Bradykinin Receptors Undergo Ligand-Induced Desensitization

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ABSTRACT: Bradykinin binds to specific cell surface receptors on Rat13 fibroblasts with a high affinity (2.1 nM). Prolonged exposure of cells to the ligand causes a concentration-dependent decline in surface levels of the 2.1 nM receptor from 40 000 receptors per cell to undetectable levels with a $t_{1/2}$ of approximately 2 h. The decline occurs in parallel with the appearance of an equal number of lower affinity binding sites (40 nM), suggesting that ligand exposure causes desensitization by an alteration in receptor affinity. The affinity change is characterized by a faster rate of ligand dissociation while the rate of association remains unaltered. The observed desensitization is dependent on the presence of active cellular metabolism since (i) it does not occur in whole cells maintained at 4 °C and (ii) membranes prepared from Rat13 cells retain their high-affinity sites at 37 °C despite extensive ligand exposure.

Bradykinin is a small peptide ligand that acts through a specific cell surface receptor to cause a variety of intracellular events [reviewed in Roberts (1989)]. These include elevations in cytoplasmic Ca^{2+} concentration and an increase in inositol phosphate metabolism. Bradykinin can also act as a mitogen in the presence of insulin (Roberts & Gullick, 1989).

Receptors for bradykinin are classified as B_1 or B_2 according to the system originally devised by Regoli and Barabé (1980). This classification distinguishes two different receptor types according to their affinity for the bradykinin fragment des-Arg^9-BK and the bradykinin analogue [Tyr(Me)^8]BK. The B_2 type of receptor has been identified on endothelial, epithelial, fibroblastic, and neuronal cells in vitro (Derian & Moskowitz, 1986; Cox et al., 1986; Roscher et al., 1983; Snider & Richelson, 1984). Various subtypes of B_2 receptors with affinities ranging from 2.1 pM to 14 nM have been reported. We have recently shown that Rat13 fibroblasts (Rat1 fibroblasts transfected with an activated HaRas oncogene) possess two types of B_2 receptor: a relatively large number of high-affinity receptors (K_d = 4.9 nM) coexist with a much smaller number of very high affinity receptors (K_d = 3 pM). The high-affinity receptors appear to be downregulated in response to ligand at physiological and at room temperature but not at 4 °C, suggesting that receptors are internalized during ligand exposure (Roberts & Gullick, 1989). This hypothesis was supported by the observation that binding of fresh ligand in ligand-pretreated cells was minimal. However, in the context of recent reports of receptor-response attenuation by desensitization (Benovic et al., 1986; Bouvier et al., 1987; Kwatra & Hosey, 1986; Sibley & Lefkowitz, 1985), it is possible that bradykinin receptors may be undergoing a similar sort of agonist-induced desensitization rather than a reduction in surface receptor numbers. We now report such a decrease in the affinity of receptors for bradykinin during ligand exposure.

Experimental Procedures

Materials. [Pro^{2,3}][3,4-^{3}H(N)]bradykinin (78.7 Ci/mmol) was obtained from New England Nuclear. Bradykinin (BK), *N*-methyl-D-glucamine, and BSA were purchased from Sigma. All salts and buffers were from Sigma unless otherwise stated. All tissue culture media and trypsin solutions were provided by ICRF Central Services unless otherwise stated.

Cell Culture. Rat13 fibroblasts were a gift from Dr. Julian Downward, ICRF, London. Rat13 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 Rat1 cells (Downward et al., 1988). However, they do display phenotypic differences from Rat1 cells when grown in culture. Stock cultures were grown in Dulbecco's modification of Eagles' medium (DMEM) supplemented with 10% fetal calf serum (FCS). For [^{3}H]bradykinin binding assays, subcultures were initiated with 10^5 cells/15-mm well in 24-well plates and used 4–7 days later at confluence.

