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DESENSITIZATION OF NORMAL RAT KIDNEY CELLS TO ADENOSINE

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Abstract—Membranes prepared from normal rat kidney fibroblasts possess an adenosine receptor of the stimulatory (A_2 or R_a) type linked to adenylate cyclase. Homologous desensitization to adenosine A_2 agonists is developed upon exposure of the cells to adenosine-NCC, a potent A_2 agonist. This desensitization process is followed by a heterologous desensitization which is manifested in a time-dependent reduction of 1-epinephrine and NaF-dependent adenylate cyclase.

Desensitization or loss of responsiveness to the activating hormone has been shown for many receptors coupled to the enzyme adenylate cyclase, and was demonstrated *in vitro* for the β-adrenergic receptor by Anderson and Jaworski [1] in normal rat kidney (NRK)† fibroblasts. Although human fibroblasts have been shown to have an adenosine-activated cyclase [2], no desensitization response to this agent has yet been reported. In the present communication the effect of incubating both intact cells and membranes derived from NRK cells to adenosine and its analogs on the subsequently measured adenylate cyclase response has been investigated.

MATERIALS AND METHODS

Materials. Adenosine-NCC was from Abbott Laboratories (North Chicago, IL); PIA from Boehringer (Mannheim, F.R.G.); 1-epinephrine from ICN Pharmaceuticals (Cleveland, OH); and RO-20-1724 [4′-(3-butoxy-4-methoxybenzyl)-2-imidazoline] from Hoffman-La Roche Inc. (Nutley, NJ). 8-Phenyltheophylline was a gift of Prof. Fumio Yoneda (Faculty of Pharmaceutical Sciences, Kumamato University, Japan). 8-(*p*-Cl)- and 8-(*p*-NH_2)-phenyltheophylline were synthesized by Dr S. Braun of this department. ATP, GTP, adenosine, theophylline, 3-isobutyl-1-methylxanthine, phosphocreatine and creatine phosphokinase were all from Sigma Chemical Co. (St. Louis, MO); and [α-^{32}P]ATP was from the Nuclear Research Center (Beer-Sheva, Israel). Dulbecco's modified Eagle's medium was from Grand Island Biological Company (Grand Island, NY); newborn calf serum from Bio-Lab Ltd (Jerusalem, Israel), and trypsin from Difco Labs (Detroit, MI).

Growth of cells. The NRK cells were those described by Anderson and Jaworski [1] and were obtained from Dr I. Pastan (NCI, NIH, Bethesda, MD). Cells were grown in Dulbecco–Vogt modified Eagle's medium with 10% newborn calf serum at 37° in a 5% CO_2 humidified atmosphere. The cells were grown in 264-ml plastic bottles until they reached confluence (10^7 cells/bottle), trypsinized and seeded onto 93-mm tissue culture dishes, and grown again to confluence (1×10^6–2×10^6 cells/dish). For preparation of membranes, cells were washed with ice-cold phosphate-buffered saline, pH 7.4, and then scraped from the dish in the presence of homogenization buffer (25 mM Tris–HCl, 2 mM $MgCl_2$, 1 mM EDTA, 0.1 mM EGTA, 0.24 M sucrose, pH 7.4), using a Teflon spatula. The cells were homogenized with 20 strokes of a tight-fitting Dounce homogenizer and the homogenate centrifuged at 17,000 *g* for 10 min in a Sorvall RC-5 refrigerated centrifuge. The pellet was washed once and resuspended in homogenization buffer. For the desensitization experiments, the growth medium was removed, the cells washed twice with serum-free medium, and then incubated with serum-free medium containing various additions before preparation of the membranes as earlier.

Adenylate cyclase assay. Assays were performed for 10 min at 30° in a medium containing 50 mM Tris–HCl (pH 7.4), 6 mM $MgCl_2$, 1 mM EDTA, 0.2 mM ATP containing 10^6 cpm [α-^{32}P]ATP, 2.2 mg/ml (8.64 mM) phosphocreatine, 2.5 mM cAMP, 1 mM EGTA, 10 μM GTP, 0.2 mg/ml creatine phosphokinase and 0.3 mM RO-20-1724, [obtained from the Roche Institute (Nutley, New Jersey)]. The final assay volume was 200 μl, and reactions were stopped by addition of 0.8 ml of a 6.25% TCA solution, to give a final concentration of 5%. Separation of cAMP and ATP on Dowex and alumina columns was performed as described by Salomon *et al.* [3]. Each experimental value quoted is the mean of duplicate or triplicate determinations. Addition of adenosine deaminase had no effect, which indicates that there is no adenosine contamination.

