Bradykinin receptor number and sensitivity to ligand stimulation of mitogenesis is increased by expression of a mutant *ras* oncogene

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Summary

Bradykinin is a nine amino acid peptide of the kinin family believed to play a role in pain mediation and in the regulation of blood pressure, fluid balance and smooth muscle contraction. Here we demonstrate that bradykinin is also a potent mitogen for a mutant Ha-*ras*-transfected cell line, Rat 13. The Rat 13 cells display two binding sites for bradykinin: a moderate number (52 000) of high affinity sites (K_d=4.9 nM) co-exist with a much smaller number (1100) of very high-affinity sites (K_d=2.7 pM). Ligand binding stimulates mitogenesis through the lower affinity receptors, which are classified as B_2. These receptors are down-regulated in response to ligand. In contrast, Rat 1 cells (the cell line from which Rat 13 was derived) have only 4000 receptors per cell in total and respond weakly to bradykinin as a mitogen. Thus, expression of a mutant *ras* protein in Rat 13 cells increases their expression of the bradykinin receptor and their sensitivity to ligand stimulation of mitogenesis. Additional binding studies demonstrate that human A431 epithelial cells and Swiss mouse 3T3 fibroblasts also possess high-affinity sites for bradykinin.

Key words: bradykinin, receptor, *ras* transfection, mitogenesis.

Introduction

Polypeptide growth factors play an important role in the growth, development, maturation and normal functioning of many mammalian cell types. Recently, it has been suggested that locally produced peptides may also play a role in the control of cell proliferation (Zachary *et al.* 1987). Several such peptides have been discovered in neural tissue where they appear to be acting in a paracrine fashion as local hormones or as neurotransmitters (Lynch and Snyder, 1986). One of these, bombesin, has been demonstrated to have a mitogenic effect on Swiss 3T3 fibroblasts (Rozengurt and Sinnett-Smith, 1983).

Bradykinin is a nine amino acid peptide of the kinin family, a class of molecules considered to be locally acting hormones (Rocha E. Silva, 1970). Bradykinin is released during tissue damage by the action of members of the kallikrein family of proteolytic enzymes on the precursor kininogen proteins (Pisano, 1975), causing localized pain, inflammation and vasodilation as well as alterations in vascular permeability (reveiwed by Regoli and Barabé, 1980). Receptors for bradykinin have been reported on uterus, intestine, aorta, kidney, heart and spinal cord (Barabé *et al.* 1977; Regoli *et al.* 1977; Innis *et al.* 1981; Steranka *et al.* 1988) as well as on endothelial, epithelial, fibroblastic and neuronal cells *in vitro* (Derian and Moskowitz, 1986; Cox *et al.* 1986; Roscher *et al.* 1983; Snider and Richelson, 1984). Interestingly, there are suggestions that transformation of cells *in vitro* by the *ras* oncogene, an oncogene found mutated in a large number of human tumours (Lemoine, 1989), is associated with increased bradykinin receptor number (Parries *et al.* 1987; Downward *et al.* 1988). Taken with the known release of bradykinin at sites of tissue injury and inflammation (Hargreaves *et al.* 1988), this may imply a role for bradykinin in cell growth control during tumorigenesis.

Bradykinin receptors are defined as B_1 or B_2, according to the classification originally devised by Regoli and Barabé (1980). This system distinguishes between two different receptor types, according to their affinity for the bradykinin fragment des-Arg^9-BK and the bradykinin analogue [Tyr-(Me)^8]BK. Despite this classification, differing reports exist on the number of distinct receptor types as well as on their tissue distribution (Francel and Dawson, 1988; Snider and Richelson, 1984; Fredrick *et al.* 1985; Regoli and Barabé, 1980). Varing reports also exist on the coupling of bradykinin receptors to second messenger systems. Bradykinin is reported to stimulate a variety of intracellular events, such as elevation of inositol phosphate metabolism (Tilly *et al.* 1987; Jackson *et al.* 1987*a*) and activation of phospholipase C (Portilla *et al.* 1988) leading to production of diacylglycerol (Francel

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and Dawson, 1988) and Ca^{2+} release from intracellular sites (Snider and Richelson, 1984; Chuang and Dillon-Carter, 1988; Olsen *et al.* 1988). These events are thought to involve a GTP-binding protein (Burch and Axelrod, 1987; Voyno-Yasenetskaya *et al.* 1989), although the ability of pertussis toxin to interfere with these responses appears to vary (Jackson *et al.* 1987*b*; Portilla *et al.* 1988). Bradykinin is also implicated in the activation of phospholipase A_2 (Burch and Axelrod, 1987) and in the release of histamine and prostaglandins (Baries *et al.* 1983) as well as the accumulation of cyclic AMP and cyclic GMP (Fahey *et al.* 1977; Stoner *et al.* 1973).

Interpretation of the events that regulate bradykinin responsiveness requires an understanding of the interaction between bradykinin and its receptor, receptor–effector coupling systems and the biological effects of bradykinin. This can best be achieved by detailed analysis of ligand binding in an intact cell system that retains its ligand-mediated biological responses. We now report measurements of affinity and receptor number for bradykinin binding to Rat 1.Ha-*ras*13 cells (Rat 1 fibroblasts transfected with a *val*13 mutant Ha-*ras*; Downward *et al.* 1988) as well as to human A431 epithelial cells, untransfected Rat 1 fibroblasts and mouse Swiss 3T3 fibroblasts. These binding data are correlated to the ability of the ligands to stimulate mitogenesis. Data on response attenuation by possible receptor down-regulation are also presented.