Preparation of Membranes. Confluent monolayers of Rat13 cells were washed in PBS at 37 °C and then equilibrated to 4 °C on ice. Cells were then scraped off into ice-cold PBS with a rubber policeman and washed by centrifugation. The cell pellet was then homogenized in homogenization buffer (10 mM Hepes buffer, pH 7.5, containing 10 mM NaCl and 2 mM EDTA) with a hand-held glass homogenizer. The homogenate was centrifuged at 50*g* for 5 min to remove whole cells and then the supernatant centrifuged at 55000*g* for 30 min to pellet the membranes. The membrane preparation was resuspended in storage buffer (50 mM Hepes, pH 7.5, containing 100 mM NaCl) and the protein concentration measured by the Bradford (1976) method. Membranes were used immediately or stored at −20 °C in aliquots until required. Fresh and frozen membranes gave identical results in the experiments described.

Preexposure to Bradykinin. Confluent monolayers of Rat13 cells or aliquots of Rat13 cell membranes were washed in DMEM and then treated with DMEM containing the appropriate concentration of unlabeled bradykinin (0.01–100 nM) at 37 or 4 °C for the indicated times. The monolayers or membrane aliquots were then washed in PBS and equilibrated on ice prior to acid washing to remove surface-bound ligand. Ligand breakdown during incubation was minimal as monitored by HPLC.

Removal of Surface-Bound Ligand. After preexposure of cells or membranes to bradykinin, surface-bound ligand was removed with the acid–salt wash technique of Haigler et al. (1988). Briefly, the samples were washed in PBS at 37 °C,

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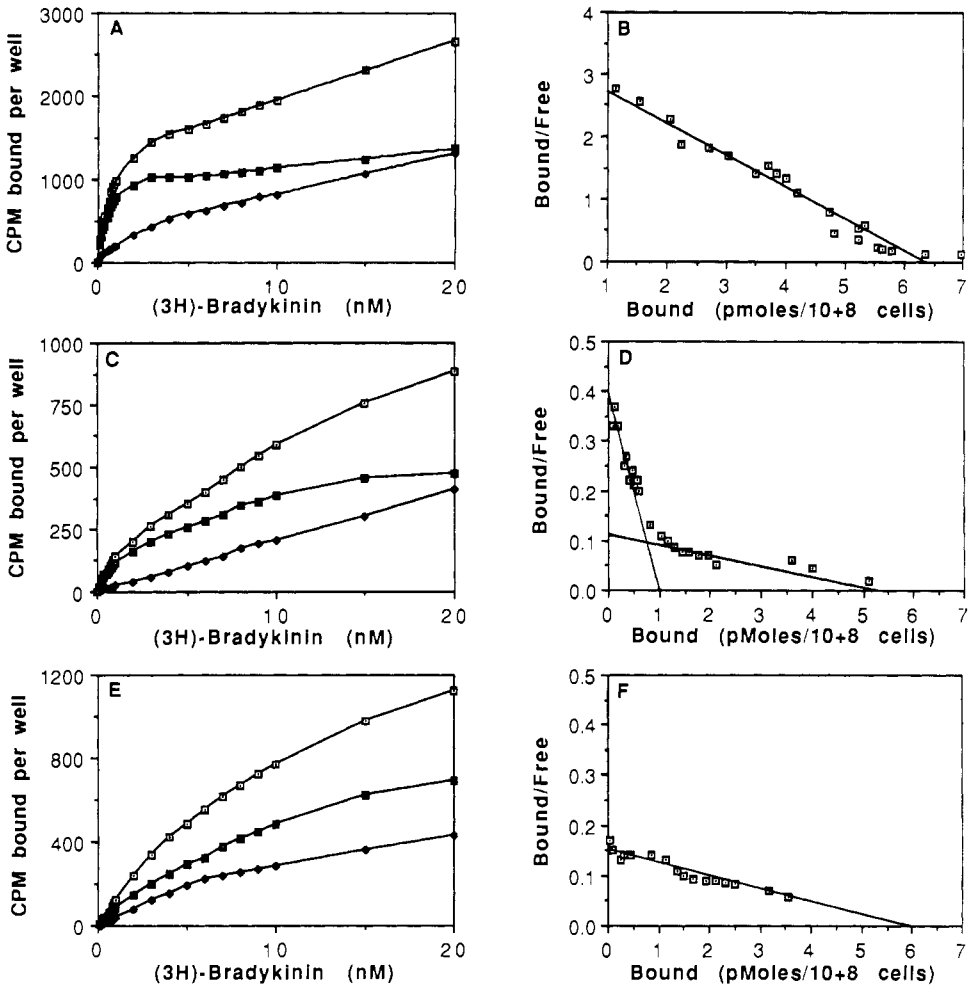

