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β-Adrenergic Receptor Levels and Function after Growth of S49 Lymphoma Cells in Low Concentrations of Epinephrine

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SUMMARY

Growth of S49 wild-type (WT) lymphoma cells for 24 hr with 3 nM epinephrine produces a very pronounced attenuation of cAMP accumulation in response to subsequent challenges with much higher concentrations of the catecholamine [*Mol. Pharmacol.* **36**:459–464 (1989)]. We report here the effects of this treatment, in S49 WT, *cyc*⁻, and *kin*⁻ cells, on the responsiveness of adenylate cyclase in partially purified membranes. The desensitization of adenylate cyclase in the S49 WT cells after 24-hr treatment was homologous, in that only responses to epinephrine were attenuated. The EC_{50} for epinephrine stimulation of adenylate cyclase was 54 ± 8% (mean ± standard error) higher in treated cells than in controls, and there was a 32 ± 3% reduction in V_{max} at supramaximal epinephrine concentrations. The treatment also caused a 34 ± 9% reduction in the levels of the β-adrenergic receptor (βAR), which was of a sufficient magnitude to account for the homologous desensitization seen. The 24-hr treatment had similar effects in S49 *kin*⁻ cells, where we observed a 28 ± 4% decrease in V_{max}, a 35 ± 6% increase in EC_{50} for epinephrine stimulation of adenylate cyclase, and a 25 ± 3% decrease in βAR. In contrast, the 24-hr treatment had no measurable effect on adenylate cyclase activity in S49 *cyc*⁻ cells. That is, the responsivity of adenylate cyclase reconstituted with G_s from S49 WT cells was not attenuated, although βAR levels were significantly decreased. The desensitization of S49 cells with the 24-hr treatment was additive with that mediated by the cAMP-dependent protein kinase (cAPK). Further, unlike the cAPK-mediated attenuation, it was relatively insensitive to the levels of free Mg^{2+} in the adenylate cyclase reaction mixture. The characteristics of the desensitization produced by 24-hr treatment with 3 nM epinephrine, together with the observation that it is similar in S49 WT and *kin*⁻ cells, demonstrates that the process in WT cells is, at least in part, independent of the rapid cAPK-mediated desensitization. It is also most likely that it is unrelated to the rapid cAMP-independent processes involving sequestration/internalization or the βAR kinase, because those mechanisms require much higher receptor occupancies than the 0.2% occurring with 3 nM epinephrine. Thus, 24-hr treatment appears to produce attenuation of adenylate cyclase by causing down-regulation of βAR, without involving any other known form of desensitization.

Over a decade ago, several investigators demonstrated that incubation of cells with low concentrations of βAR agonists resulted in the desensitization of cells to challenges with βAR agonists (1, 2). We confirmed and extended these findings in studies examining the desensitization of cellular cAMP responses in S49 cells grown in 1–3 nM epinephrine for 24 hr (3), and we subsequently demonstrated that the attenuation of cAMP responses after the 24-hr treatment is matched by an attenuation of the epinephrine-induced activation of the cellular cAPK activity ratio (4). These observations suggested that, in S49 WT cells, profound desensitization could be produced by prolonged incubation with epinephrine at concentrations on the same order of magnitude as those found for circulating levels in resting rats (5). Thus, this form of desensitization should be considered in any assessment of the pharmacological or even physiological consequences of cellular βAR desensitization. In contrast, many previous studies exploring desensitization as a function of prolonged treatment with catecholamines have used very high concentrations of agonists, e.g., 1–10 μM epinephrine or isoproterenol (6–10).

An obvious next step was to determine how the desensitization produced by the 24-hr treatment with 3 nM epinephrine differed from or was similar to the kinds of desensitization of βAR previously described for the S49 cells. There are several known mechanisms (Table 1) that might be involved in the attenuation of adenylate cyclase responses in S49 lymphoma cells to subsequent epinephrine challenges after the 24-hr treatment. These are 1) the rapid cAPK-mediated desensitization, which in the S49 WT cell exhibits an EC_{50} of 5–10 nM (11); 2) the rapid cAMP-independent homologous desensitization, which appears to involve βARK and/or sequestration/internalization and requires concentrations of agonist of ≥100 nM (11–15); and 3) the slower down-regulation or loss of the βAR which

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ABBREVIATIONS: βAR, β-adrenergic receptor(s); cAPK, cAMP-dependent protein kinase; WT, wild-type; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PGE_1, prostaglandin E_1; βARK, β-adrenergic receptor kinase; ICYP, iodocyanopindolol.

