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Growth of S49 Cells in Low Concentrations of β-Adrenergic Agonists Causes Desensitization

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SUMMARY

Epinephrine at concentrations approximating circulating levels in resting subjects produced significant desensitization in wild type S49 lymphoma cells after long term treatment. Desensitization by such low levels of catecholamines was measured by examining subsequent responses of the cells to higher agonist concentrations and was quantified by comparing the integral cAMP accumulations with time in naive and epinephrine-treated cells challenged with the higher epinephrine concentrations. The cells were significantly desensitized after 8 hr of treatment with 3 nM epinephrine or 3 nM terbutaline and were essentially maximally refractory after 24 hr. The 3 nM epinephrine treatment resulted in a small right shift of the EC_{50}. Responses to epinephrine were partially restored by incubating desensitized cells for 8 hr or longer in growth medium that was free of epinephrine. The attenuation of cAMP responses was largely specific, in that the decrease in the response to prostaglandin was small and the response to forskolin was unchanged. This, together with small increases in cAMP destruction in cell-free preparations from treated cells, suggested that higher phosphodiesterase activity contributed in a minor way to the desensitization. However, the response of the adenylate cyclase system to epinephrine was dramatically attenuated, and very significant changes in the properties of the β-adrenergic receptors were also obvious. That is, the number of binding sites for epinephrine was reduced by about 65% while the number of sites for [^{125}I]iodocyanopindolol was unchanged. The affinity for the radioactive ligand was significantly reduced. Wild type S49 cells remained viable after several days of continuous treatment with 3 nM epinephrine or terbutaline but responded to subsequent increases in cellular cAMP levels with the expected growth arrest and cytolysis. Involvement of cAMP-dependent protein kinase in this type of desensitization was suggested by the observation that S49 kin$^-$ cells were not desensitized by long term incubation with 3 nM epinephrine. Further, low concentrations of dibutyryl cAMP mimicked the effect of low level epinephrine treatment. We conclude that circulating levels of epinephrine in intact animals are sufficiently high to cause desensitization in cells with sensitivities to the catecholamines in the same range as that of the S49 lymphoma cell *in vitro*. We would predict that cells with those characteristics would always be at least partially desensitized *in vivo*.

In the years since desensitization of the cAMP responses accompanying agonist action was first described by Kakiuchi and Rall (1), many experiments have been carried out in an effort to understand how desensitization occurs. In a minority of cases, increased phosphodiesterase activity has been demonstrated (2) and, more commonly, the activity of the adenylate cyclase system has been attenuated. As described by Clark in a recent review (3), depending on the cell type, the agonist(s) used, the conditions of the experiment, and the investigators involved, this decreased activity has been ascribed to a variety of causes. These included decreased receptor availability, decreased receptor activation, changes in the activities of the GTP-binding proteins transducing the receptor-cyclase interaction, and several others as well. Receptor translocation and/or phosphorylation by a variety of phosphoprotein kinases have also been suggested as mechanisms (4). However, the pharmacological implications of desensitization are not much clearer than they were in 1964 because many of the *in vitro* experiments in which desensitization was studied employed agonist concentrations that were out of the pharmacological range and well into the toxicological (5, 6).

We have reported that, when intact cultured cells were continuously exposed to an agonist and the extent of desensitization was determined, the attenuation was more pronounced at the lower concentrations of the agonist (7, 8). The implication of this finding for the state of cells *in vivo* is obvious, because circulating concentrations of most agonists are quite low. For example, in resting rats, plasma catecholamine levels are in the range of 1–3 nM (9), and only rarely do catecholamine levels rise higher than 30 nM. More recently, we showed that incubation of intact S49 or C6 cells with low concentrations of epinephrine for 2 hr caused significant attenuation of the effect of 50 or 100 nM epinephrine (10). Although desensitization

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ABBREVIATIONS: DMEM, Dulbecco's modified Eagle's medium; ICYP, [^{125}I]iodocyanopindolol; PGE_1, prostaglandin E_1.

invariably appeared, quantitative aspects of cAMP levels, adenylate cyclase activity, and the activity of the PDEs were quite variable. Because there was more consistency when the incubation period with these low concentrations of agonist was increased to 18 hr, it seemed likely that the process as measured at 2 hr was incomplete. Therefore, we adopted protocols examining desensitization in detail after longer time periods.