In vitro *desensitization.* The method used followed that of Anderson and Jaworski [1], in which membranes prepared in the usual way were incubated with all components of the adenylate cyclase assay cocktail, excluding [α-^{32}P]ATP, and either water or the agent proposed to cause desensitization, at 30°.

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† Abbreviations: adenosine-NCC, 5′-*N*-cyclopropyl carboxamide adenosine; PIA, N^6-phenylisopropyl adenosine; NRK, normal rat kidneys.

Aliquots of this mixture were then added directly to tubes containing [α-^{32}P]ATP and agents to be tested in the assay, for a second stage of incubation. In the first (desensitization) stage of incubation the reagents present were 91 mM Tris–HCl (pH 7.4), 10.9 mM $MgCl_2$, 1.8 mM EDTA, 0.52 mM ATP, 4 mg/ml phosphocreatine, 4.5 mM cAMP, 1.8 mM EGTA, 17.8 μM GTP, 0.36 mg/ml creatine phosphokinase, and 0.54 mM RO-20-1724. One hundred and ten microlitre aliquots of this mixture, containing approximately 50 μg protein, were added after specified times to tubes containing 1 μCi [α-^{32}P]ATP diluted in a solution of 140 μM ATP, to give a final concentration of 15.5 μM in a volume of 10 μl, and reagents to be tested in the assay in a volume of 80 μl, so that the final concentration of all reagents in the total volume of 200 μl corresponded to those normally present in the adenylate cyclase assay.

RESULTS

Response to adenosine analogs

Basal adenylate cyclase activity was stimulated two-fold by GTP and a further two-fold by epinephrine in the presence of GTP, concentrations required for half-maximal activation being 0.4 μM for GTP and 0.3 μM for epinephrine. Stimulation by adenosine and its analogs was also dependent on the presence of GTP and resulted in a four-fold elevation of activity over basal, which is determined in the presence of GTP. Eight millimolar NaF gave a five-fold elevation over the basal activity. Both basal and NaF-stimulated activities were linear for up to 20 min of incubation, while epinephrine- and adenosine-stimulated activities trailed off after about 5–10 min. Therefore, in routine desensitization experiments, short adenylate cyclase assays were performed (2 min). The order of potency of various adenosine analogs is shown in Fig. 1, and was: adenosine-NCC > adenosine > PIA, characteristic of the A_2 or R_a stimulatory receptor [4, 5].

The response to adenosine-NCC was antagonized by methylxanthine derivatives: concentrations required for half-maximal inhibition of the response to 20 μM adenosine-NCC being 1 mM for theophylline, 80 μM for 3-isobutyl-1-methylxanthine, 0.06 μM for 8-phenyltheophylline, and 0.035 μM for 8-(*p*-Cl)-phenyltheophylline. A concentration of 8 μM 8-(*p*-Cl)-phenyltheophylline or its analog 8-(*p*-NH_2)-phenyltheophylline was routinely used to antagonize the effects of adenosine derivatives in assays in which these were carried over from desensitization incubations, and thus establish a true basal activity.

Desensitization in intact cells

In the majority of experiments, incubation of the cells with serum-free medium for periods of up to 40 min had no effect on subsequently measured activities. When cells were incubated for 30 min with serum-free medium containing various concentrations of adenosine-NCC, prior to isolation of membranes, a 70% decline in the response to adenosine-NCC was found with a smaller (25%) decline in the response to l-epinephrine (Fig. 2). In order to compensate for the decreases in NaF-stimulated activities due to heterologous desensitization (see Discussion), results were also expressed as percentages of activities obtained in corresponding incubations with NaF.

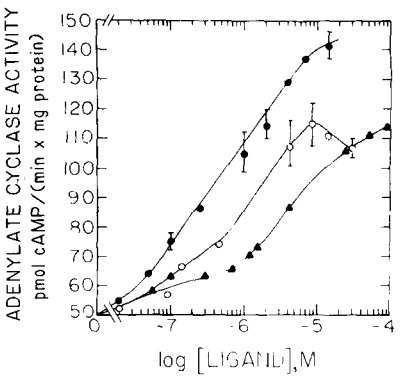

Fig. 1. Stimulation of adenylate cyclase by various concentrations of adenosine analogs. Activities were measured for 10 min at 30° in the presence of 10 μM GTP and various concentrations of compounds, as follows: (○—○) adenosine, (●—●) adenosine-NCC and, (▲—▲) PIA. Bars indicate S.E.M. of triplicate determinations.