TABLE 1
Agonist-mediated desensitization of β_2-AR in S49 lymphoma cells

Proposed mechanism	cAPK phosphorylation of Ser^{262} of β_2AR	Down-regulation of β_2AR	Sequestration/ internalization	βARK/βARRESTIN phosphorylation of Ser/Thr on carboxyl-terminal domain of β_2AR
Minimal epinephrine concentration for significant effect	5–10 nM	1–3 nM	100 nM[a]	>200 nM
$t_{½}$	1–3 min	3–6 hr	1–3 min	1–3 min
Desensitization for PGE_1 stimulation of adenylate cyclase	+[b]	–	–	–
Mg^{2+} sensitivity	+	–	–	–
Desensitization observed in				
kin$^-$	–	+	+	+
cyc$^-$	–	?	+	+
Change in total β_2AR levels	–	Decrease	–[c]	–

[a] For *kin*$^-$ the concentration is about 3-fold lower than for the WT cells (R. B. Clark and R. Barber, unpublished observations).
[b] +, yes; –, no.
[c] There is a change in receptor distribution but not in total levels.

occurs in response to prolonged incubation with βAR agonists and involves primarily a cAMP-independent process (2, 10, 16–18). The attenuation produced by 24-hr treatment with 3 nM epinephrine could involve one or more of these mechanisms. Alternatively, it is not inconceivable that there is a completely different pathway to desensitization that is apparent only after the 24-hr treatment with low concentrations of epinephrine.

In an effort to obtain an understanding of the desensitization produced by the 24-hr treatment, we have studied the effects of the treatment on the basic kinetic parameters of epinephrine, forskolin, and PGE_1 stimulation of adenylate cyclase in membranes from S49 WT, *kin*$^-$, and *cyc*$^-$ cells. Further, we have examined the changes in βAR levels, as measured using [^{125}I] ICYP, and have looked for additivity of the desensitization observed with the 24-hr treatment and with cAPK-mediated attenuation. We also have contrasted the magnesium sensitivity of the desensitization of adenylate cyclase observed after the 24-hr treatment with that observed with cAPK-dependent (11, 21) and -independent desensitization. Our results clearly distinguish the desensitization produced with the 24-hr treatment from the acute cAPK-mediated desensitization. Further, it seems unlikely that acute cAMP-independent homologous desensitization can explain the attenuation seen after 24-hr treatment. However, the desensitization observed with 24-hr treatment with 3 nM epinephrine can be largely explained by the down-regulation of βAR. These results are important because they reinforce earlier studies in other cell lines, which demonstrated that physiological concentrations of βAR agonists may produce down-regulation of receptors (2).

Materials and Methods

Cell culture and treatment. WT S49 cells, *cyc*$^-$ mutants, which lack the α-subunit of the stimulatory guanine nucleotide-binding protein G_s, and *kin*$^-$ clones 24.6.1 and U200/19, which lack functional cAPK, were grown in Dulbecco's modified Eagle's medium with antibiotics, as previously described (11), and with 20 mM HEPES, pH 7.4. The Dulbecco's modified Eagle's medium was supplemented with 5% horse serum for the WT cells and 10% serum for the mutants. Cells were maintained at populations of 0.5–2.0 × 10^6 cells/ml, with daily feedings. cAPK activity in the S49 *kin*$^-$ and WT clones was routinely measured, to ensure that there was no significant activity in the *kin*$^-$ cells.

In all experiments requiring 24-hr treatment, epinephrine or its vehicle (1.0 mM thiourea, 0.1 mM ascorbate) was added to the growth medium. The vehicle had no measurable effect on cAMP metabolism or adenylate cyclase (data not shown). In experiments in which cells were exposed to a second and higher concentration of epinephrine for 5–15 min at the end of the 24-hr treatment period, the cells were harvested, split into the appropriate groups, and resuspended in the original growth medium, at 20 × 10^6 cells/ml, before the addition of epinephrine or vehicle.

For the cell-free desensitization experiments, the one-stage protocol of Kunkel *et al.* (19) was followed. The membranes, the purified cAPK catalytic subunit, and the adenylate cyclase incubation mixture without [α-^{32}P]ATP were incubated together at 30° for 6 min. After this incubation, [α-^{32}P]ATP was added and the incubation was continued for an additional 10 min before the stop solution was added.

Membrane preparation and adenylate cyclase assays. Membranes for assay of adenylate cyclase were prepared as previously described (11), with slight modifications. After the epinephrine or vehicle pretreatment, cells were collected by centrifugation, washed twice with ice-cold buffer A (137 mM NaCl, 5.36 mM KCl, 1.1 mM KH_2PO_4, 1.08 mM Na_2HPO_4, 3 nM epinephrine or vehicle, at pH 7.2), and resuspended in buffer B (150 mM NaCl, 20 mM HEPES, 1 mM EDTA, 1 mM benzamidine, 3 nM epinephrine or vehicle, at pH 7.4) for lysing by nitrogen cavitation. The cell lysate was centrifuged at 600 × *g*, and the supernatant was layered over a gradient of 25% and 43% sucrose in 20 mM HEPES, pH 8.0, 1 mM EDTA. The gradient was centrifuged for 45–60 min at 80,000 × *g*, after which the band at the 25/43 interface was removed, frozen in liquid nitrogen, and maintained at −70° until the time of the assay.