Materials and methods

Materials

2,3-Prolyl-3,4-^{3}H(*N*)bradykinin (78.7 Ci mmol^{-1}) was obtained from New England Nuclear. [^{3}H]thymidine (26 Ci mmol^{-1}) was from Amersham. Bradykinin (BK), Lys-bradykinin (Lys-BK), Met-Lys-bradykinin (Met-Lys-BK), Tyr^0-bradykinin (Tyr^0-BK), Tyr^5-bradykinin (Tyr^5-BK), Tyr^8-bradykinin (Tyr^8-BK) and des-Arg^9-bradykinin (des-Arg^9-BK) were purchased from Sigma (see Table 1 for peptide structures). *N*-methyl-D-glucamine, bovine serum albumin (BSA), transferrin and MDCB 100 medium were from Sigma. Insulin solution (3.6 mg ml^{-1}) was from NOVO, Denmark. All salts and buffers were from Sigma unless stated otherwise. All tissue culture media and trypsin solutions were provided by ICRF Central Services unless stated otherwise.

Table 1. *Estimations of the binding affinity for a variety of bradykinin analogues compared with the concentration of peptide required for stimulation of mitogenesis*

Peptide	Structure	K_d (nM)	Half-maximal stimulation (nM)
BK	R-P-P-G-F-S-P-F-A	1.4	1.1
Lys-BK	K-R-P-P-G-F-S-P-F-A	1.1	2.2
Met-Lys-BK	M-K-R-P-P-G-F-S-P-F-A	2.5	3.6
Tyr^0-BK	Y-R-P-P-G-F-S-P-F-A	3.2	5.0
Tyr^5-BK	R-P-P-G-Y-S-P-F-A	15.8	22.4
Tyr^8-BK	R-P-P-G-F-S-P-Y-A	40	31.6
des-Arg^9-BK	R-P-P-G-F-S-P-F	>10 000	>1000

Affinities are taken from the competition curves shown in Fig. 3, and half-maximal stimulation of mitogenesis is taken from the thymidine incorporation curves shown in Fig. 6.

Cell culture

Rat 1 and Rat 13 fibroblasts were gifts from Dr Michael Fried ICRF, London, and Dr Julian Downward, Whitehead Institute, USA, respectively. Rat 13 cells are not transformed, in that they give no colonies in soft agar assays and are unable to form foci on a monolayer of parental Rat 1 cells (Downward *et al.* 1988). However, they do display phenotypic differences from Rat 1 cells when grown in culture. Swiss 3T3 fibroblasts and A431 epithelial cells were obtained from ICRF Central Services, London. Stock cultures were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% foetal calf serum (FCS), with the exception of A431 cells, which were grown in RPMI supplemented with 10% FCS. For [^{3}H]-bradykinin binding assays, subcultures were initiated with 10^5cells/15 mm well in 24-well plates and used 4–7 days later at confluence. For [^{3}H]thymidine incorporation assays, subcultures of Rat 13 cells were initiated with 5×10^4 cells per 15 mm well in 10% FCS in DMEM. After 24 h cells were changed into MDCB 100 medium supplemented with 1 μg ml^{-1} insulin and 10 μg ml^{-1} transferrin. Culture media were changed on a daily basis for 5 days, at which point the cultures were partially growth arrested and approximately 80% confluent. For Rat 1 cells, subcultures were initiated with 10^5 cells/well. After around 7 days these cultures were confluent and quiescent.

[^{3}H]bradykinin binding studies

Unless otherwise stated, all binding studies were carried out at 0–4°C, with the cell culture dishes standing on ice. Growth medium was removed and 1 ml of binding buffer (Hanks' Balanced Salts Solution with 120 mM-NaCl replaced by *N*-methyl-D-glucamine: 17 mM-NaCl, 5.4 mM-KCl, 0.44 mM-KH_2PO_4, 0.63 mM-$CaCl_2$, 0.205 mM-$MgSO_4.H_2O$, 0.338 mM-Na_2HPO_4, 110 mM-*N*-methyl-D-glucamine, 20 mM-Hepes, pH 7.3, supplemented with 0.1% bovine serum albumin and 0.2 mM-bacitracin) was added per well. Plates were then equilibrated on ice for 10 min, after which the binding buffer was removed and replaced with 0.15 ml of binding buffer containing the appropriate concentration of [^{3}H]bradykinin with or without a 500-fold excess of unlabelled bradykinin. For competition studies, binding medium containing 1 nM-[^{3}H]bradykinin was added to each well together with the appropriate concentration of competing ligand. At the indicated time thereafter, binding medium was removed and cells were rinsed twice with 1 ml of binding medium at 4°C. Cells were then dissolved in 0.2 ml of 2% Na_2CO_3, 1 M-NaOH and total activity per well was assessed by scintillation counting using 4 ml of Optiphase 'Safe' (LKB products).

Determination of cellular distribution of [^{3}H]bradykinin

Membrane-bound *versus* intracellular ligand was measured using the acid–salt wash technique of Haigler *et al.* (1980). After incubation with [^{3}H]bradykinin, cultures of Rat 13 cells were washed twice in medium, then treated for 6 min at 4°C with 0.2 ml of 0.2 M-acetic acid containing 0.5 M-NaCl, pH 2.5, to remove membrane-bound [^{3}H]bradykinin. This solution was then removed and the cells dissolved as described previously. Intracellular and membrane fractions were assessed by scintillation counting as described previously.