It appears that the desensitization phenomenon induced by adenosine-NCC is composed largely of an agonist-specific component with a small contribution of a heterologous form of desensitization reflected in the significant desensitization to β-agonists. This phenomenon was investigated further by incubation of the cells with 10 μM adenosine-NCC for various times (Fig. 3). In these experiments all activities were lower than those found previously, but it was found possible to maintain the same-fold activation of adenylate cyclase by all the agents tested by pre-incubating the cells with serum-free medium for 30 min before applying medium containing the desensitizing agent.

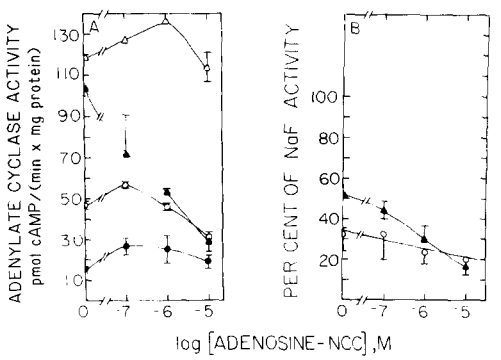

Fig. 2. Concentration dependence of the desensitization to adenosine-NCC. Cells were untreated or incubated with serum-free medium containing various concentrations of adenosine-NCC for 30 min before preparation of membranes. Results are expressed as absolute activities (A) and as ratios of activities to those obtained in the corresponding incubation with NaF, after subtraction of basal activities from both (B). (●—●) Basal activity in the presence of 8 μM 8-(*p*-Cl)-phenyltheophylline; (○—○) activity with 1 mM epinephrine; (▲—▲) activity with 20 μM adenosine-NCC; (△—△) activity with 8 mM NaF. Bars indicate S.E.M. of triplicate determinations. These experiments were repeated numerous times with very similar results.

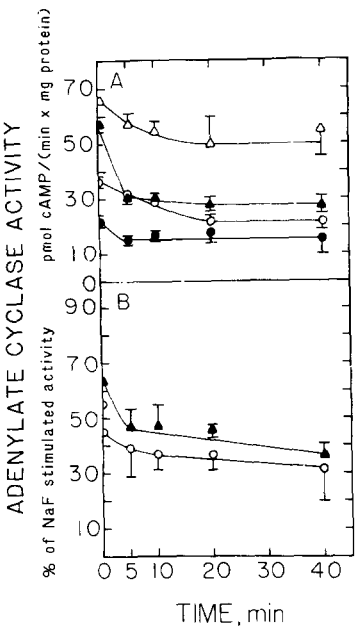

Fig. 3. Time course for *in vivo* desensitization to 10 μM adenosine-NCC. Cells were incubated with serum-free medium for 30 min, and then either used for preparation of membranes (control) or incubated with serum-free medium containing 10 μM adenosine-NCC for the periods indicated. Results are expressed as in Fig. 2. (●—●) Basal activity in the presence of 8 μM 8-(*p*-Cl)-phenyltheophylline; (○—○) activity with 1 mM epinephrine; (▲—▲) activity with 20 μM adenosine-NCC; (△—△) activity with 8 mM NaF. The time course described is very reproducible and was repeated frequently. The bars indicate the S.E.M. of triplicate determinations.

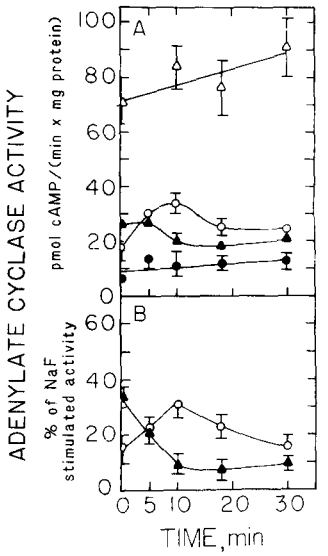

Fig. 4. Time course for *in vitro* desensitization to adenosine-NCC. Membranes were preincubated with 1.78 μM adenosine-NCC and assay reagents, except [α-^{32}P]ATP, as described in Materials and Methods, for the periods indicated, and then assayed for adenylate cyclase activity in a 2.5-min incubation. Results are expressed as in Fig. 2. (A) (●—●) Basal activity in the presence of 8 μM 8-(*p*-Cl)-phenyltheophylline; (○—○) activity with 1 mM 1-epinephrine; (▲—▲) activity with 20 μM adenosine-NCC; (△—△) activity with 8 mM NaF. (B) (○—○) 1-Epinephrine; (▲—▲) adenosine-NCC.

It is apparent that the adenosine-NCC-dependent activity is already reduced after 5 min, whereas the 1-epinephrine-stimulated activity remains essentially unchanged. Desensitization to a β-agonist proceeded as described earlier [1] and was found not to affect the response of the systems to adenosine agonists or NaF (Table 1).