Adenylate cyclase was assayed according to the method of Salomon *et al.* (20), with the modifications of Clark *et al.* (11). The assay solution contained 50 mM HEPES, pH 7.7, 1 mM EDTA, 1.47 mM $MgCl_2$ (0.3 mM final free Mg^{2+}) unless indicated otherwise, 8 mM creatine phosphate, 16 units/ml creatine phosphokinase, 0.2 mM ATP, 10 μM GTP, 0.1 mM 1-methyl-3-isobutylxanthine, 2 μCi of [α-^{32}P]ATP, and 40–50 μg of protein, in a final volume of 100 μl. The incubation was for 10 min at 37°. Unless otherwise indicated, all experimental values are the mean ± standard error of triplicate determinations.

The adenylate cyclase activity in S49 *cyc*$^-$ cells was assayed using a slight modification (21) of the previously described reconstitution procedure of Sternweis and Gilman (22). Crude cholate extracts of G_s were prepared from the S49 WT cell membranes (22), and the final concentration of free Mg^{2+} in the adenylate cyclase assay was 1.0 mM.

Determination of βAR levels. Levels of βAR in membrane preparations and in intact cells were determined with [^{125}I]ICYP, as previously described (21). The decreases in βAR levels after the 24-hr treatment were similar whether measurements were made with membrane preparations or intact cells.

Results

Effect of 24-hr treatment with 3 nM epinephrine on adenylate cyclase activity in S49 WT membranes. As shown in Fig. 1, membranes from S49 WT cells grown for 24 hr in medium containing 3 nM epinephrine demonstrated attenuated adenylate cyclase responses to epinephrine, compared with cells grown for 24 hr in medium containing the thiourea and ascorbate diluent. The V_{max} for epinephrine stimulation of adenylate cyclase was decreased from 330 pmol/min/mg to 210 pmol/min/mg, and the EC_{50} was increased from 170 nM to 300 nM. In 17 similar experiments performed over 1 year, the mean ± standard error for increase in EC_{50} was 54 ± 8% and the mean decrease in V_{max} was 32 ± 3%. The 24-hr treatment did not alter the responses of adenylate cyclase to PGE_1, forskolin, or NaF. Furthermore, S49 *cyc*⁻ membranes reconstituted with G_s from S49 WT cells previously exposed to 3 nM epinephrine for 24 hr responded similarly to increasing concentrations of epinephrine (data not shown). These results were consistent with homologous desensitization of adenylate cyclase and suggested that the desensitization did not involve changes in G_s or the catalytic unit.

Effect of 24-hr treatment with 3 nM epinephrine on adenylate cyclase activity of S49 *cyc*⁻ and *kin*⁻ variants. Fig. 2 illustrates that the adenylate cyclase activity in the *cyc*⁻ S49 variant (which lacks functional G_s) was unaffected by 24-hr treatment with 3 nM epinephrine. This was demonstrated by measuring adenylate cyclase activity in membranes prepared from S49 *cyc*⁻ cells treated with 3 nM epinephrine or vehicle for 24 hr and reconstituted with cholate extracts of G_s prepared from untreated S49 WT membranes. Epinephrine-stimulated adenylate cyclase activity was similar in control and epinephrine-treated cells, suggesting that receptor occupancy alone was not sufficient to produce the attenuation seen after the 24-hr treatment. This was clearly different from the case in which high epinephrine concentrations were used to produce cAMP-independent desensitization in S49 *cyc*⁻ cells. Green and Clark (13) and Clark *et al.* (12) previously showed that treatment of S49 *cyc*⁻ cells with 10 μM epinephrine for short periods caused a 50–70% decrease in epinephrine-stimulated adenylate cyclase activity, and those results were confirmed during the course of this study. That is, when epinephrine-stimulated adenylate cyclase was measured in membranes from S49 *cyc*⁻ cells that had been subjected to the 24-hr treatment with 3 nM epinephrine or a 15-min incubation with vehicle or 1 μM epinephrine, attenuation of epinephrine stimulation of adenylate cyclase occurred only in cells treated with 1 μM epinephrine. Thus, the ability of S49 *cyc*⁻ cells to be desensitized when pretreated with high concentrations of epinephrine was confirmed, and the absence of attenuation after the 24-hr treatment suggested that the two processes differ. However, one clear effect of the 24-hr treatment on S49 *cyc*⁻ cells was apparent. βAR concentration, as measured by [^{125}I]ICYP binding to membrane fractions from S49 *cyc*⁻ cells in four independent experiments, was reduced from 114 ± 14 fmol/mg of protein in cells treated for 24 hr with thiourea and ascorbate to 85 ± 9 fmol/mg of protein (a reduction of about 25%) in cells treated with 3 nM epinephrine for 24 hr.

