDA, A. (1988). Inositol 1,4,5- trisphosphate and diacylglycerol mimic bradykinin effects on mouse neuroblastoma x rat glioma hybrid cells. *J. Physiol.*, **397**, 185–207.

BUCK, S.H. & BURKS, T.F. (1986). The neuropharmacology of capsaicin: review of some recent observations. *Pharmacol. Rev.*, **38**, 179–226.

BURCH, R.M. & DEHAAS, C. (1990). A bradykinin antagonist inhibits carageenan edema in rat. *Naunyn-Schmiedebergs Arch Pharmacol.*, **342**, 189–193.

BURGESS, G.M. & MCNEILL, M. (1989). Homologous desensitization of bradykinin-induced inositol polyphosphate formation in neonatal rat dorsal root ganglion neurones in culture. *Soc Neurosci.*, **15**, 1007.

BURGESS, G.M., MULLANEY, I., MCNEILL, M., COOTE, P.R., MINHAS, A. & WOOD, J.N. (1989a). Activation of guanylate cyclase by bradykinin in rat sensory neurones is mediated by calcium influx: possible role of the increase in cyclic GMP. *J. Neurochem.*, **53**, 1212–1218.

BURGESS, G.M., MULLANEY, I., MCNEIL, M., DUNN, P.M. & RANG, H.P. (1989b). Second messengers involved in the mechanism of action of bradykinin in sensory neurones in culture. *J. Neurosci.*, **9**, 3314–3325.

CASTAGNA, M., TAKAI, Y., KAIBACHI, K., SANO, K., KIKKAWA, U. & NISHIZUKA, Y. (1982). Direct activation of calcium-activated phospholipid dependent protein kinase by tumor promoting phorbol esters. *J. Biol. Chem.*, **257**, 335–363.

CHAOUHAN, V.P.S. & BROCKERHOFF, H. (1987). Phenobarbital competes with diacylglycerol for protein kinase C. *Life Sci.*, **40**, 89–93.

CONKLIN, B.R., BURCH, R.M., STERANKA, L.R. & AXELROD, J. (1988). Distinct bradykinin receptors mediate stimulation of prostaglandin synthesis by endothelial cells and fibroblasts. *J. Pharmacol. Exp. Ther.*, **244**, 646–649.

COSTELLO, A.H. & HARGREAVES, K.M. (1989). Suppression of carageenin induced hyperalgesia, hyperthermia and edema by a bradykinin antagonist. *Eur. J. Pharmacol.*, **171**, 259–263.

DALSGAARD, C.-J., RYDH, M. & HAEGERSTRAND, A. (1989). Cutaneous innervation in man visualized with protein gene product 9.5. (PGP 9.5) antibodies. *Histochem.*, **92**, 385–390.

DIXON, M., JACKSON, D.M. & RICHARDS, I.M. (1980). The actions of sodium chromoglycate on 'C' fibre endings in the dog lung. *Br. J Pharmacol.*, **70**, 11–13.

DOLPHIN, A.C. (1987). Nucleotide binding proteins in health and disease. *Trends Neurol. Sci.*, **10**, 53–57.

DRAY, A., BETTANEY, J., FORSTER, P. & PERKINS, M.N. (1988a). Bradykinin-induced stimulation of afferent fibres is mediated through protein kinase C. *Neurosci. Lett.*, **91**, 301–307.

DRAY, A., BETTANEY, J., FORSTER, P. & PERKINS, M.N. (1988b). Activation of bradykinin receptor in peripheral nerve and spinal cord in the neonatal rat *in vitro*. *Br. J. Pharmacol.*, **95**, 1008–1010.

DRAY, A., BETTANEY, J. & FORSTER, P. (1989). Capsaicin desensitization of peripheral nociceptive fibres does not impair sensitivity to other noxious stimuli. *Neurosci. Letts.*, **99**, 50–54.

DRAY, A., BETTANEY, J. & FORSTER, P. (1990a). Action of capsaicin on peripheral nociceptors of the neonatal rat spinal cord-tail preparation in vitro: dependence of extracellular ions and independence of second messengers. *Br. J. Pharmacol.*, **101**, 727–733.

DRAY, A., FORBES, C.A. & BURGESS, G.M. (1990b). Ruthenium red blocks capsaicin-induced increase in intracellular calcium and activation of membrane currents in sensory neurones as well as the activation of peripheral nociceptors in vitro. *Neurosci. Letts.*, **110**, 52–59.

DUNN, P.M., COOTE, P.R., WOOD, J.N., BURGESS, G.M. & RANG, H.P. (1991). Bradykinin evoked depolarization of a novel neuroblastoma x DRG neurone hybrid cell line (ND 7/23). *Brain Res.*, **545**, 80–86.

ERDOS, E. (1979). *Bradykinin, Kallidin and Kallikrein. Handbook of Experimental Pharmacology*. Supplement to vol 25, Berlin: Springer Verlag.

FITZGERALD, M. (1987). Cutaneous primary afferent properties in the hind limb of the neonatal rat. *J. Physiol.*, **383**, 79–92.

FRANZ, M. & MENSE, S. (1975). Muscle receptors with Group IV afferent fibres responding to application of bradykinin. *Brain Res.*, **92**, 369–383.

FULLER, R.W., DIXON, C.M.S., CUSS, F.M.C. & BARNES, P.J. (1987). Bradykinin-induced bronchoconstriction in man. *Am. Rev. Respir. Dis.*, **135**, 176–180.