Inhibitory response

NRK adenylate cyclase is not inhibited by adenosine or its analogs, and therefore lacks functional inhibitory (A_1) adenosine receptors. Such activity also does not appear subsequent to desensitization; namely, an inhibitory adenosine response is not involved in the desensitization process.

In vitro *desensitization*

Membranes incubated with 1.78 μM adenosine-NCC in the presence of GTP and other components of the adenylate cyclase assay mixture, except [α-^{32}P]ATP, showed a reduced response to stimulation by 20 μM adenosine-NCC in the subsequent assay stage (Fig. 4), which was maintained at all time periods studied up to 30 min. Epinephrine-stimulated activity was not reduced and, indeed, increased

Table 1. The absence of an effect of 1-epinephrine desensitization on the adenosine response*

Treatment	Basal activity	Assay in the presence of: Adenosine-NCC (20 μM)	1-Epinephrine (1 mM)	NaF (8 mM)
		pmoles/min/mg protein		
Control	26.4 ± 4.2	97.7 ± 12.8	54.1 ± 5.1	145 ± 2
1-Epinephrine-desensitized	35.0 ± 4.7	98.5 ± 5.2	41.2 ± 1.3	157.8 ± 10.1
Adenosine-NCC-desensitized	30.5 ± 1.7	43.4 ± 1.7	54.0 ± 4.5	147 ± 16.2

* The cells were untreated or incubated with serum-free medium containing 10 μM 1-epinephrine or adenosine-NCC for 30 min at 37°. Desensitization was performed for 5 min. During that incubation period very little heterologous desensitization occurs. The data are of three separate experiments run in triplicate samples, and are expressed with the S.E.M.

in some incubations, whereas NaF-stimulated activity was unchanged. The concentration of adenosine-NCC was carried over from the desensitization stage to the assay stage of the incubation, was 1 μM, and thus not likely to appreciably alter the degree of stimulation due to the reagents added in the second stage.

DISCUSSION

The characteristics of the adenosine receptor linked to adenylate cyclase in human fibroblasts have been extensively investigated by Bruns [6], and present results show that NRK fibroblasts possess an essentially similar receptor, both in terms of agonist [2] and antagonist [6] potency. Desensitization of the enzyme in these cells to β-adrenergic agonists after exposure to isoproterenol, both in intact cells and *in vitro*, has been shown by Anderson and Jaworski [1]. A similar effect was shown in the present experiments for l-epinephrine in intact cells. The response to epinephrine in control cells was small but in some experiments was increased by pre-treatment of the cells with serum-free medium (cf. Figs 2B and 3B). This may be due to low concentrations of β-agonists present in the serum, inducing a state of partial desensitization to epinephrine, as has recently been found for C_6 glioma cells grown in medium containing fetal bovine serum [7]. The desensitization of β-receptors observed in the present work had no effect on the properties of the adenosine receptor (Table 1).

In addition, adenosine-NCC, a potent adenosine analog in the activation of adenylate cyclase (Fig. 1), was shown to induce specific desensitization of the adenylate cyclase both in intact cells and in a cell-free preparation. The effect in intact cells was already significant at 1.0 μM adenosine-NCC (Fig. 2), suggesting an apparent K_D for desensitization similar to that observed for activation of adenylate cyclase (Fig. 1). The desensitization effect to adenosine-NCC was already obtained after 5 min of incubation (Fig. 3), in keeping with the time courses of specific desensitization to epinephrine observed both in NRK cells [1] and WI-38 fibroblasts [8]. A similar time course was observed *in vitro* with a cell-free preparation (Fig. 4), where 1.8 μM adenosine-NCC was effective in producing specific desensitization. Incubation of the cells for longer times resulted in the phenomenon of heterologous desensitization, first observed in human astrocytoma cells [9, 10], which can be mimicked by cAMP and where all activities including that due to stimulation by NaF are reduced. The time of onset of this effect was variable, requiring 40 min in some experiments but only 10 min in others (data not shown), although in all cases it followed a phase of specific desensitization to the agonist present.

During preparation of this manuscript, homologous desensitization to A_2 adenosine agonists was reported for neuroblastoma × glioma hybrid N9-108-15 [11]. The effect described here seems to be dissimilar to that produced in brain slices by prolonged incubation in the presence of endogenous adenosine [12], where subsequent cAMP responses to adenosine, norepinephrine and histamine were all reduced. In that case no homologous desensitization was reported.

It seems that the nature of adenosine-induced desensitization in NRK cells is similar to that described for the β-receptor-dependent phenomenon. The fact that the desensitization to either receptor is independent of the other receptor further suggests that the component modified during desensitization is the receptor itself [13].

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