As demonstrated in the data from a typical experiment shown in Fig. 3, the effects of the 24-hr 3 nM epinephrine treatment on S49 *kin*⁻ cells were similar to those observed with S49 WT cells. The 24-hr treatment caused a decrease in V_{max} (from 97 to 70 pmol/min/mg) and an increase in EC_{50} (from 140 to 230 nM). It did not significantly affect the responses to forskolin, NaF, or PGE_1 (data not shown). Over the course of several

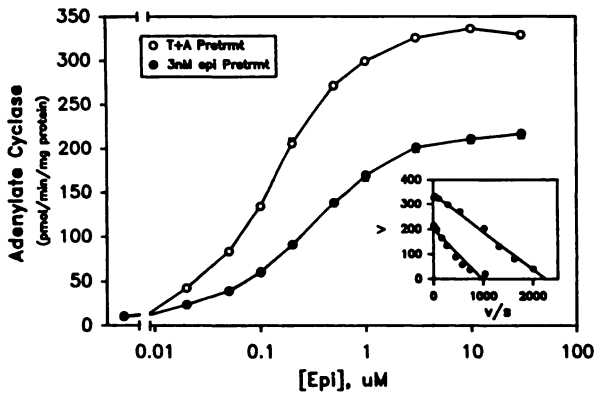

Fig. 1. Effect of long term treatment with 3 nM epinephrine on epinephrine stimulation of adenylate cyclase in S49 cells. Epinephrine (*Epi*) stimulation of adenylate cyclase was measured in membranes prepared from S49 cells pretreated with 3 nM epinephrine (●) or thiourea-ascorbate (*T+A*) (○) for 24 hr. *Inset*, Eadie-Hofstee plots of the data (basal activity is subtracted for *V*).

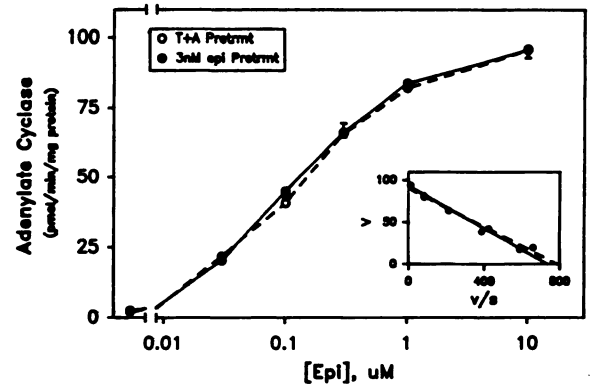

Fig. 2. Effect of long term treatment with 3 nM epinephrine on epinephrine stimulation of adenylate cyclase in *cyc*⁻ cells. Epinephrine (*Epi*) stimulation of adenylate cyclase was measured in membranes prepared from *cyc*⁻ cells pretreated with 3 nM epinephrine (●) or thiourea-ascorbate (*T+A*) (○) for 24 hr. Membranes were reconstituted with WT $G_{sα}$, as described in Materials and Methods. *Inset*, Eadie-Hofstee plots of the data (basal activity is subtracted for *V*).

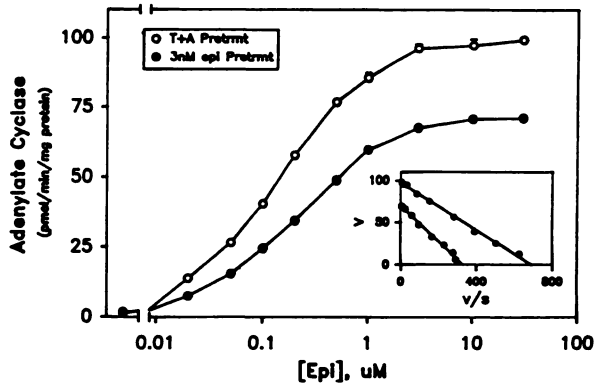

Fig. 3. Effect of long term treatment with 3 nM epinephrine on epinephrine stimulation of adenylate cyclase in *kin*⁻ cells. Epinephrine (*Epi*) stimulation of adenylate cyclase was measured in membranes from *kin*⁻ cells pretreated with 3 nM epinephrine (●) or thiourea-ascorbate (*T+A*) (○) for 24 hr. *Inset*, Eadie-Hofstee plots of the data (basal activity is subtracted for *V*).

months, results from nine similar experiments with S49 *kin*$^-$ cells showed that the 24-hr treatment caused a mean ± standard error of decrease in V_{max} of 28 ± 4% and a mean increase in EC_{50} of 35 ± 6%. When a second S49 *kin*$^-$ strain, U200 *kin*$^-$, was examined in three experiments, the 24-hr treatment produced a mean decrease in V_{max} of 17 ± 7% and an increase in EC_{50} of 35 ± 3%.