GAMMON, C.M., ALLEN, A.C. & MORELL, P. (1989). Bradykinin stimulates phosphoinositide hydrolysis and mobilization of arachidonic acid in dorsal root ganglion neurons. *J. Neurochem.*, **53**, 95–101.

GREGA, D.S. & MACDONALD, R.L. (1987). Activators of adenylate cyclase and cyclic AMP prolong calcium-dependent action potentials of mouse sensory neurons in culture by reducing a voltage-dependent potassium conductance. *J. Neurosci.*, **7**, 700–707.

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

GROSS, R.A. & MACDONALD, R.L. (1989). Activators of protein kinase C selectively enhance inactivation of a calcium current component of cultured sensory neurons in a pertussis toxin-sensitive manner. *J. Neurophysiol.*, **61**, 1259–1269.

HALEY, J.E., DICKENSON, A.H. & SCHACHTER, (1989) Electrophysiological evidence for a role of bradykinin in chemical nociception. *Neurosci. Letts.*, **97**, 198–202.

HARGREAVES, K.M., TROULLOS, E.S., DIONNE, R.A., SCHMIDT, E.A., SCHAFER, S.C. & JORIS, J.L. (1988). Bradykinin is increased during acute and chronic inflammation: therapeutic implications. *Clin. Pharmacol. Ther.*, **44**, 613–621.

HIDAKA, H. & HAGIWARA, M. (1987). Pharmacology of the isoquinoline sulfonamide protein kinase C inhibitors. *Trends Pharmacol. Sci.*, **8**, 162–164.

HOCK, F.J., WIRTH, K., ALBUS, U., LINZ, W., GERHARDS, H.J., WIEMAR, G., HENKE, ST., BREIPOL, G., KONIG, W., KNOLLE, J. & SCHOLKENS, B.A. (1991). Hoe 140 a new potent and long acting bradykinin-antagonist: *in vitro* studies. *Br. J. Pharmacol.*, **102**, 769–773.

KEELE, C.A. & ARMSTRONG, D. (1964). *Substances Producing Pain and Itch*. London: Edward Arnold.

LANG, E., NOVAK, A., REEH, P.W. & HANDWERKER, H.O. (1990). Chemosensitivity of fine afferents from rat skin in vitro. *J Neurophysiol.*, **63**, 887–901.

MCGEHEE, D.S. & OXFORD, G.S. (1989). Bradykinin-induced currents in rat DRG neurons and F-11 cells are insensitive to pertussis toxin. *Soc. Neurosci.*, **15**, 217.

MCGUIRK, S.M., VALLIS, PASTERNAK, C.A. & DOLPHIN, A.C. (1989). Bradykinin enhances excitability in cultured rat sensory neurons by a GTP-dependent mechanism. *Neurosci. Lett.*, **99**, 85–89.

MENSE, S. & SCHMIDT, R.F. (1974). Activation of Group IV afferent units from muscle by algesic agents. *Brain Res.*, **72**, 305–310.

MILLER, R.J. (1987). Bradykinin highlights the role of phospholipid metabolism in the control of nerve excitability. *Trends Neurol. Soc.*, **10**, 226–228.

RANE, S.G., WALSH, M.P., MCDONALD, J.R. & DUNLAP, K. (1989). Specific inhibitors of protein kinase C block transmitter-induced modulation of sensory neuron calcium current. *Neuron.*, **3**, 239–247.

RANG, H.P. & RITCHIE, M. (1988). Depolarization of non-myelinated fibres of the rat vagus nerve produced by activation of protein kinase C. *J. Neurosci.*, **8**, 2606–2617.

REGOLI, D. & BARABE, J. (1980). Pharmacology of bradykinin and related kinins. *Pharmacol. Rev.*, **32**, 1–46.

REYNOLDS, M.L., FIZGERALD, M. & BENOWITZ, L.I. (1991). GAP-43 expression in developing cutaneous muscle nerves in the rat hindlimb. *Neuroscience*, **41**, 201–211.

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

STERANKA, L.R., FARMER, S.G. & BURCH, R.M. (1989). Antagonists of B_2 bradykinin receptors. *FASEB J.*, **3**, 2019–2025.

TAIWO, Y.O. & LEVINE, J.D. (1991). Further confirmation of the role of adenyl cyclase and of cAMP dependent protein kinase in primary afferent hyperalgesia. *Neuroscience*, **44**, 131–135.

TAMAOKI, T., NOMOTO, H., TAKAHASHI, I., KATO, Y., MORIMOTO, M. & TOMITA, F. (1986). Staurosporine, a potent inhibitor of phospholipid/Ca^{2+} dependent protein kinase. *Biochem. Biophys. Res. Commun.*, **135**, 397–402.

THAYER, S.T., EWALD, D.A., PERNEY, T.M. & MILLER, R.J. (1988). Regulation of calcium homeostasis in sensory neurons by bradykinin. *J. Neurosci.*, **8**, 4089–4097.

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

WEINREICH, D. (1986). Bradykinin inhibits a slow spike afterhyperpolarization of visceral sensory neurons. *Eur. J. Pharmacol.*, **132**, 61–63.

WEINREICH, D. & WONDERLIN, W.F. (1987). Inhibition of calcium-dependent spike after-hyperpolarization increases excitability of rabbit visceral sensory neurones. *J. Physiol.*, **394**, 415–427.

WILHELM, D.L. (1973). Chemical mediators. In *The Inflammatory Process*. 2nd edition. ed. Zweifach, B.W., Grant, L. & McLusky, R.T. pp. 251–301. New York: Academic Press.

(Received April 7, 1992
Revised July 14, 1992
Accepted August 12, 1992)