In this report, we detail the results and provide evidence that the degree of cellular attenuation was profound. Our data suggest that the mechanisms underlying this form of desensitization are multiple. Finally, we predict that catecholamine-responsive cells *in vivo* may be significantly desensitized under basal conditions.

Materials and Methods

Cell cultures. Wild type S49 clone 22 cells were grown in DMEM supplemented with 5% horse serum and antibiotics, as previously described (10). Cells were maintained at population densities of 0.5–2.0 × 10^6 cells/ml with daily feedings. Viabilities were greater than 90%.

Long term exposure to epinephrine or terbutaline. S49 wild type cells were adapted to fresh roller bottles by growing them for 24–72 hr before experimental additions were made. At the appropriate time, epinephrine or terbutaline in thiourea and ascorbate or the thiourea-ascorbate solution alone was added to the cultures. Epinephrine and terbutaline were prepared so as to be diluted 1000-fold in the roller bottles. In all cases, the final thiourea and ascorbate concentrations were 1.0 and 0.1 mM, respectively.

These experiments were carried out with three sorts of protocols for epinephrine addition. Most commonly, single additions of the catecholamine or the control solution were employed. For experiments longer than 24 hr, protocols involving either the addition of fresh medium and the appropriate addition to cells that had been pelleted by centrifugation at 600 × *g* for 10 min or the addition of fresh epinephrine or control solution without removing the old medium were employed. No differences in the outcome of these experiments were detected.

In all cases, the operator(s) for the intact cell or cell-free experiments described below were blinded with regard to which cell populations had been treated with epinephrine, terbutaline, or the control additions until after the calculations were final.

Intact cell experiments. Cells were harvested by centrifugation at 600 × *g* and resuspended in their own growth medium at a cell population density of 20 × 10^6 cells/ml. The adenine nucleotides were prelabeled by incubating the cells for 1 hr with 7.5 μCi of [^{3}H]adenine/ml. (11). Excess extracellular radioactivity and epinephrine or control pretreatments were removed by washing the cells with serum-free DMEM through centrifugation and resuspension. The elapsed time between removal of the pretreatments and beginning of the experimental protocol was between 1 and 1.5 hr. Experimental protocols were followed as described in the text, with reactions terminated as described earlier (11).

Cell-free assays. Cells were harvested and washed in ice-cold buffers as described previously (12). The cells were ruptured after a 20-min equilibration at 500 psi with nitrogen in a Parr cell disruption bomb. Adenylate cyclase activities were measured using membranes prepared and assayed as previously described (12).

ICYP binding. ICYP was prepared as described by Hoyer *et al.* (13). Binding of ICYP to intact S49 cells was determined by incubation of 2–5 × 10^6 cells in 0.5-ml samples of the same DMEM used for the cAMP accumulation experiments. Phentolamine (0.1 mM) was added in the present case so as to minimize nonspecific binding. Incubations of 45 min were used for the determination of equilibrium binding of ICYP. The binding parameters for epinephrine were determined using the nonequilibrium technique described by Toews *et al.* (14), with ICYP as the slowly dissociating radioactive ligand. The addition of 3 ml of a solution containing 50 mM Tris-HCl (pH 7.4), 10 mM magnesium chloride, and 0.1 mM phentolamine terminated the incubation. This was followed immediately by filtration of the cells through Whatman GF/C filters with an Amicon filtration manifold and washing with the same solution four times while the cells were on the filters. Nonspecific binding was defined using 0.1 μM alprenolol.