Additivity of the desensitization produced with 24-hr treatment with 3 nM epinephrine and via cAPK. Clark *et al.* (11) previously showed that brief treatment of S49 WT cells with concentrations of epinephrine ranging from 5 to 100 nM caused a heterologous desensitization mediated by the cAPK, and in this study we demonstrated, using several approaches, that the attenuations produced with the 24-hr treatment and with cAPK were additive. First, as shown in Fig. 4 for a representative experiment, adenylate cyclase activity was measured in membranes prepared from S49 WT cells grown with 3 nM epinephrine for 24 hr and then exposed to short term cAPK-mediated desensitization by incubation with 100 nM epinephrine for 5 min. The 24-hr treatment alone caused a decrease in V_{max} (219 ± 61 to 94 ± 27 pmol/min/mg; $p = 0.019$; mean ± standard deviation; four experiments) and an increase in the EC_{50} (197 ± 25 to 413 ± 120 nM; $p = 0.060$) for epinephrine stimulation of the adenylate cyclase system, whereas the short term treatment resulted in a highly significant increase in the EC_{50} (197 ± 25 to 457 ± 31 nM; $p = 0.002$), with little change in V_{max} (219 ± 61 to 179 ± 38 pmol/min/mg; $p = 0.155$). Exposure of cells to both the 24-hr treatment and short term treatment resulted in an increase in EC_{50} that was significantly greater than the increase observed with either treatment alone (from 413 ± 120 nM with the 24-hr treatment and 457 ± 31 nM with the 15-min treatment to 783 ± 149 nM with the combined treatment; $p = 0.002$ for the 24-hr group and $p = 0.009$ for the 15-min group). This demonstrated that the two types of attenuation were additive and provided support for the idea that the processes are distinct.

A second approach involved the use of a cell-free system mimicking cAPK-dependent desensitization, developed by Kunkel *et al.* (19), in which membranes from control or treated cells were exposed to the catalytic unit of cAPK before the measurement of adenylate cyclase activity. As shown in Fig. 5,

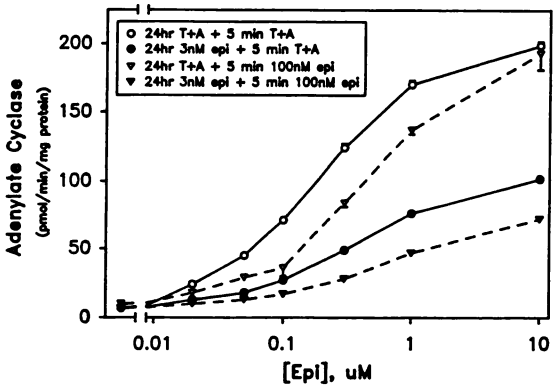

Fig. 4. Effect of short term treatment with 100 nM epinephrine on epinephrine stimulation of adenylate cyclase in S49 cells exposed to long term treatment with 3 nM epinephrine. Epinephrine (*Epi*) stimulation of adenylate cyclase was measured in membranes from S49 cells pretreated with 3 nM epinephrine (●, ▼) or thiourea-ascorbate (*T+A*) (○, ▽) for 24 hr and then exposed to 100 nM epinephrine (▽, ▼) or thiourea-ascorbate (○, ●) for 5 min.

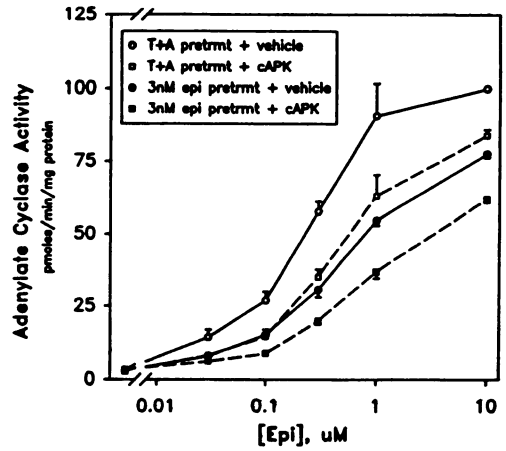

Fig. 5. Effect of cAPK on epinephrine stimulation of adenylate cyclase in S49 cells exposed to long term treatment with 3 nM epinephrine. Epinephrine (*Epi*) stimulation of adenylate cyclase was measured in membranes from S49 cells pretreated with 3 nM epinephrine (●, ■) or thiourea-ascorbate (*T+A*) (○, □) for 24 hr and exposed to cAPK (■, □) or vehicle (●, ○) during the adenylate cyclase assay. *Points*, means ± standard error (six experiments), with the data collected in two independent experiments.