Assay of DNA synthesis

Quiescent cultures of Rat 13 or Rat 1 cells were washed twice with MDCB 100 medium, then incubated at 37°C in MDCB

100 medium containing 1 μCi ml^{-1} of [^{3}H]thymidine ([^{3}H]dThd). Other additions were as indicated. Positive controls included 10% FCS and negative controls had no additions (except transferrin and insulin). After the indicated times, cells were washed twice in PBS, then extracted for 30 min in trichloroacetic acid. The remaining acid-insoluble material was then counted for [^{3}H]thymidine content.

Analysis of binding data

Data from [^{3}H]bradykinin association experiments were analysed using the 'EBDA/LIGAND' collection of radioligand binding analysis programmes (Elsevier-BIOSOFT, Cambridge, UK) written by P. J. Munson and D. Rodbard and modified for microcomputers by G. A. McPherson. These programmes use a weighted non-linear least-squares curve-fitting technique to give final parameter estimates with associated errors for K_d and number of binding sites (Munson and Rodbard, 1980; McPherson, 1985). A partial F-test is incorporated into the programme, which gives an indication of the best fit (i.e. one-site *versus* two-site). Data intended for analysis by these programmes were collected as disints min^{-1}, due to the requirements of the programme. In the β-counter used for these experiments, 1 ct min^{-1} (^{3}H)=4.9 disints min^{-1}.

Results

Bradykinin binds to Rat 13 cells in a specific and saturable manner

[^{3}H]bradykinin bound to Rat 13 cells in a specific and saturable manner (Fig. 1A), with binding reaching a maximum after around 40 min (Fig. 1B). The [^{3}H]-bradykinin dissociated very slowly from its receptor ($t_{\frac{1}{2}}$=180 min, data not shown), displaying the same dissociation rate in either the presence or absence of excess cold ligand. This indicates no apparent cooperativity of binding. Scatchard transformation of specific binding data gave a curvilinear plot (Fig. 1C). A one-line model fitted to these data gave a K_d of 1.25 nM and 36 000 receptors per cell but with a very poor fit. A two-line model fitted to these data as shown gave a significant (P=0) improvement in fit and indicated two binding sites with K_d values of 4.9 nM and 2.7 pM, and 51 800 and 1000 receptors per cell, respectively. The existence of two binding sites was confirmed by Scatchard analysis of competition data, a technique that gives more accurate results for the first few points of a Scatchard plot (Klotz, 1982). These experiments (Fig. 2) indicated two classes of binding site with K_d values of 5.2 nM and 1.8 pM, and 48 000 and 2200 receptors per cell, respectively. Averaging the results from the two different types of experiments gives K_d values of 5.1 nM and 2.3 pM, with 50 000 and 1600 receptors per cell, respectively.

Bradykinin analogues compete for binding

In order to determine the receptor type, the order of affinity for a variety of bradykinin analogues was determined by competition with [^{3}H]bradykinin for binding to Rat 13 cells (Fig. 3). Analysis of these competition curves indicated a binding affinity order of Lys-BK>BK>Met-Lys-BK>Tyr-0-BK>Tyr-5-BK>Tyr-8-BK≥des-Arg-BK (Table 1), indicating that the receptor was of the B_2 type. To provide additional confirmation of the

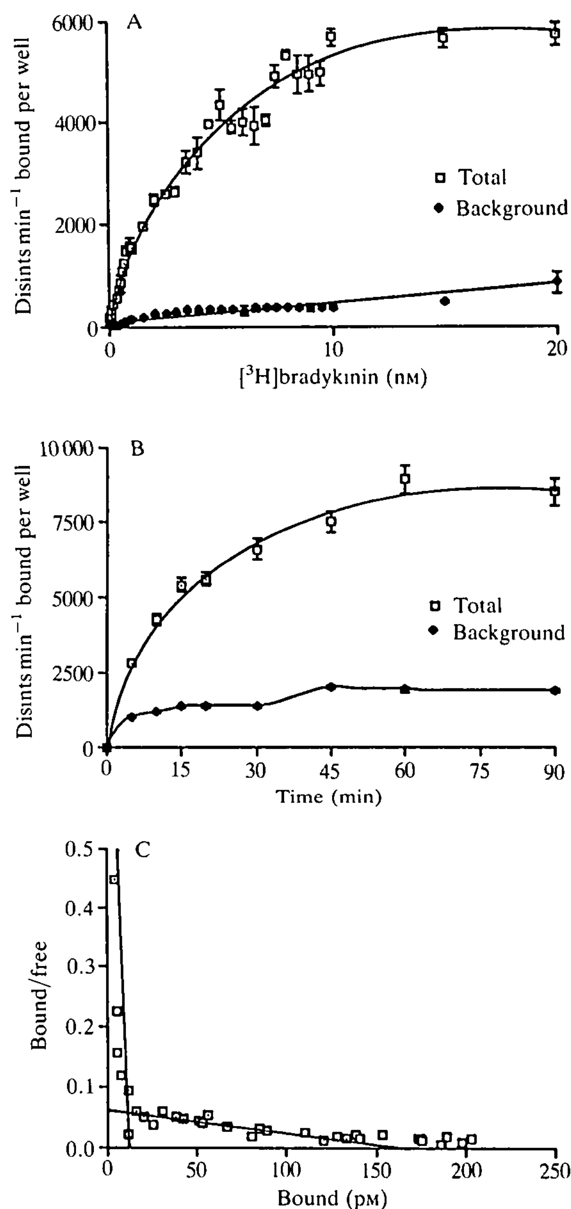
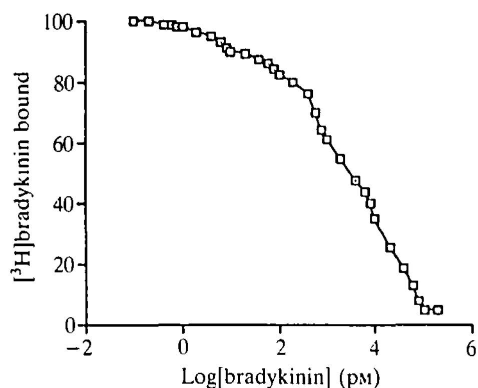