Phosphodiesterase assays. Cells destined for phosphodiesterase assay were washed twice in an ice-cold solution containing 0.25 M sucrose, 20 mM Tris·HCl (pH 7.4), and 7 mM β-mercaptoethanol. They were harvested by centrifugation and resuspended in a solution containing 0.25 M sucrose, 20 mM Tris-HCl (pH 7.4), 5 mM β-mercaptoethanol, 2 mM $MgCl_2$, 0.4 mM phenylmethanesulfonyl fluoride, 5 μM leupeptin, 25 μg/ml trypsin inhibitor, and 2 mM benzamidine. Cells were lysed in a Parr cell disruption bomb after allowing 20 min for equilibration at 500 psi. The lysate was not further purified before assay.

Assay incubations were based on the method of Thompson and Appleman (15). The incubation volume was 250 μl and contained 40 mM Tris·HCl (pH 7.4), 5 mM $MgSO_4$, 5 mM β-mercaptoethanol, and 1 mg/ml bovine serum albumin. Typically, each incubate contained 10^5 dpm of exogenous [^{3}H]cAMP. The enzyme was adjusted to a dilution such that substrate conversion was never greater than 10% for the 10-min period of the incubation (37°). The reaction was terminated by the addition of 50 μl of 3 mM cAMP, followed by heating in a boiling water bath for 1 min. A 100-μl aliquot of a solution of *Crotalus atrox* venom (1 mg/ml) in water was added and the samples were incubated for 15 min at 30°. The action of the venom was stopped by the addition of 600 μl of a solution containing 20 mM ammonium formate (pH 7.4), 15 mM EDTA, and 0.1 mM adenosine (16).

Adenosine and other dephosphorylated products were separated from cAMP by chromatography on QAE-Sephadex A25 (17). The samples (1 ml) were added to individual QAE-Sephadex columns and the eluant was collected. A further 2 ml of ammonium formate (20 mM) was run through and the additional eluate was combined with the first. The total eluate was counted in 10 ml of Liquiscint (National Diagnostics).

Viability studies. Cells were placed in T-75 tissue culture flasks with the appropriate additions and were allowed to equilibrate overnight before they were divided into two groups. One group had no further addition other than the pretreatment. The other groups received single additions of 1 mM dibutyryl cAMP or 5 μM forskolin or daily additions of 1 μM epinephrine. Each day after the initial treatment, the viable cells were counted in each group and compared. The ratios of cells surviving in treated groups and control groups were measures of survival.

Results

Test system for measuring desensitization in intact cells. Measurements of the cAMP levels achieved at any single time cannot provide unique quantitative information about desensitization. One useful parameter for quantifying desensitization is the area under a plot of cAMP accumulation versus time following exposure to a stipulated epinephrine concentration. The usefulness of this approach is shown by the data in Fig. 1. In those experiments, cells were treated for 22 hr with 3 nM epinephrine or the thiourea-ascorbate control and then assayed for cAMP accumulation by stimulation with 20, 100, or 1000 nM epinephrine. In all cases, the pretreatment with 3 nM epinephrine resulted in reduced responses, but the reduction in the integral cAMP accumulations (on a percentage basis) was clearly greater when the challenge was performed with 20 nM epinephrine (93% desensitized) rather than with 100 nM or 1 μM (77 and 62%, respectively). Fig. 1 also illustrates the effect that time of assay, as well as the concentration of assaying agonist, has on the quantitation of desensitization. Inspection

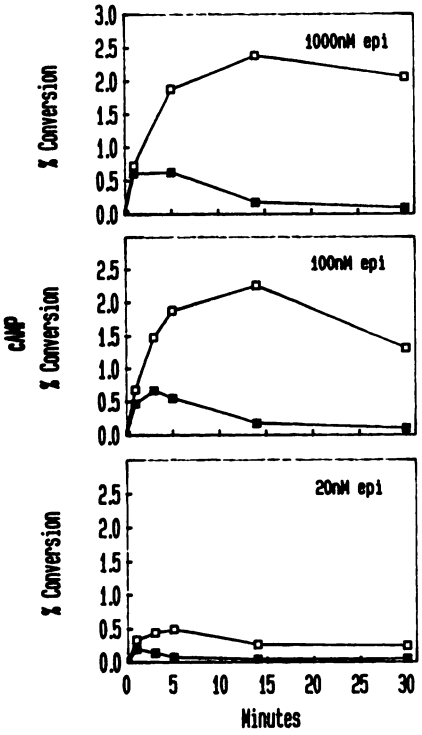

Fig. 1. The effect of assay time and concentration of challenge agonist on estimated extent of desensitization. The challenge concentrations of epinephrine (epi) are indicated in the figure. □, Control cells; ■, cells treated for 24 hr with 3 nM epinephrine before challenge with the indicated epinephrine concentration. Incubations were at 37°.

