DESENSITIZATION OF BETA-ADRENERGIC RECEPTORS OF GLIOMA CELLS: STUDIES WITH INTACT AND BROKEN CELL PREPARATIONS

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1. Introduction

It is commonly observed that the exposure of cells and tissues, possessing a beta receptor linked cyclic 3′,5′-AMP response, with beta adrenergic agonists results in a diminished cyclic AMP response to a subsequent beta adrenergic challenge [1–8]. Among the various explanations that have been proposed to account for this phenomenon are the following: (i) Prolonged beta adrenergic stimulation results in elevated intracellular cyclic AMP levels which induce an increase in intracellular phosphodiesterase activity thereby diminishing the net synthesis of cyclic AMP [3]. (ii) There is a loss of beta adrenergic binding sites in sensitized cells [6] which may result from an agonist-induced change in the conformation of the receptor. We describe experiments which suggest that beta adrenergic agonist-induced desensitization of the cyclic AMP response of the cyclic AMP response of rat glioma (C6) cells may not involve elevation of intracellular cyclic AMP. Further evidence is presented that beta adrenergic desensitization is not dependent on enhanced phosphodiesterase.

2. Materials and methods

2.1. *Rat glioma C6 cell cultures*

We routinely cultured the cells (A.T.C.C. C6/38) in Medium 199 supplemented with 10% (v/v) foetal bovine serum, 500 units/ml penicillin, 100 μg/ml streptomycin, 4.1 mM glutamine and buffered to pH 7.5 with 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Medium A). The cells usually grew to confluency in 5–7 days. An atmosphere of air was used throughout. Prior to beginning all experiments the cultures were equilibrated with Eagle's minimal essential medium supplemented as previously described [2] (Medium B) at 37°C for at least 1 h.

2.2. *Assay of cyclic AMP cell cultures*

This was performed by the method of Brown et al. [9] modified as previously described [8].

2.3. *Assay of adenylate cyclase activity in broken cell preparations*

Cells were grown to confluency in large flat-sided glass bottles. The growth medium was poured away and the cell sheet rinsed three times with ice-cold 0.9% NaCl solution. The cells were scraped off the glass into a hypotonic solution containing 5 mM Tris-HCl, pH 7.8 and 75 mM sucrose, left for 15–30 min at 0°C and then homogenized at 2000 rev/min in a glass–Teflon homogenizer allowing 1 min for each 5 ml of suspension. Any cells remaining intact were broken by a single cycle of freezing and thawing after diluting the homogenate with 1/4 volume of a buffer containing 20 mM Tris-HCl, pH 7.4, 30 mM $MgCl_2$, 5 mM EDTA, 5 mM dithiothreitol and 0.95 M sucrose. Adenylate cyclase was assayed by adding 100 μl of the homogenate (1 mg of protein/ml) to 1 ml of a solution containing 50 mM Tris-HCl pH 7.4, 10 mM KCl, 5 mM $MgCl_2$, 10 mM creatine phosphate, 15 μg/ml creatine kinase, 3 mM dithiothreitol, and 0.1 mM ATP. The reaction was stopped by transferring the tubes to a boiling water bath for 5 min and the cyclic AMP content of the heated solutions determined.

2.4. *Assay of phosphodiesterase activity in broken cell preparations*

The phosphodiesterase activity (low K_M enzyme) of the broken cell preparations of C6 cells was assayed by the method of Brooker et al. [10].

2.5. *Chemicals*

DL-Isoproterenol was purchased from Sigma Chemical Company; creatine phosphate and creatine kinase from Boehringer, Mannheim. The phosphodiesterase inhibitor 2-amino-6-methyl-5-oxo-4-*n*-propyl-4,5-dihydro-*s*-triazolo (1,5-α) pyrimidine (ICI 63,197) is a product of ICI Ltd. [2,8].

3. Results

3.1. *Isoproterenol-induced loss of cyclic AMP response in C6 cells*

After preincubation with 1 μM isoproterenol for 2.5 h the response of C6 cells to a subsequent challenge with 1 μM isoproterenol was 120 pmoles cyclic AMP/mg protein/10 min compared with a control response of 2190 pmoles cyclic AMP/mg protein/10 min. This was the maximal level of desensitization achieved. The response to isoproterenol was also depressed after incubating the cells with norepinephrine, epinephrine or salbutamol (not shown). Although the time course of development of desensitization is rather slow ($t_{\frac{1}{2}}$ = 36 min for all concentrations of agonists, Franklin and Twose, unpublished observations), its induction shows considerable sensitivity to beta adrenergic stimulation (fig.1). The apparent dissociation constant (K_d), i.e. the concentration of agonist required for half maximal desensitization, for desensitization of C6 cells over 2 hr by isoproterenol was 2.6 nM whereas the K_d for activation of the cyclic AMP response by isoproterenol over the same time period was 30.2 nM. The K_d for activation of the cyclic AMP response over 10 min was slightly lower but probably not significantly different at 18 nM.