Fig. 1. [^{3}H]bradykinin binding to Rat 13 cells at 4°C using 4×10^5 cells per well. Background binding was measured in the presence of a 500-fold excess of cold ligand and subtracted from total binding to give specific binding. A. Increasing binding with increasing ligand concentration measured at 1½ h. The concentration range used was 0.01 nM (total disints min^{-1} bound=130±24; background disints min^{-1} bound=20±1) to 20 nM (total disints min^{-1} bound=5736±239; background disints min^{-1} bound=854±200). Data points represent triplicate points from three separate experiments. B. Time course of association of 10 nM-[^{3}H]bradykinin at 4°C. C. Scatchard transformation of the specific binding data shown in A. A one-site model fitted to these data indicates a K_d=1.3±5.13 nM and 36 000±4000 receptors/cell. A two-site model fitted to these data gives an improved fit and indicates K_d values of 4.9±2.4 nM and 2.7±1.4 pM, and 52 000±2900 and 1000±180 receptors per cell, respectively.



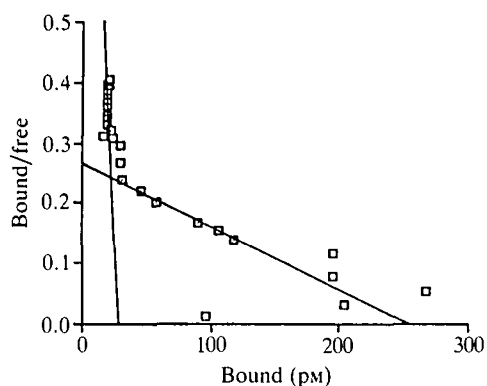


Fig. 2. Competition for [^{3}H]bradykinin binding to Rat 13 cells using increasing concentrations of cold bradykinin. A. Experiments were carried out at 4°C and binding of the 1 nM tracer measured at 1½ h. Points represent triplicate determinations from two separate experiments. Error bars have been omitted for clarity but were no more than ±4 % at any point. Maximum and background binding averaged 2250 and 110 disints min^{-1} per well, repectively. B. Scatchard transformation of the dissociation data. A two-line model gave a good fit, indicating two sites with K_d values of 5.2 nM and 1.8 pM, and 48 000 and 2200 sites per cell, respectively.

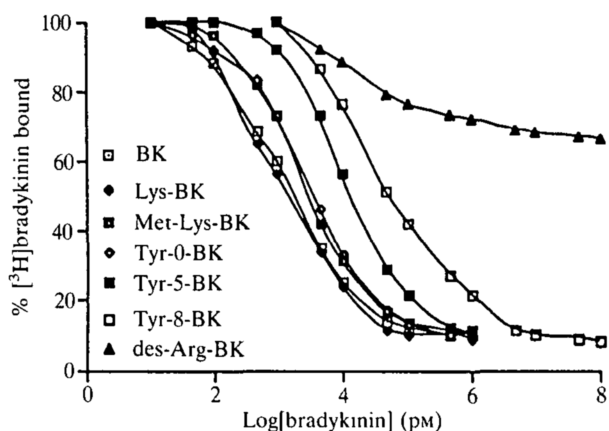


Fig. 3. Competition of binding of [^{3}H]bradykinin to Rat 13 cells by increasing concentrations of bradykinin analogues. Experiments were carried out at 4°C and binding of the 1 nM tracer was measured at 1½ h. Points are means of three determinations. Error bars have been omitted for clarity but were no more than ±4 % at any point. Maximum and background binding were 1900 and 100 disints min^{-1} per well, respectively.

Table 2. *Assessment of factors for ability to compete for binding with [^{3}H]bradykinin*

Competing ligand (10^{-7} M)	% [^{3}H]bradykinin bound
None	100
BK	14±2
EGF	97±3
PDGF	81±7
Alpha-FGF	91±5
TGF-alpha	93±4
TGFβ	75±11
IGF$_1$	83±7
Oxytocin	99±5
Bombesin	96±4
Gastrin releasing peptide	92±6
Insulin	92±4

specificity of bradykinin binding, several structurally unrelated peptides and growth factors were also assessed for their ability to compete for binding (Table 2). These were all unable to compete in this assay.

Rat 1 cells have fewer binding sites for bradykinin

[^{3}H]bradykinin also binds to the parental Rat 1 fibroblasts in a specific and saturable manner (Fig. 4). However, Rat 1 cells display around ten times fewer receptors (approximately 4000 per cell) than Rat 13 cells as calculated from total disints min^{-1} bound to Rat 1 cells at saturation. This low number of receptors makes Scatchard transformation very difficult, preventing an accurate estimate of the K_d and number of receptors per cell. However, from the concentration of bradykinin required to give half-maximal binding, the K_d was estimated to be around 5 nM.

Bradykinin also binds to A431 epithelial cells and 3T3 fibroblasts

In order to compare the affinity and number of receptors on other cell types and in different species we analysed bradykinin binding to human A431 epithelial cells and mouse Swiss 3T3 fibroblasts. Specific binding was observed, indicating that these two cell lines possess 40 000 and 82 000 receptors per cell, respectively (Fig. 5). In both cases, a one-site model gave the best fit after Scatchard transformation with affinities of 7.3 and 3.1 nM, respectively. Neither cell type appears to possess the second very high-affinity site displayed by Rat 13 cells.