FIGURE 1: Association curves showing [^{3}H]bradykinin binding to Rat13 cells at 4 °C with 4 × 10^5 cells per well after (A) 0, (C) 2.5, and (E) 5 h of ligand preexposure carried out at 37 °C. Background binding (◆) was measured in the presence of a 500-fold excess of cold ligand and subtracted from total binding (⊡) to give specific binding (■). Error bars have been omitted for clarity but were no more than ±6%. Data points represent triplicate points from three separate experiments. (B), (D), and (F) show the Scatchard transformations of the data shown in (A), (C), and (E), respectively. A one-site model gives the best fit after 0 h of preexposure (B), indicating 38 500 sites per cell with an affininity of 2.1 nM. A two-site model gives the best fit after 2.5 h of preexposure (D), indicating 6000 and 31 900 sites per cell with affinities of 2.5 and 49 nM, respectively. A one-site model gives the best fit after 5 h of preexposure (F), indicating 36 700 sites per cell with an affinity of 41 nM.

equilibrated to 4 °C on ice, and then incubated at 4 °C for 6 min in 0.2 mL of 0.2 M acetic acid containing 0.5 M NaCl, pH 2.5. Our own control experiments indicate that this technique removes 95–99% of surface-bound [^{3}H]bradykinin from these cells.

[^{3}H]Bradykinin Binding Studies. Cell monolayers or membrane aliquots were washed in 1 mL of ice-cold binding buffer (20 mM Hepes, pH 7.3, containing 17 mM NaCl, 5.4 mM KCl, 0.44 mM KH_2PO_4, 0.63 mM $CaCl_2$, 0.21 mM $MgSO_4$, 0.34 mM Na_2HPO_4, and 110 mM *N*-methyl-D-glucamine supplemented with 0.1% bovine serum albumin and 0.2 mM bacitracin). No kininase II (the enzyme involved in bradykinin breakdown) inhibitors were included since their addition to early pilot experiments made no difference to the outcome and HPLC analysis indicated minimal ligand breakdown. The samples were placed on ice, and the buffer was replaced with 0.15 mL of binding buffer containing the appropriate concentration (0.1–20 nM) of [^{3}H]bradykinin with or without a 500-fold excess of unlabeled bradykinin. At the indicated time thereafter, binding medium was removed, and the monolayers or membranes were rinsed twice with 1 mL of binding medium at 4 °C. These samples were then dissolved in 0.2 mL of 2% Na_2CO_3 and 1 M NaOH, and total activity per sample was assessed by scintillation counting in 4 mL of Optiphase Safe (LKB Products).

Ligand Dissociation Studies. To measure the rate of ligand dissociation, cell monolayers or aliquots of membranes were allowed to reach equilibrium binding at 4 °C with 20 nM [^{3}H]bradykinin (t = 1.5 h) as described above. Samples were then washed, and fresh ice-cold binding buffer was added. At the indicated times thereafter, the binding buffer was separated from the cell monolayer by aspiration or membrane pellet by centrifugation, and both fractions were dissolved for counting as described above.

RESULTS

Exposure of Cells to Bradykinin Causes Receptor Desensitization. Bradykinin bound to confluent, untreated Rat13 cells in a specific and saturable manner (Figure 1A). Scatchard transformation of the binding data obtained in the concentration range 0.3–60 nM indicated 35 500 high-affinity binding sites (K_d = 2.1 nM) per cell (Figure 1B). We have previously reported that Rat13 cells also possess a small number of very high affinity sites for bradykinin (K_d = 3 pM) (Roberts & Gullick, 1989). The concentration ranges used for the present study are chosen to give an optimum analysis of sites with an affinity of 1 nM and above and, as expected, do not detect the very high affinity site.