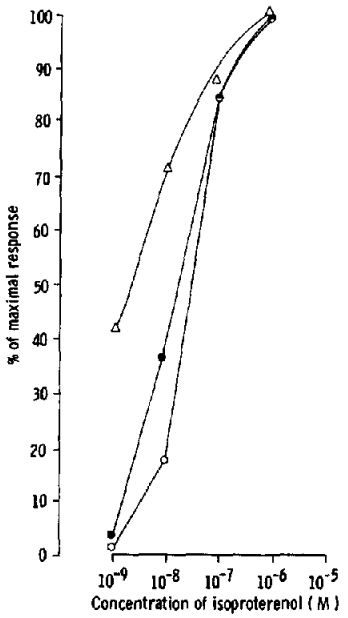

Fig.1. Dose–response curves for the activation of the cyclic AMP response and the desensitization of C6 cells by isoproterenol. DL-Isoproterenol was used to stimulate the cyclic AMP response of confluent cultures of C6 cells over 10 min (•) and 2 h (○). Parallel cultures were incubated for 2 h with the indicated concentrations of DL-isoproterenol, rinsed three times with fresh medium B and challenged for 10 min DL-isoproterenol (1 μM) (△). All challenge incubations contained I.C.I. 63,197 (25 μM). The results (mean of duplicate assays on each of two or three cultures) were normalized to give % of maximal response, i.e. either of stimulation or desensitization.

3.2. *Adenylate cyclase and phosphodiesterase activity in C6 cell homogenate*

Homogenates were prepared from control C6 cells and from cells that had been incubated for 2–2.5 h with 1 μM isoproterenol. There was a marked stimulation of cyclic AMP production by isoproterenol in homogenates of control cells and the synthesis of cyclic AMP continued linearly for at least 1 h. The homogenates from cells exposed to isoproterenol produced significantly less cyclic AMP in response to isoproterenol (table 1). The responses of these two different preparations to NaF were not significantly different. The response of mixed homogenate, containing equal parts from desensitized and control cells, to isoproterenol was approximately the mean of the responses of the individual homogenates (table 1). The K_d (1.26 μM) for the activation of the adenylate cyclase of homogenates of control cells (fig.2) was significantly higher than that for the activation of the cyclic AMP response of intact cells by isoproterenol. The K_d of the desensitized cell

Table 1
Cyclic AMP responses of homogenates of control and desensitized C6 cells

System	Cyclic AMP produced (pmoles/mg protein/10 min)
Control cell homogenate	114
Control cell homogenate, 10 μM isoproterenol	494
Control cell homogenate, 10 mM NaF	415
Desensitized cell homogenate	96
Desensitized cell homogenate, 10 μM isoproterenol	233
Desensitized cell homogenate, 10 mM NaF	466
Mixed homogenate	93
Mixed homogenate, 10 μM isoproterenol	341

Homogenates were prepared from confluent sheets of control C6 cells and cells previously incubated for 2 h with 1 μM DL-isoproterenol. The homogenates were assayed for adenylate cyclase activity over 10 min. The mixed homogenate contained equal parts of the homogenates of control and desensitized cells. All the homogenate incubations contained 25 μM I.C.I. 63,197. Each value is the mean of duplicate assays carried out on each of two incubations.

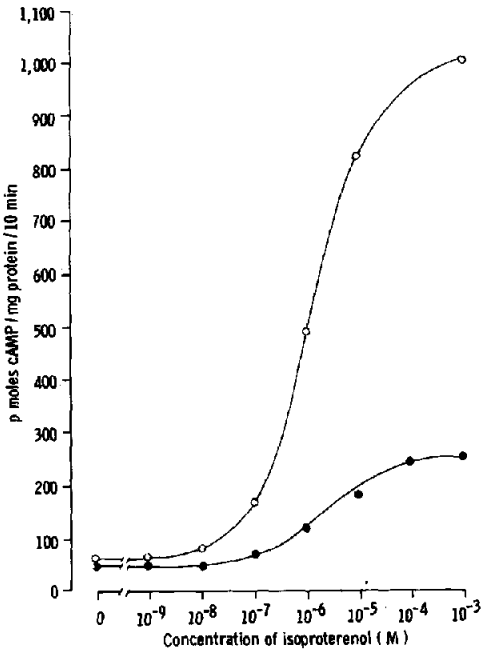

Fig.2. Dose–response curves for the cyclic AMP response of homogenates of control and desensitized C6 cells to isoproterenol. Homogenates were prepared from control cells (○) and cells incubated for 2.5 h with DL-isoproterenol (1 μM) (●). The homogenates were challenged with graded concentrations of DL-isoproterenol for 10 min in the presence of I.C.I. 63,197 (25 μM). The cyclic AMP produced was determined at the end of the incubation.