of the 1-min points of the three panels shows that there were not significant differences between naive and 3 nM epinephrine-treated cells challenged with 100 nM or 1 μM epinephrine, but that there was a decrease of more than 50% when the challenge was performed with 20 nM epinephrine. On the other hand, although pretreatment had no significant effect on cAMP accumulated in response to 100 nM or 1 μM epinephrine at 1 min, the accumulation diverged dramatically at longer times. The use of the area under the cAMP accumulation curve thus provided a useful expression of desensitization as a single number, because it reflected ratios of accumulation in control cells and in cells exposed to the specified treatment. Obviously, however, the duration of the challenge incubation and the concentration of agonist employed in the measurement of cAMP accumulation must be chosen carefully. It should be mentioned here that 3 nM epinephrine per se had only small and short-lived effects on cAMP accumulation in naive S49 wild type cells (data not shown).

Concentration response following desensitizing treatment. As pointed out above, desensitization occurs not only during the long term incubation of the cells with 3 nM epinephrine but also throughout the challenge incubation as well. Measurements of the cAMP accumulation in naive and desensitized cells at short times after the start of the epinephrine challenge were useful because they were less influenced by challenge-phase desensitization. Also, at early times, cAMP accumulation in intact cells was a reasonable reflection of the initial rate of adenylate cyclase activity (7, 8). The results of 1-min incubations of S49 wild type cells with increasing concentrations of epinephrine following 24 hr of preliminary incubation, with or without 3 nM epinephrine, are presented in Fig. 2.

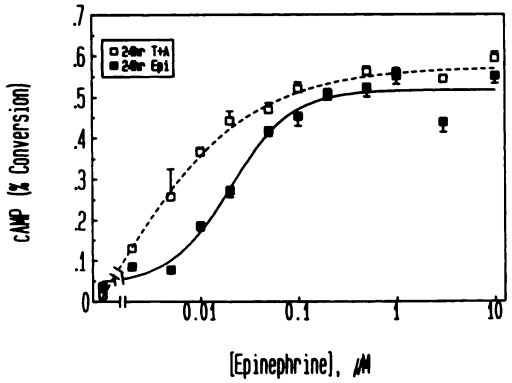

Fig. 2. The concentration response of S49 cells to epinephrine. □, Control cells; ■, cells treated for 24 hr with 3 nM epinephrine before challenge. Incubations were for 1 min at 37°.

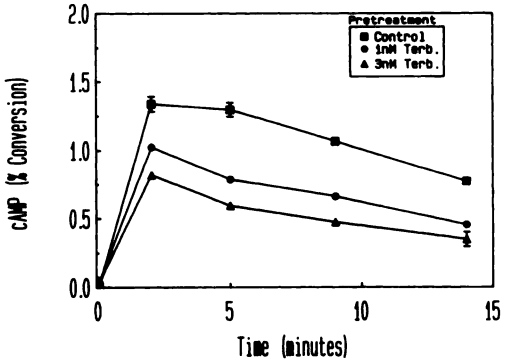

Fig. 3. The effect of prior treatment with terbutaline on the time course of cAMP accumulation in S49 cells stimulated by 100 nM epinephrine. ■, Control cells; ●, cells treated for 6 days with 1 nM terbutaline; ▲, cells treated for 6 days with 3 nM terbutaline. The terbutaline concentrations were estimated initially by single additions and were maintained by having the appropriate concentration present in fresh growth medium that was added at the daily feedings of the cells.

There was a clear right shift in the response of the desensitized cells to epinephrine. This shift in EC_{50} to higher concentrations has been associated with reduced β-receptor numbers and/or receptor efficiency (8). At higher epinephrine concentrations, however, initial rates of cAMP accumulation were much less attenuated.