treatment with the catalytic subunit for 6 min before the adenylate cyclase assay resulted in decreased epinephrine-stimulated activity. The decrease in activity was of similar magnitude in the control and treated cells. This, again, suggested that the attenuation due to the 24-hr treatment was additive with the cAPK-dependent desensitization.

Magnesium sensitivity of desensitization. Because it is well established that the cAPK-mediated short term desensitization of adenylate cyclase is masked by the presence of high concentrations of free Mg^{2+} (3–10 mM) in the adenylate cyclase assay (11, 21, 23), we compared the effect of varying Mg^{2+} concentrations on various forms of desensitization. Fig. 6A illustrates the effects of Mg^{2+} concentration on epinephrine stimulation of adenylate cyclase in membranes prepared from S49 WT cells exposed to 3 nM epinephrine for 24 hr or to short term treatment with 100 nM epinephrine (5-min exposure, causing cAPK-dependent heterologous desensitization). The attenuation produced by the 24-hr treatment was less sensitive to the free Mg^{2+} concentration than was that produced by short term treatment (see Fig. 6A, *inset*). That is, the desensitization measured after 100 nM epinephrine fell to zero as the concentration of free Mg^{2+} was increased from 0.1 to 10 mM. On the other hand, the desensitization observed with the 24-hr treatment was quantitatively similar at Mg^{2+} concentrations up to 1.0 mM and never fell below 25%. We also examined the Mg^{2+} sensitivity of the cAMP-independent homologous desensitization, by measuring epinephrine-stimulated adenylate cyclase activity in membranes from S49 *kin*$^-$ cells pretreated with or without 1 μM epinephrine for 15 min (Fig. 6B). Desensitization was unaffected by Mg^{2+} concentrations up to 1 mM, above which it began to decrease. However, the decrease in desensitization was much less dramatic than that observed with the short term cAPK-mediated desensitization and even the attenuation caused by 24-hr treatment with 3 nM epinephrine.

Changes in βAR with 24-hr treatment with 3 nM epinephrine. The data presented above suggest that, at least in some ways, the attenuation produced by 24-hr treatment with 3 nM epinephrine was different from the cAPK-dependent or cAMP-independent desensitization mechanisms previously de-

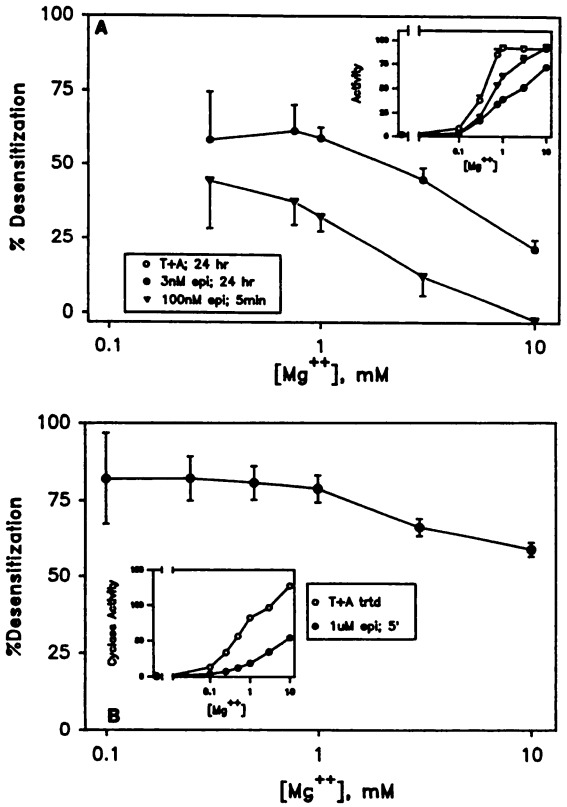

Fig. 6. Effect of magnesium concentration on the desensitization measured in S49 and *kin*$^-$ cells exposed to long term or short term treatment with epinephrine. The percentage of desensitization of adenylate cyclase activity to stimulation by 100 nM epinephrine (*Epi*) was measured at various concentrations of free magnesium, in membranes prepared from S49 cells pretreated with 3 nM epinephrine for 24 hr (●) or 100 nM epinephrine for 5 min (▼) (A) or from *kin*$^-$ cells pretreated with 1 μM epinephrine for 15 min (●) (B). *Insets*, adenylate cyclase data for percentage of desensitization measurement. Adenylate cyclase activity (pmol/min/mg of protein) was measured with 100 nM epinephrine at various concentrations of free magnesium, in membranes from S49 cells pretreated with 3 nM epinephrine for 24 hr (●), 100 nM epinephrine (▼), or thiourea-ascorbate (*T+A*) for 5 min (○) (A) or from *kin*$^-$ cells pretreated with 1 μM epinephrine (●) or thiourea-ascorbate (○) for 15 min (B).