homogenate was not significantly different from that for the control cell preparation (fig.2).

Measurements of phosphodiesterase activities in cell homogenates revealed only a small increase (3.6%) in phosphodiesterase activity (low K_m enzyme) in homogenates from desensitized cells (table 2) compared with homogenates of control cells.

4. Discussion

We have been struck by the apparently greater sensitivity of the desensitization phenomenon to beta adrenergic agonists when compared with the activation of the cyclic AMP response by these agents. In other experiments with a variety of agonists using C6 cells and also human embryonic fibroblasts we have noted that concentrations of agonists that barely elicit a rise in the cyclic AMP content of the cultures in 2–3 h induce a marked depression in the cyclic AMP response to a subsequent isoproterenol challenge. It is possible that the induction of desensitization is very highly geared to cellular cyclic AMP levels. Alternatively, desensitization may not depend on the elevation of cyclic AMP but could be triggered by interaction of the beta receptor with agonist alone. This could occur if the activation of adenylate cyclase by beta adrenergic agonists depends on a co-operative interaction between

Table 2
Phosphodiesterase and adenylate cyclase activities in homogenates of control and isoproterenol-desensitized C6 cells

Homogenate	Phosphodiesterase activity (pmoles cyclic AMP hydrolyzed /10 min/mg protein)	Adenylate cyclase activity (pmoles cyclic AMP formed /10 min/mg protein)	
		Basal	Isoproterenol stimulated
Control C6 cells	469	62	577
Desensitized C6 cells	486	65	120

Homogenates were prepared from control C6 cells and from cells desensitized by incubation with 1 μM DL-isoproterenol for 2 h. Assays of phosphodiesterase and adenylate cyclase activities were carried out over 10 min in parallel. DL-Isoproterenol (1 μM) was used to stimulate adenylate cyclase activity. Each value is the mean of duplicate assays carried out on each of two incubations.

a minimal number of agonist–receptor complexes. Below the necessary threshold level of complexes partial desensitization of the receptor pool would be possible without activation of adenylate cyclase.

The results with homogenates of C6 cells strongly reinforce our view [2,8] that increased phosphodiesterase activity does not provide a general explanation for the phenomenon of agonist-induced desensitization of the beta-receptor-coupled cyclic AMP response. An earlier study from this laboratory [2] found that desensitization of cultures of human embryonic fibroblasts is not accompanied by any significant rise in phosphodiesterase activity. A similar result was obtained by DeVellis and Brooker [5] with the 2B subclone of C6 cells. The results of experiments in which homogenates from control and desensitized cells were mixed also indicate that increased phosphodiesterase activity is unlikely to account for the decline in the cyclic AMP response. The result of the mixed homogenate study suggests that, if an inhibitor of the beta adrenergic coupled cyclic AMP response is formed during desensitization, it must either by tightly associated with the receptor-adenylate cyclase complex or be in close stoicheometric equivalence to it. The beta-receptor–adenylate cyclase complexes that remain responsive after desensitization are apparently unchanged in their affinity for the agonist. A similar conclusion was reached by Mukherjee et al. [6] working with desensitized frog erythrocytes.

The mechanism of the agonist induced loss of the cyclic AMP response remains uncertain. It may be that in some cells phenomena such as increased phosphodiesterase activity or the formation of an inhibitory protein [5] or some other chemical [7] are superimposed on a basic mechanism of desensitization which probably involves a loss of available receptor sites as suggested by the work of Lefkowitz's group [6,11] possibly induced by agonist-induced receptor activation. We suggest, however, that elevated intracellular cyclic AMP may not be necessary for desensitization to occur.

During the preparation of this paper, a report appeared [12] describing a marked increase (53%) in phosphodiesterase activity in C6 cells desensitized with norepinephrine. This suggests that there may be considerable differences between the various lines of C6 cells. However, Browning et al. [12] also concluded that increased phosphodiesterase does not provide a complete explanation for the desensitization phenomenon.

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