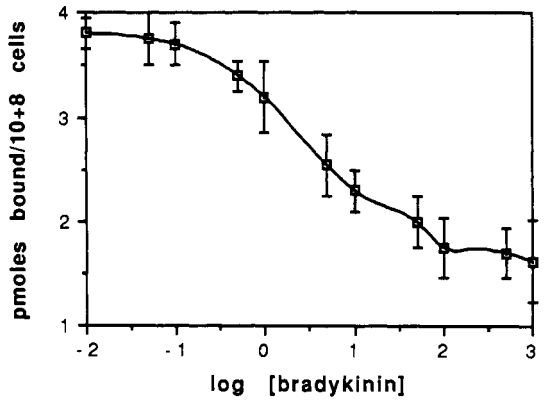

FIGURE 2: Concentration dependence of the desensitization reaction. Cells were preexposed to 0.01–1000 nM bradykinin for 5 h at 37 °C before being tested for their ability to bind [^{3}H]bradykinin. The half-maximal desensitization occurs around a log [bradykinin] of 0.5, equivalent to a concentration of 3.2 nM. Data points represent triplicate points from two separate experiments.

Panels C–F of Figure 1 indicate that preexposure of the confluent Rat13 cells to unlabeled bradykinin at 37 °C causes a progressive change in the pattern of ligand binding with time. After 2.5 h of preexposure, only 6000 of the high-affinity sites remain (Figure 1C,D). This number is reduced to a negligible level after 5 h of pretreatment (Figure 1E,F). In parallel with this change, a previously undetected lower affinity site (K_d = approximately 40 nM) can be detected at a level of 31 900 sites per cell after 2.5 h increasing to 36 700 after 5 h. Despite these changes in affinity the total number of receptors remained at 37 700 ± 1000 (Table I), suggesting that bradykinin receptors are desensitized during ligand exposure by a ligand-induced reduction in receptor affinity.

Desensitization Is Concentration Dependent. Figure 2 demonstrates that the loss of high-affinity bradykinin binding sites is concentration dependent. The ability of the preexposed cells to bind 5 nM radioactive bradykinin declines with increasing concentration of the unlabeled ligand used for the 5-h pretreatment. The concentration causing half-maximal desensitization is approximately 3.2 nM, a figure very similar to the K_d of the high-affinity receptors (2.1 nM).

Table I: Values for Number of Receptors per Cell (or per Cell Equivalent) and Their Affinities[a]

sample	time	concn (nM)	sites/ng of protein	sites/cell	total
cell monolayer	0	2.1		38 500	38 500
	2.5	2.1		6000	37 900
		49		31 900	
	5			0	36 700
		41		36 700	
membrane prepn	0	1.9	36 700	40 200	40 200
	2.5	2.3	37 900	41 700	41 700
	5	2.5	37 600	41 400	41 400

[a] For this cell type, 10^6 cells gave 1.1 mg of protein in the membrane preparation, implying that 1 ng of protein is equivalent to 0.909 cell.

Desensitization Does Not Occur at 4 °C. In order to determine if active cellular metabolism is required for desensitization, the preexposure experiments were repeated at 4 °C. Figure 3A shows the association curve and Scatchard analysis for Rat13 cells after their preexposure to 10 nM bradykinin for 5 h at 4 °C. The ability of these cells to bind bradykinin is essentially unaltered by this preexposure. Scatchard transformation indicates that they show around the same number (37 300) of high-affinity binding sites (K_d = 2.0 nM) as the untreated cells shown in Figure 1A,B.

Desensitization Does Not Occur in a Membrane Preparation of Rat13 Cells. A similar series of experiments was carried out in Rat13 cell membranes to determine whether cytoplasmic constituents are necessary for the desensitization response. Untreated membranes gave a ligand binding profile similar to that seen for untreated intact cells (association curves are not shown but were virtually identical with Figure 1A).