Bradykinin is a potent mitogen for Rat 13 cells

Bradykinin and its various analogues stimulate DNA synthesis in Rat 13 cells, but only in the presence of insulin. DNA synthesis was observed approximately 8 h after ligand addition and was maximal after 32 h (Fig. 6A). The ability of the bradykinin analogues to stimulate mitogenesis shows a clear correlation with their relative affinities for the bradykinin receptor (Fig. 6B). In all cases, the concentration of peptide required for

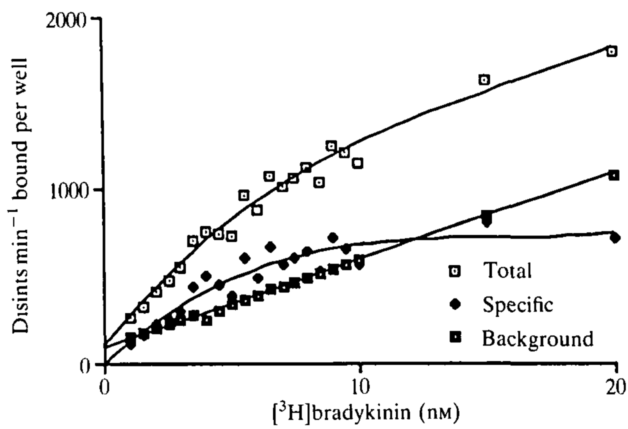


Fig. 4. [^{3}H]bradykinin binding to Rat 1 cells. Binding was measured after $1\frac{1}{2}$ h at 4°C using 4×10^5 cells per well. Each point is the mean of three observations. Error bars have been omitted for clarity, but were no more than ±9 % in any case. The specific binding curve indicates a K_d of around 5 nM and maximum binding of around 620 disints min^{-1} per 4×10^5 cells, which is equivalent to around 4000 receptors per cell.

half-maximal stimulation of [^{3}H]thymidine incorporation (Table 1) is similar to the K_d for ligand binding to the high-affinity site, indicating that the mitogenic signal is being transmitted through specific receptor binding. However, it should be noted that no mitogenic stimulus is transmitted at bradykinin concentrations sufficiently high to give 100 % occupation of the very high-affinity site found on these cells.

In contrast to Rat 13 cells, the ability of bradykinin to stimulate mitogenesis in Rat 1 cells is limited (Fig. 6C). Maximal stimulation gives only 21 % of the positive control (10 % FCS) compared with 103 % seen in Rat 13 cells.

Prolonged exposure of cells to bradykinin causes temperature-dependent receptor down-regulation and ligand internalization

Exposure of cells to bradykinin caused a sharp increase in the amount of surface-associated ligand (Fig. 7), which peaked at a higher level in cells at 22°C than at 4°C, with binding at 37°C intermediate between the two. This was followed by a slow decline in surface binding coupled with internalization of the ligand. A steady accumulation of cell-associated ligand at 22°C was seen, whereas at