Terbutaline as the desensitizing agent. Obviously, epinephrine was the catecholamine of choice in these experiments, given that it is a natural adrenergic agonist. However, effects of oxidation of epinephrine have been demonstrated even at much higher concentrations (18). As a result, it seemed appropriate to use the nonoxidizing β-adrenergic agonist terbutaline. Terbutaline is a full agonist in S49 wild type cells, with an EC_{50} shifted approximately 1 order of magnitude to the right of that of epinephrine (data not shown), but with qualitatively identical actions. Fig. 3 illustrates the effects of growth of S49 wild type cells in 1 or 3 nM terbutaline for 6 days. Even at concentrations as low as 1 and 3 nM, terbutaline produced 38 and 54% desensitization, respectively, in cells challenged with 100 nM epinephrine.

Time courses of desensitization and recovery. The time course by which desensitization occurred is illustrated in Fig. 4A. Desensitization was obvious within 2 hr after the addition of 3 nM epinephrine, but was not maximal until 24 hr or longer. On the other hand, recovery from desensitization occurred over

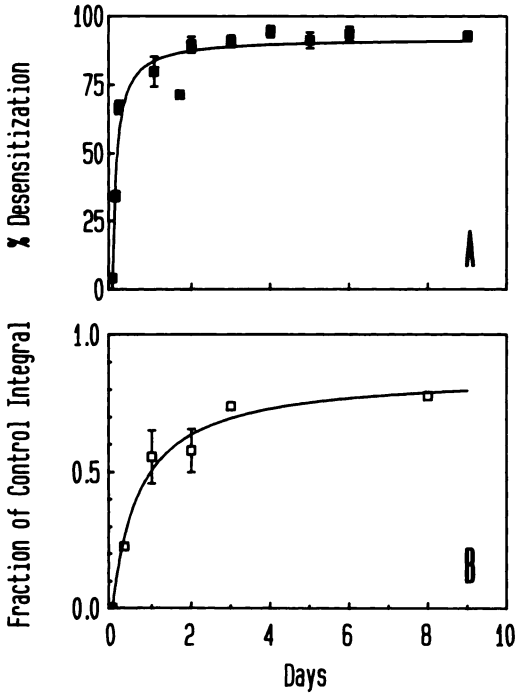

Fig. 4. Time courses of desensitization and recovery from desensitization. In A, cells were treated with 3 nM epinephrine for the indicated times. Time courses of response to 100 nM epinephrine were integrated and % *desensitization* is the percentage of control integral that was lost due to pretreatment. The results are the averages ± standard errors of the means from 11 experiments. In B, cells were treated for 8 days with 3 nM epinephrine. The epinephrine was removed by washing and recovery from desensitization was ascertained by the same assay described in A. The *ordinate* gives the integral accumulation for cells stimulated by 100 nM epinephrine as a fraction of the integral for control cells in identical assays. The results are the averages ± standard errors of the means from five experiments.

a slower time course (Fig. 4B). In these experiments, after desensitization had become well established, aliquots of the cells were washed free of epinephrine-containing medium and incubation was continued in growth medium with or without 3 nM epinephrine. There was apparent recovery to the extent of 50% after 24 hr, after which it slowed. In no case did recovery exceed 75%.

Heterologous desensitization. The effects of long term incubation with 3 nM epinephrine on subsequent responses to PGE_1 and forskolin are shown in Fig. 5. Although the epinephrine-treated cells showed significantly diminished responses to PGE_1, the attenuation was always minor compared with the effect on responses to 100 nM epinephrine. Effects on the response to forskolin were not detected.

Association of changes in synthesis and degradation of cAMP with long term desensitization. To examine the roles of synthesis and hydrolysis as components of the expression of desensitization under the long term-low hormone conditions used in these studies, adenylate cyclase and phosphodiesterase were measured in cell-free preparations. Membranes prepared from cells pretreated with 3 nM epinephrine for 3 days were significantly less responsive than control membranes when assayed at a variety of epinephrine concentrations (Fig. 6). There were no differences in the responses to PGE_1 or forskolin. Thus, as judged by adenylate cyclase data, the desensitization was catecholamine specific.