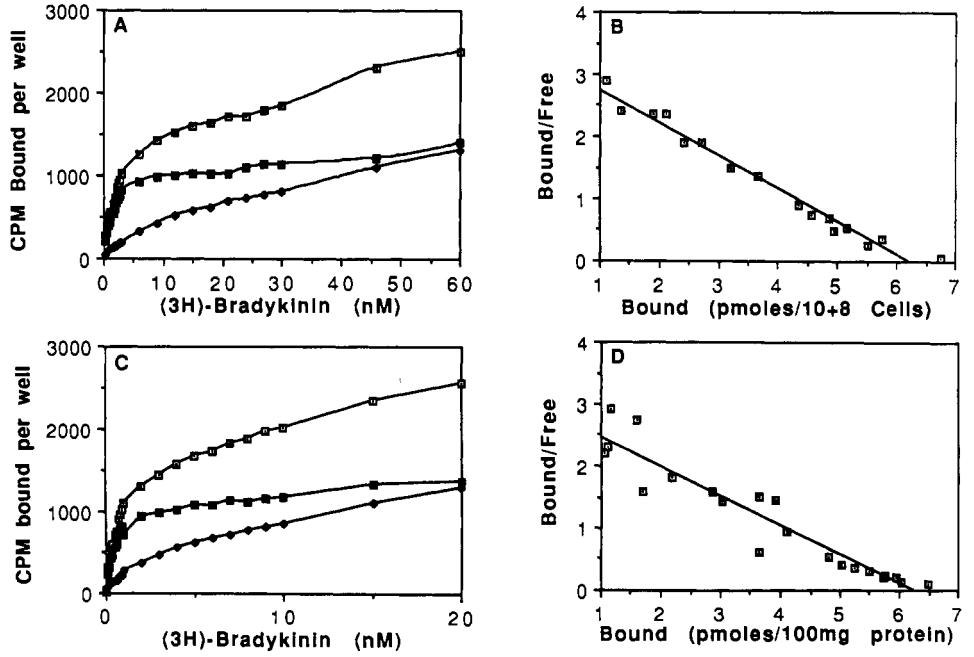

FIGURE 3: (A) Association curve showing [^{3}H]bradykinin binding to Rat13 cells after 5 h of ligand preexposure carried out at 4 °C. Background binding (◆) was measured in the presence of a 500-fold excess of cold ligand and subtracted from total binding (⊡) to give specific binding (■). Data points represent triplicate points from two separate experiments. Error bars have been omitted for clarity but were no more than ±6%. (B) Scatchard transformation of the data indicates a single class of receptor with a K_d of 2.0 nM at a level of 37 300 sites per cell. (C) shows the association curve for [^{3}H]bradykinin binding to Rat13 cell membranes after 5 h of ligand preexposure carried out at 37 °C. Data points represent triplicate points from two separate experiments. Error bars have been omitted for clarity but were no more than ±8%. (D) Scatchard transformation of the data indicates that the membranes possess 37 600 receptors/ng of total cell protein with an affinity of 2.5 nM. For this cell type, this is equivalent to 41 400 receptors per cell.