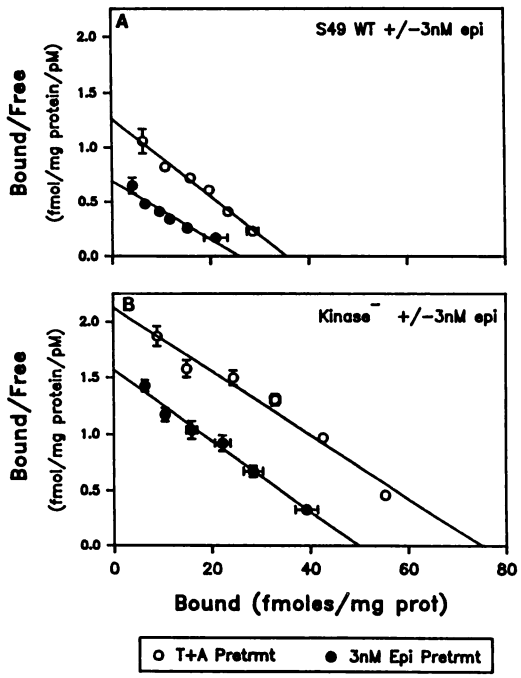

Fig. 7. Scatchard plots showing the effect of long term treatment with 3 nM epinephrine on ICYP binding to S49 WT membranes. [^{125}I]ICYP binding was measured in S49 membranes prepared from S49 (A) or *kin*$^-$ (B) cells after long term treatment with 3 nM epinephrine (*Epi*) (●) or thiourea-ascorbate (*T+A*) (○) for 24 hr.

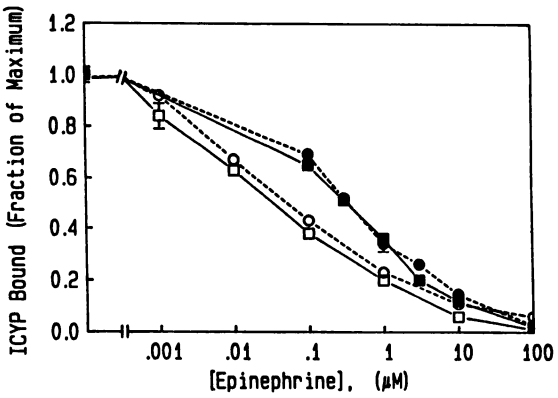

Fig. 8. Displacement of [^{125}I]ICYP binding to S49 membranes by epinephrine. The concentration of [^{125}I]ICYP was 69 pM. ——, Membranes isolated from control (thiourea-ascorbate-treated) cells; – – –, membranes isolated from cells treated for 24 hr with 3 nM epinephrine. To maximize the difference between treated and untreated preparations, the assays contained no added Mg^{2+}. □, Control membranes without GTP; ■, control membranes with GTP; ○, desensitized membranes without GTP; ●, desensitized membranes with GTP.

scribed. This difference was also apparent in receptor binding studies. Previous studies of the rapid forms of desensitization in S49 WT membranes demonstrated that there were no changes in levels of βAR, as measured by [^{125}I]ICYP binding. However, as shown in Fig. 7A with S49 WT membranes and in Fig. 7B with S49 *kin*$^-$ membranes, 24-hr treatment with 3 nM epinephrine caused very significant loss of binding sites available for [^{125}I]ICYP but little change in affinity for the ligand of the remaining βAR. The mean ± standard error of decrease in βAR was 34 ± 9% (three experiments) in S49 WT cells and 25 ± 3% (nine experiments) in S49 *kin*$^-$ cells, with a $t_{1/2}$ of approximately 4–6 hr. The 24-hr treatment with 3 nM epinephrine did not significantly affect epinephrine affinity, as measured by epinephrine displacement of ICYP in the presence or absence of GTP (Fig. 8).