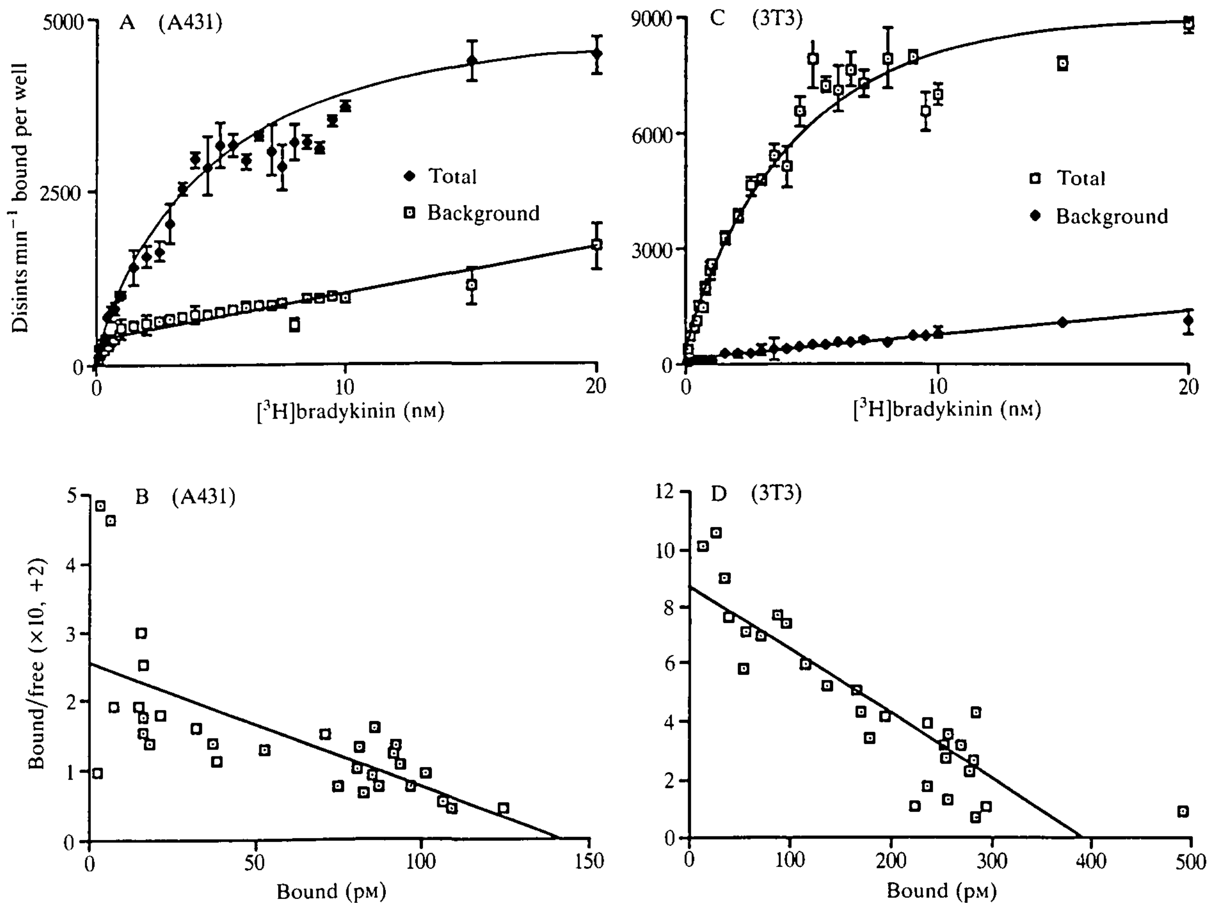


Fig. 5. [^{3}H]bradykinin binding to: A, A431 cells; and C, 3T3 cells. Binding was measured after $1\frac{1}{2}$ h at 4°C using 4×10^5 cells/well. Each point is the mean of three measurements ±S.E.M. B. Scatchard transformation of data for A431 cells indicates a single class of binding site with a K_d=7.3±3.0 nM and 40 000±7000 receptors cell. D. Scatchard transformation of data for 3T3 cells indicates a single class of binding site with a K_d=3.1±3.0 nM and 82 000±5000 receptors per cell.

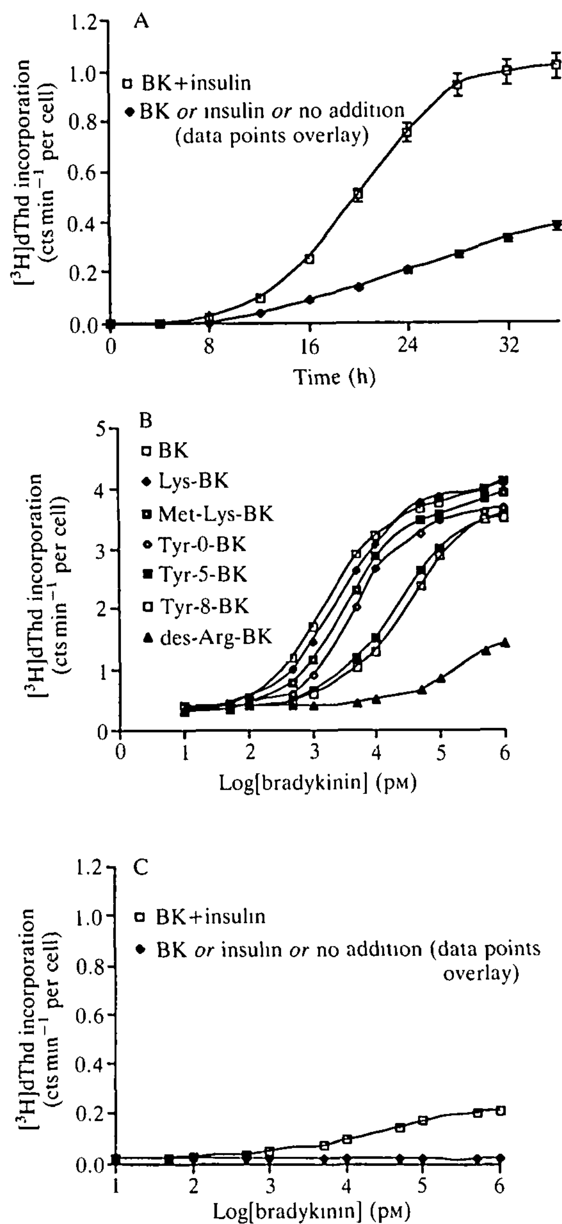


Fig. 6. Bradykinin (BK) stimulation of DNA synthesis in Rat 13 and Rat 1 cells. A. Time course of [^{3}H]thymidine (dThd) incorporation in Rat 13 cells in the presence or absence of 10 nM-BK or 1 μg ml^{-1} insulin. Points are averages of three determinations ±S.E.M. B. [^{3}H]dThd incorporation in Rat 13 cells after 32 h in the presence of 1 μg ml^{-1} insulin and increasing concentrations of bradykinin analogues. Positive control (10% FCS)=1.012 cts min^{-1} per cell and negative control (no addition)=0.085 cts min^{-1} per cell. Error bars have been omitted for clarity but were no more than ±6%. C. [^{3}H]dThd incorporation in Rat 1 cells after 32 h in the presence of 1 μg ml^{-1} insulin and increasing concentrations of bradykinin. Positive control (10% FCS)=1.120 cts min^{-1} per cell and negative control (no addition)=0.021 cts min^{-1} per cell. Points are average of three determinations ±S.E.M.