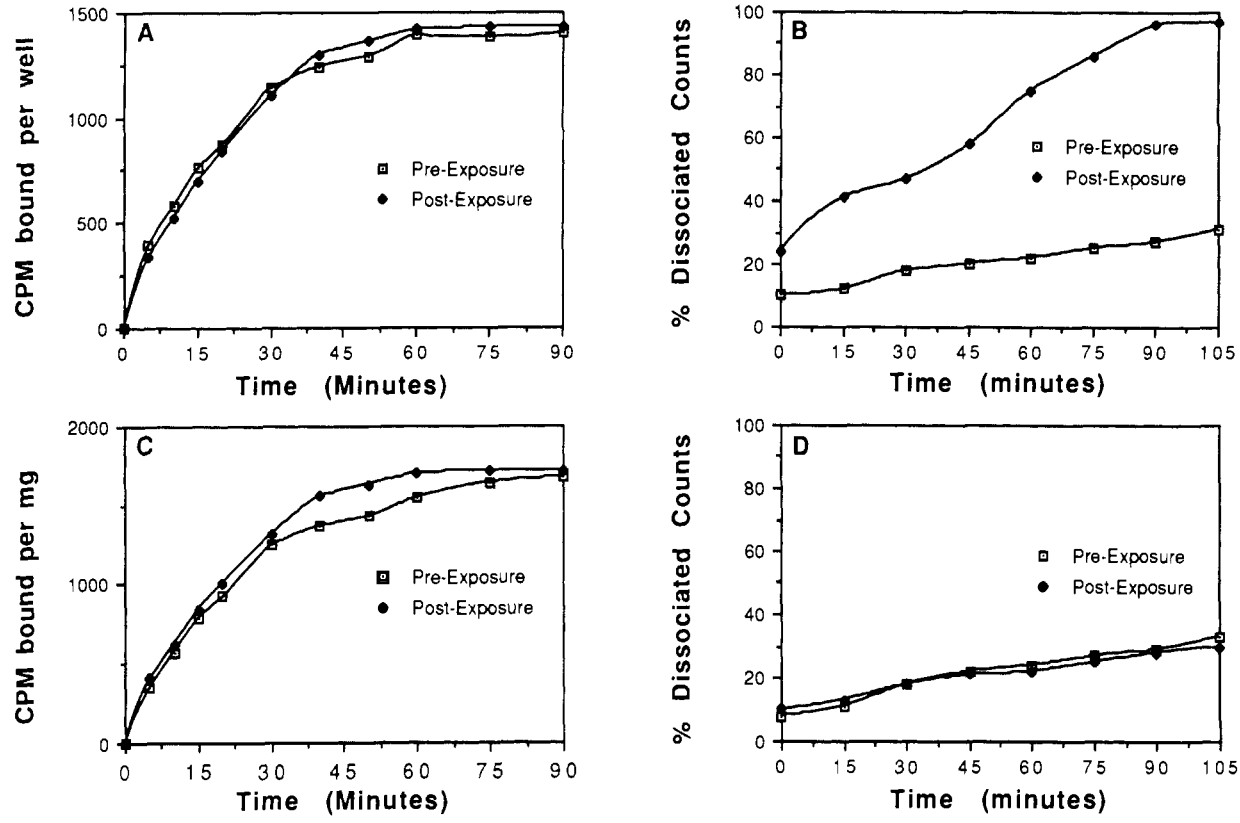

FIGURE 4: Kinetics of [^{3}H]bradykinin association and dissociation from cells or membranes at 4 °C. (A) Kinetics of association to cells. $t_{1/2}$(association) = 30 min both before and after ligand preexposure. (B) Kinetics of dissociation from cells. $t_{1/2}$(dissociation) = 2.5 h before but decreases to 45 min after ligand preexposure. (C) Kinetics of association to a membrane preparation. $t_{1/2}$(association) = 25 min both before and after ligand preexposure. (D) Kinetics of dissociation from membranes. $t_{1/2}$(dissociation) = 2.5 h both before and after ligand preexposure.

Scatchard transformation of these data (see Table I) gave 36 700 high-affinity sites (K_d = 1.9 nM) per nanogram of cell protein, which is equivalent to 40 200 receptors per cell for this cell type. This value is very close to the figures obtained for untreated intact cells (38 500 receptors per cell of K_d = 2.1 nM). In contrast to the results seen for intact cells, however, preexposure of Rat13 cell membranes to bradykinin had no effect on their subsequent ligand binding ability (Figure 3C). Scatchard transformation (Figure 3D) indicates that the membranes retain a constant number (see Table I) of high-affinity receptors for bradykinin despite 5 h of ligand preexposure.

Receptor Desensitization Is Associated with an Increased Rate of Ligand Dissociation. Since the affinity of a ligand for its receptor is a balance between association and dissociation, we investigated whether changes in these parameters were the mechanism by which the affinity of binding was reduced. Figure 4A shows that intact cells have the same $t_{1/2}$ for ligand association (approximately 30 min) both before and after preexposure to ligand. However, cells preexposed at 37 °C show a much faster rate of ligand dissociation ($t_{1/2}$ = ~45 min) when compared to untreated cells ($t_{1/2} \geq$ 2.5 h) (Figure 4B). In contrast to this, Rat13 cell membranes exhibit the same slow rate of ligand dissociation ($t_{1/2}$ = ≥2.5 h) despite extensive ligand preexposure (Figure 4B,D).

Discussion

The data presented here demonstrate that exposure of the bradykinin receptor to its specific ligand causes a time-, concentration-, and temperature-dependent reduction in receptor–ligand affinity. Presumably, this change represents a mechanism of receptor desensitization in response to chronic ligand exposure. These findings are the first demonstration of such an effect for the bradykinin system.