Discussion

As noted in the introduction and Table 1, several mechanisms (cAPK, βARK, internalization/sequestration, and down-regulation) have been implicated in the attenuation of adenylate cyclase responses to epinephrine in S49 lymphoma cells, and the attenuation produced by 24-hr treatment with 3 nM epinephrine characterized in this paper may involve one or more of these mechanisms. Although it is also possible that there is a completely different pathway responsible for the desensitization observed with the 24-hr treatment, the results presented here suggest that at least part of the desensitization due to the 24-hr treatment is a consequence of the down-regulation of βAR.

The observation that the attenuation associated with the 24-hr treatment was apparent in S49 *kin*$^-$ as well as WT cells demonstrated that at least a part of the desensitization was due to a cAPK-independent process, thus distinguishing it from the

cAPK-dependent desensitization characterized by Clark *et al.* (11). The idea that these are two distinctly different processes was further supported by the findings that the desensitization observed with the 24-hr treatment was additive with that observed after treatments associated with cAPK-dependent desensitization and was much less sensitive to the presence of high concentrations of Mg^{2+} in the adenylate cyclase assay than was cAPK-dependent desensitization.

There is also evidence that the attenuation produced by the 24-hr treatment with 3 nM epinephrine is not mechanistically identical to the very rapid cAMP-independent homologous desensitization that can be observed after brief treatment with high concentrations of epinephrine. Under those conditions, two major cAMP-independent routes to desensitization have been described in S49 WT, *kin*$^-$, and *cyc*$^-$ cells, i.e., sequestration/internalization (10, 12) and the βARK pathway (14, 23). They have a $t_{1/2}$ of <5 min and require substantial receptor occupancy by agonist (see Table 1). Neither is accompanied by significant down-regulation, but sequestration/internalization is distinguished by a redistribution of βAR to a light vesicle fraction in sucrose gradients. In contrast, desensitization observed with the 24-hr treatment had a $t_{1/2}$ of 4–6 hr, an EC_{50} of ≈3 nM, and a receptor occupancy of <0.2% with the 3 nM epinephrine used with the 24-hr treatment. We also observed no effect of the 24-hr treatment on reconstituted adenylate cyclase activity from S49 *cyc*$^-$ cells, but there was considerable down-regulation of βAR. Thus, the data suggest that the mechanism associated with desensitization after the 24-hr treatment differs from that associated with the sequestration/internalization and βARK pathways. The possibility remains that one of these two pathways, i.e., sequestration/internalization or βARK, is involved in the attenuation produced by the 24-hr treatment. However, because both occurred in S49 *cyc*$^-$ cells (10–12) after treatment with high concentrations of agonists, whereas there was no effect of the 24-hr 3 nM epinephrine treatment on adenylate cyclase activity, one would be forced to postulate either a downstream defect in S49 *cyc*$^-$ cells or an artifact due to reconstitution. Further, the finding that the 24-hr treatment caused a significant reduction in βAR on intact S49 *cyc*$^-$ cells is paradoxical. This is because that decrease should have been sufficient to have caused a measurable attenuation of the adenylate cyclase system. It is possible that the reconstitution protocol rendered the attenuation produced by the 24-hr treatment occult.

In contrast to the differences between the desensitization with the 24-hr treatment and the desensitization associated with cAPK, sequestration/internalization, and βARK, the reduction of epinephrine-stimulated adenylate cyclase observed with the 24-hr treatment can be largely explained by the down-regulation of βAR. The 24-hr treatment with 3 nM epinephrine produced a 34% mean loss of βAR in S49 WT cells, and 10 nM epinephrine (data not shown) produced losses approaching the 70–90% loss of receptor previously reported after treatment of cells for 16–24 hr with 1–100 μM β_2AR agonists (10, 16, 17). We routinely used the lower concentration because it is close to the EC_{50} and is also closer to the circulating levels of epinephrine reported in rats by Siri and Kauer (5).

There appear to be at least two major routes to down-regulation. The fact that it occurs in S49 *kin*$^-$ cells (present study and Ref. 10) and in the S49 coupling mutant H21a (9, 10) argues that there must be a cAMP-independent mechanism. On the other hand, Bouvier *et al.* (24) have reported extensive down-regulation by dibutyryl-cAMP in Chinese hamster fibroblast cells in the absence of any βAR agonist, indicating a cAMP-dependent mechanism that does not require receptor occupancy. In our experiments, because the losses of βAR in the S49 WT and *kin*$^-$ cells were similar, it does not seem unlikely that the process is largely cAMP independent.

The data presented here demonstrate that growth of S49 cells with concentrations of epinephrine approximating those reported in resting rats (5) caused significant βAR loss. Regardless of the mechanism by which this down-regulation occurred, the result was a marked attenuation of adenylate cyclase responses to later challenges by higher epinephrine concentrations. This suggests that in any *in vivo* system, in which a finite level of epinephrine is always present, a significant degree of βAR down-regulation may be present.

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