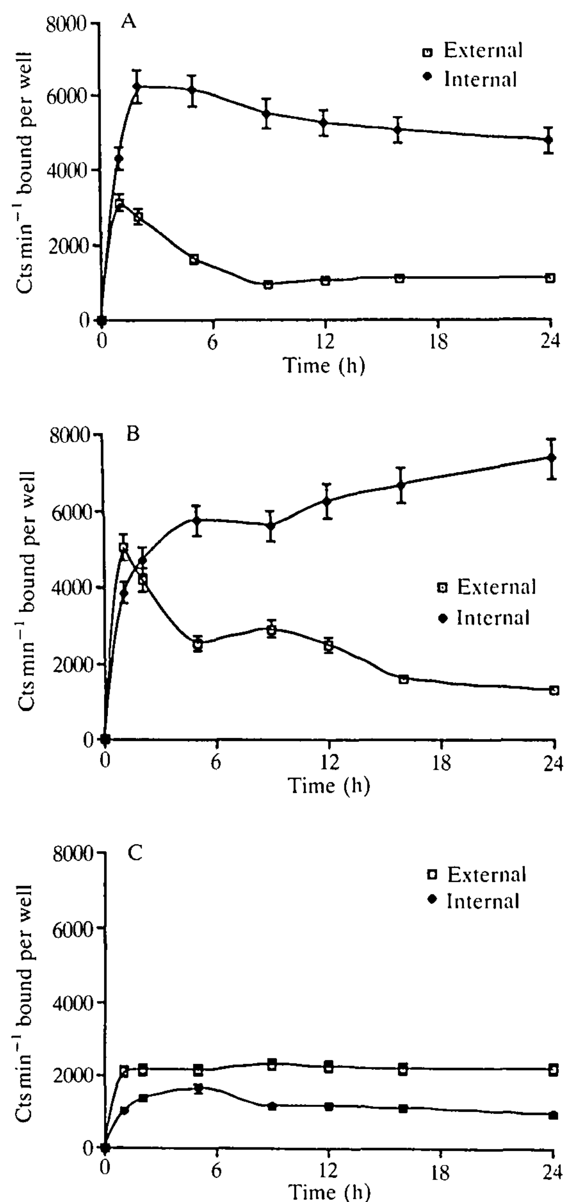


Fig. 7. Comparison of internalized and membrane-bound bradykinin in Rat 13 cells incubated at: A, 37°C; B, 22°C; C, 4°C. Each point is the average of three determinations ±S.E.M.

37°C there was a net degradation. The ligand internalization seen at 4°C was minimal.

Since endocytotic uptake of environmental ligands is diminished at 4°C (King and Cuatrecasas, 1981), these data suggest that bradykinin is being rapidly internalized by the Rat 13 cells in a temperature-dependent manner. The observed differences in ligand accumulation suggest that the amount of ligand measured in the cells is a balance between internalization and degradation, the latter process also being temperature dependent.

In order to determine whether this decline in surface binding was due to receptor down-regulation or to proteolytic degradation of the ligand, fresh ligand was added to cells maintained in the presence of bradykinin at 37°C for 9 h. This led to no new ligand binding (Fig. 8), suggesting that the pre-treatment causes receptor down-regulation.

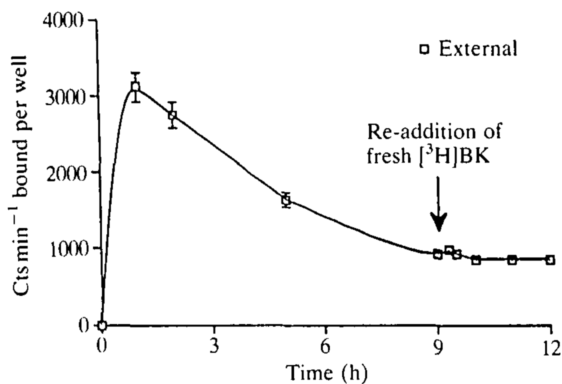


Fig. 8. Binding of bradykinin to Rat 13 cells at 37°C during a 9-h incubation, followed by a further 3-h incubation after addition of fresh ligand. Each point is the average of three determinations ±S.E.M.

Discussion

The data presented here clearly demonstrate that bradykinin binds to Rat 13 cells in a specific and saturable manner. This binding is mediated through two classes of receptor: a moderate number of binding sites with an affinity of around 5 nM co-exists with a much smaller number of very high-affinity sites (K_d=2 pM). Studies of relative affinity for a series of bradykinin analogues indicate that the category of receptors forming the majority of the binding sites are of the B_2 type, according to the classification of Regoli and Barabé (1980).

Mouse 3T3 fibroblasts and human A431 epithelial cells also display high-affinity receptors (K_d=5 nM) for bradykinin at a number comparable to that seen on Rat 13 cells. However, the very high-affinity site (K_d=2 pM) seen on Rat 13 cells appears to be absent or expressed at a level below the detection thresholds of the binding technique. Thus, there appears to be variability in the number and type of receptors expressed on the different cell lines tested. This finding is consistent with the available information: the majority of reports from other laboratories indicate only one class of B_2 bradykinin receptor with a K_d of 0.7–5 nM, although there are reports of two types of B_2 receptor on intestinal epithelia and primary rat brain cultures (Manning *et al.* 1986; Lewis *et al.* 1985) and three B_2 receptor types on a neuroblastomal cell line (Snider and Richelson, 1984).

Pharmacological evidence supports the idea of more than one type of B_2 bradykinin receptor (reviewed by Plevin and Owen, 1988). This evidence falls into two main categories: (1) experiments where different biological effects of bradykinin, such as phospholipase C and phospholipase A_2 activation, can be shown to be totally independent in the same cell (Burch and Axelrod, 1987; Conklin *et al.* 1988); and (2) experiments where specific bradykinin antagonists can be shown to be agonists in some B_2 receptor systems (Braas *et al.* 1988).