Data for phosphodiesterase activity in S49 cell lysates from

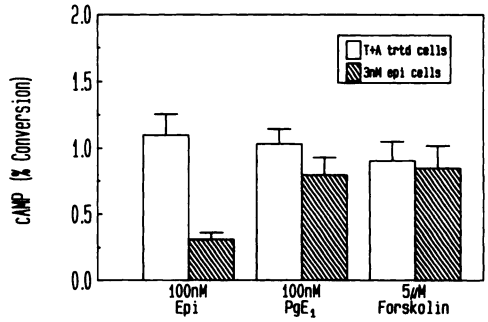

Fig. 5. Effect of epinephrine treatment on response of S49 cells to PGE_1 and forskolin. Cells were grown with 3 nM epinephrine or thiourea-ascorbate for 72 hr. Following the procedures for intact cell experiments described above, the cells were challenged with 100 nM epinephrine (*Epi*), 100 nM PGE_1, or 5 μM forskolin. The *ordinate* gives the cAMP accumulation for 2 min of stimulation by the indicated assay agonist. The results are the averages ± standard errors of the means from eight experiments.

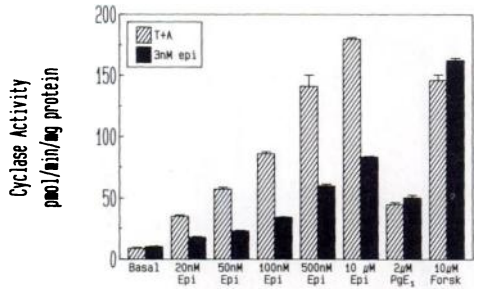

Fig. 6. Adenylate cyclase activities in membranes prepared from S49 cells treated for 72 hr with 3 nM epinephrine. The control membranes were prepared from cells treated for the same time with thiourea-ascorbate vehicle. *Epi*, epinephrine; *Forsk*, forskolin.

TABLE 1

Effect of pretreatment with or without 3 nM epinephrine on cAMP phosphodiesterase activity

S49 cells treated with 3 nM epinephrine from 1 to 4 days were lysed using the procedure described in Materials and Methods and were assayed at 37° for phosphodiesterase activity at the indicated cAMP concentrations. Control lysates were prepared from cells treated with ascorbate and thiourea for the same periods. Activities are means ± the standard errors from eight experiments.

[cAMP]	Phosphodiesterase activity		Increase over control
	Control lysate	3 nM Epinephrine-treated Lysate	
μM	*nmol of cAMP hydrolyzed/mg/min*		%
1	30.0 ± 0.9	37.4 ± 1.2	25
10	62.6 ± 3.0	87.0 ± 4.1	39
100	119.5 ± 4.2	154.9 ± 6.2	30

epinephrine-pretreated and control cells are shown in Table 1. These relatively small but highly significant increases in phosphodiesterase activity were consistent with the observation in Fig. 5 that desensitization of the PGE_1 response was minor.

Effect of epinephrine pretreatment on β-receptors. The effect of pretreatment for 8 days with 3 nM epinephrine on ICYP binding to S49 cells is shown in Fig. 7. There was a small decrease in affinity for the antagonist but no significant change in the extrapolated estimate of the amount that binds at saturating concentrations of ICYP. This is a different result from those obtained from shorter treatment with much higher concentrations of agonist, where it has generally been concluded that there is no change in affinity for the antagonist (14).

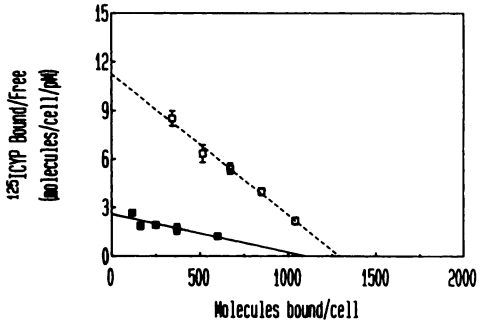

Fig. 7. Scatchard plots to show the effect of 3 nM epinephrine treatment on ICYP binding to S49 cells. The duration of the epinephrine treatment was 8 days. □, Control cells; ■, treated cells.