Information has recently emerged on the mechanisms of ligand-induced receptor downregulation or desensitization in other polypeptide ligand systems. The receptors investigated so far appear to fall into two categories: (i) those receptors that undergo downregulation by internalization in response to ligand and (ii) those receptors that become desensitized during ligand exposure. The former category is represented by one of the most widely studied of the growth factor receptors, that for epidermal growth factor (EGF). The EGF receptor is internalized in response to ligand in a temperature-dependent, pH-dependent, and ligand concentration dependent manner and is subsequently degraded (Cohen & Fava, 1986).

The second category of receptors (those that become desensitized by ligand exposure) is represented by the β-adrenergic receptors, the serotonin, and the α-muscarinic receptors (Sibley & Lefkowitz, 1985; Benovic et al., 1986; Kwatra & Hosey, 1986). These have all been demonstrated to undergo desensitization during lipid exposure by a mechanism involving phosphorylation of the cytoplasmic domain of the receptor protein by serine and threonine kinases (Bouvier et al., 1987). In the case of the α-muscarinic receptor, this desensitization takes place within 15 min of exposure to ligand. In contrast, desensitization of the β-adrenergic receptor and of the "light receptor", rhodopsin, in response to agonist appears to be a chronic effect taking place over 2–3 h. Our data indicate that the bradykinin receptor falls into the second category of receptors (those that are desensitized in response to ligand) and displays desensitization kinetics similar to those for the β-adrenergic system. Information gained from the recent cloning of a β-adrenergic receptor specific kinase (β-ARK; Benovic

et al., 1989) suggests that several such specific kinases may exist since the primary structure of the β-ARK delineates a multigene family. This information suggests the possible presence of an as yet undiscovered bradykinin receptor specific kinase.

Although receptor desensitization is the most likely interpretation of the data presented, several other explanations could be considered. First, ligand exposure may unmask a latent carboxypeptidase activity giving rise to the B_1 agonist, des-Arg^9-bradykinin, which could bind to any B_1 receptors present. This seems unlikely since our earlier data (Roberts & Gullick, 1989) show no evidence for the presence of the B_1 receptor type and HPLC analysis indicates minimal ligand breakdown by the cell monolayer. Second, ligand exposure may enhance kininase II activity, which could alter the binding kinetics. This possibility appears remote since the inclusion of kininase II inhibitors has no effect on binding or desensitization. The third possibility is that the preexposure is unmasking the putative B_3 bradykinin receptor (Farmer et al., 1989). This latter possibility cannot easily be ruled out and would require careful analysis of analogue binding affinities during the time course of preexposure.

Although we do not yet have any evidence for bradykinin receptor phosphorylation, the temperature dependency of the effect and the absence of a desensitization response in membrane preparations demonstrate that the desensitization is dependent upon the presence of active cellular machinery. This rules out the possibility that the observed affinity shift is the result of a simple ligand-induced conformational change within the receptor protein. The role of bradykinin receptor protein phosphorylation in the desensitization mechanism is currently under investigation.

The data presented in this paper may be relevant to the debate concerning the behavior of the bombesin receptor in response to chronic ligand exposure. This receptor system is often thought of as being similar to that for bradykinin and has been investigated by several groups. While Zachary and Rozengurt (1987) and Brown et al. (1988) report no internalization or downregulation of bombesin receptors, Swope and Schonbrunn (1987) and Moody et al. (1989) report that bombesin receptors are lost during ligand preexposure since no freshly added ligand binds to these treated cells. Our demonstration of bradykinin receptor desensitization may resolve these apparent discrepancies since it could be that a similar biological regulatory mechanism is operating in the bombesin receptor system. Experimentally, this could result in diminished binding of any freshly added ligand.

We have previously suggested (Roberts & Gullick, 1989) that the bradykinin receptor may be an additional member of the family of receptor proteins typified by the β-adrenergic receptor and characterized by their seven α-helical transmembrane regions and their ability to interact with G-proteins (Dohlman et al., 1987). The data presented here demonstrate an additional property of the bradykinin receptor in common with this receptor family.

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