Bradykinin and a series of ligand analogues are able to stimulate DNA synthesis in Rat 13 cells at a concentration clearly correlated to their binding affinities. This suggests that bradykinin is acting on Rat 13 fibroblasts to cause mitogenesis and that this biological effect is being mediated principally through the receptor, which displays a K_d of around 5 nM. However, it is difficult so far to ascribe a physiological role to the very high-affinity receptors, since there is no positive evidence for their involvement in this stimulation of cell proliferation.

The stimulation of DNA synthesis seen in response to bradykinin only takes place in the presence of insulin. Neither of these ligands alone constitutes a mitogenic stimulus, but when added together a synergistic effect is seen. It has previously been suggested that ligands whose receptors act through a G-protein may synergise with ligands that act to elevate cyclic AMP levels to produce a combined signal, leading to DNA synthesis (Rozengurt and Mendoza, 1985). Although insulin is not thought to elevate cyclic AMP levels (Rozengurt, 1986), it may be acting to stimulate some other second messenger pathway that can synergize with bradykinin. Interestingly, both interleukin 1 (IL1) and epidermal growth factor (EGF) have also been demonstrated to synergise with bradykinin. Thus it appears that the bradykinin receptor may be involved in a series of inter-receptor and messenger transmodulation events that combine to mediate a cellular response. A thorough analysis of the synergistic activities of other ligands on the biological effects of bradykinin needs to be undertaken in order to understand these effects fully.

The data presented here support the idea that multiple bradykinin receptors may be involved in the various bradykinin-mediated responses. Recently, a pattern has been emerging whereby receptors for small peptide ligands belong to a family of structurally related molecules that interact with the same or structurally related ligands. Examples of this include adrenergic, muscarinic and serotonin receptors. The possibility that multiple bradykinin receptors exist, taken together with the evidence that they are coupled to G-proteins (Burch and Axelrod, 1987; Voyno-Yasenetskaya *et al.* 1989), suggests that receptors for bradykinin may belong to this super family of homologous receptors, characterized by their seven alpha-helical transmembrane regions (Dohlman *et al.* 1987; Libert *et al.* 1989) and their ability to couple to guanosine nucleotide-binding proteins.

The observation that *ras* transfection increases the number of bradykinin receptors has previously been reported by Parries *et al.* (1987) and by Downward *et al.* (1988). The *dbl* (*h*uman *d*iffuse *B*-cell *l*ymphoma) oncogene is also reported to increase the expression of bradykinin receptors in NIH 3T3 cells, whereas other oncogenes (v-*src*, v-*abl*, v-*mos*, v-*raf*, v-*fos*) did not have this effect (Ruggiero *et al.* 1989). Downward *et al.* (1988) did not investigate whether increased receptor expression leads to increased stimulation of mitogenesis, whereas Ruggiero *et al.* (1989) did examine this effect and found ligand stimulation of phosphatidylinositol turnover but no stimulation of mitogenesis. The omission of insulin from the incubation medium may have been responsible for the failure to observe mitogenic stimulation by bradykinin in this case. Although we have only demonstrated increased sensitivity to ligand stimulation of mitogenesis in one *ras*-transfected cell line so far, the

increase in receptor number occurs for several such cell lines (Downward *et al.* 1988) and is reported to be a *ras*-specific event (rather than a general effect of transformation), occurring after overexpression of each of the three normal *ras* gene products (Ha-, K- and N-) as well as after expression of the three activated *ras* gene products. We are currently studying receptor number effects and ligand stimulation of mitogenesis in several other *ras*-transfected cell lines.

It has been suggested that *ras* transformation improves the responsiveness of cells to certain ligands by improving receptor–G-protein coupling (Wakelam *et al.* 1986; Chiarugi *et al.* 1986), although the mechanism of this is not clear. However, in the case of Rat fibroblasts, *ras* transfection appears to effect the actual number of bradykinin receptors expressed on the cell surface. A similar observation has been reported for the *dbl* oncogene (Ruggiero *et al.* 1989). This raises the possibility that both the *ras* protein and p66 *dbl* protein (Srivastava *et al.* 1986) are having a direct influence on the regulation of receptor expression at the cell surface and/or a direct effect on receptor gene expression. A potential mechanism for this latter possibility is provided by recent findings that the c-Ha-*ras* oncogene activates both the transcription factor PEA1 and the polyoma virus (Py) enhancer (Wasylyk *et al.* 1987, 1988). Wasylyk and coworkers propose that this activation may lead to altered transcription of transformation-related genes and could play a key role in tumorigenesis. Our findings suggest that the bradykinin receptor gene may be one of the many possible targets for this effect.

Whatever the mechanisms of the *ras*-induced increase in receptor number, the net result is that Rat 13 cells respond strongly to bradykinin as a mitogen whereas Rat 1 cells do not. Basal levels of bradykinin in the blood or those measured during various models of inflammation (15 pM and 67 pM respectively; Bouscarel *et al.* 1988) are well below the measured K_d for bradykinin receptor-mediated mitogenesis in Rat 13 cells. However, the local concentrations of bradykinin in inflamed tissue (where levels in subcutaneous perfusates reach 1 nM during the course of inflammation; Joris *et al.* 1987) are in the correct range to trigger a mitogenic response to bradykinin. This raises the possibility that bradykinin released in the reactive inflammation to malignant invasion could stimulate proliferation of some tumour cells. This may be particularly pertinent to those containing activated *ras* or *dbl* oncogenes. Many antagonists for bradykinin exist that might be exploited as specific therapeutic agents in such cases. An analysis of the kinetics of receptor number increase during *ras* transfection may contribute to an understanding of these processes. Studies are underway using cells transfected with an inducible *ras* oncogene to examine this effect and the processes by which the 5 nM-bradykinin binding site disappears during ligand exposure.

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