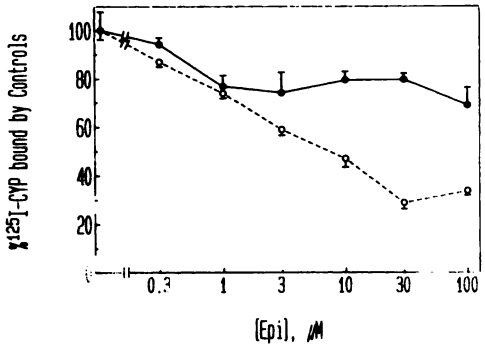

Fig. 8. Inhibition of ICYP binding to S49 cells by epinephrine (*Epi*). Test cells were treated with 3 nM epinephrine; control cells were treated for the same time with thiourea-ascorbate acid. The concentration of ICYP was the same in both assays (80 pM). Initial values of ICYP bound for control and epinephrine-treated cells were 2.9×10^{-7} and 1.0×10^{-7} nmol/10^6 cells, respectively. ○, Control cells; ●, treated cells.

The effect of the same pretreatment on epinephrine binding is even more striking. The data in Fig. 8 show ICYP binding to S49 cells during a 1-min incubation period with ICYP and epinephrine added simultaneously. This nonequilibrium procedure, originally described by Toews *et al.* (14), avoids the problems associated with changes in the receptor-epinephrine affinity, which complicate equilibrium binding studies. The extent of ICYP binding during a 1-min incubation in the presence of epinephrine is proportional to the fraction of receptors that are free of epinephrine. The inhibition of ICYP binding by epinephrine under these conditions, therefore, gives a direct estimate of epinephrine binding without recourse to a Cheng-Prusoff correction (19). The figure demonstrates the observation, made by many authors, that in control cells epinephrine can bind to the same site as ICYP and can largely prevent its binding. The K_d for epinephrine estimated in the control cells was about 3 μM, which agrees with previous studies (8). In the epinephrine-treated cells, however, only about 35% of the ICYP binding could be prevented by competition with epinephrine.

Changes in ICYP binding to membrane fractions were also concomitant with long term epinephrine treatment. Membranes were prepared from S49 cells that had been incubated for 20 hr with or without 3 nM epinephrine and ICYP binding studies were carried out (20); K_d values for binding to the membranes from control cells were 47 pM and were 133 pM for binding to membranes from epinephrine-treated cells.

Effects of long term growth in epinephrine. Tomkins and his co-workers (21, 22) demonstrated that S49 wild type cells would enter growth arrest and ultimately undergo cytolysis following elevated cellular cAMP levels, Indeed, it was this observation that made possible the development and isolation of S49 mutant cell lines like cyc^- and kin^-. Thus, S49 wild type cells are so phenotypically unstable that it was important to show that cells grown in epinephrine were not variants. We incubated the cells in high concentrations of agents that are known to elevate cellular cAMP levels and cause cytolysis in naive S49 wild type cells. Dibutyryl cAMP (1 mM) included in the culture medium produced cytolysis in both the control and 3 nM epinephrine-treated cells, and similar results were seen when both were stimulated with 5 μM forskolin. The treated cells were less sensitive to high (1 μM) concentrations of epinephrine than the controls. This was probably due to desensitization and, in any case, was overcome by the additions of phosphodiesterase inhibitors.

Possible role of cAMP-dependent protein kinase. Experiments by Clark *et al.* (12) suggested that the cAMP-dependent protein kinase played a significant role in acute desensitization of S49 cells. In our experiments involving 3 nM epinephrine, there were also strong suggestions that the cAMP-dependent protein kinase was involved. For example, when S49 kin^- cells were treated for 24 hr with 3 nM epinephrine, there was no desensitization. Integral cAMP accumulations in control and 3 nM epinephrine-treated cells exposed to 100 nM epinephrine were identical in several representative experiments. Additionally, growth of S49 wild type cells in low concentrations of dibutyryl cAMP caused attenuation of the response to 100 nM epinephrine. For example, when cells were incubated for 24 hr in complete medium including 3 μM dibutyryl cAMP or 6 μM sodium butyrate and 3 μM cAMP, desensitization of the former amounted to 53%. Presumably, the effect of dibutyryl cAMP involved phosphorylation by the cAMP-dependent protein kinase.

Discussion

The data presented here demonstrate that significant desensitization of S49 wild type cells occurred when the cells were grown in epinephrine concentrations that themselves caused only minor and transitory effects on cAMP levels. Although 3 nM epinephrine is at the upper limit of resting circulating catecholamine levels, this was the initial and thus highest concentration to which the cells were exposed during the desensitizing incubation. Further, experiments with terbutaline (like those in Fig. 3) demonstrated that significant desensitization was present after several days with the nonoxidizable agonist at an initial concentration of 1 nM. This was significant, given the fact that the EC_{50} for terbutaline is about 1 order of magnitude higher than that for epinephrine.

The kind of desensitization produced by prolonged incubation with low concentrations of catecholamine appeared to be at least partially reversed by incubation in growth medium in the absence of the agonist. However, it has not been possible to study the return of the response in cells that were not dividing. As a result, it is not clear whether the return of cAMP responses towards normal is due to better responses in the cells that had been desensitized or to the appearance of new cells.

Experiments with intact cells (Fig. 5) and with cell-free preparations (Fig. 6 and Table 1) suggested that, at least under the conditions employed in these experiments, attenuated responses of the adenylate cyclase system were quantitatively

more important than the increased levels of phosphodiesterase activity. That alterations in both activities underlie the effects of prolonged incubation with low concentrations of epinephrine suggested that the attenuation is an integrated response. The intact cell experiments also suggested that the egress of cAMP, which is minor in S49 wild type cells (data not shown), was not a major factor in this desensitization.

The implication to be drawn from the data in Figs. 7 and 8 is that the pretreatment caused a large fraction of the β-adrenergic receptors to bind ICYP with lower affinity and reduced the number of epinephrine receptors by 65%. From the aspect of cAMP accumulation in response to epinephrine, the relevant point is that the number of receptors capable of involvement in the activation of adenylate cyclase was reduced. It has been suggested previously (8) that reduced receptor number should be associated (a) with a right shift in the concentration-response curve and (b) with a more rapid decline in the rate of cAMP synthesis during a time course, as the result of desensitization that occurs during the time course itself. The data in Figs. 1 and 2 are entirely consistent with these ideas.

Our experiments are entirely consistent with the involvement of altered receptor functions as a component of long term, low agonist-induced desensitization. Fig. 8 showed that, relative to control cells, the number of epinephrine-receptor complexes was reduced at any concentration of epinephrine employed. That is, there was a reduced affinity for epinephrine under the conditions of the incubation, which were identical to the conditions of the cAMP accumulation assays. However, this particular experiment did not address any of the possible molecular changes in the receptor. The experiments with S49 kin$^-$ cells and those with dibutyryl cAMP made it seem likely that the cAMP-dependent protein kinase was involved. The effect on the protein kinase and the altered receptor state may be dependent or independent.

The data presented here suggest that cells exposed to low concentrations of catecholamines exhibited very significant desensitization. Extrapolations of data from mouse lymphoma cells grown *in vitro* to cells *in vivo* can be no more than suggestive, but we would speculate that the naive state commonly used as a starting point for studies of desensitization is probably an artifact of experiment design. In the *in vivo* situation, β-adrenergic-responsive cells would be always at least partially desensitized, and it would follow that cAMP responses to agonists would be attenuated. Further, the very low effective receptor concentrations demonstrated in Figs. 7 and 8 would predict, on the basis of calculations previously reported by Barber (8), that the rate of attenuation of adenylate cyclase in such cells exposed to an epinephrine challenge might be much faster than that observed in naive and receptor-replete cells.

Acknowledgments

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