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β_2-Adrenergic Receptor Mutants Reveal Structural Requirements for the Desensitization Observed with Long Term Epinephrine Treatment

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SUMMARY

In the present study wild-type and various mutant hamster β_2-adrenergic receptors (βARs) expressed in L cells were used to examine potential molecular mechanisms involved in the desensitization of hormonal stimulation of adenylyl cyclase observed after long term exposure to low concentrations of epinephrine. The mutant βARs included deletion mutants, D(259–262)βAR and D(343–348)βAR, that lack the consensus sites for cAMP-dependent protein kinase (cAPK) and protein kinase C (PKC) and a truncation mutant, T(354)βAR, that lacks the putative consensus sites for βAR kinase. Epinephrine stimulation of adenylyl cyclase was desensitized in all four cell lines after growth for 24 hr in the presence of 3 nM epinephrine, and this desensitization was characterized by a 3–4-fold increase in the EC_{50} for epinephrine stimulation of adenylyl cyclase. In addition, the V_{max} was significantly decreased in the cells with the wild-type βAR and the D(343–348)βAR. The desensitization was not masked by high concentrations of magnesium and was accompanied by a 40–70% down-regulation of βARs. In the cells treated with 3 nM epinephrine, prostaglandin E_1 stimulation of adenylyl cyclase was decreased 11% in cells with the wild-type βAR and forskolin stimulation was decreased 25–36% with all but the D(259–262)βAR mutant. These results demonstrated that phosphorylations of the cAPK/PKC consensus sites and the serine- and threonine-rich segment of the carboxyl-terminal tail of the βAR were not required for the desensitization caused by 3 nM epinephrine, thus further differentiating it from cAPK- or PKC-mediated desensitization and the desensitization attributed to βAR kinase.

It is now apparent from numerous studies that there are a number of distinct mechanisms for desensitization of βAR stimulation of adenylyl cyclase. Most thoroughly documented are those involving (i) cAPK- and PKC-mediated phosphorylation of the third intracellular loop consensus site (1, 2), (ii) internalization of the βAR (3, 4), (iii) the βARK/β-arrestin pathway, for which there is extensive cell-free evidence (5), and (iv) down-regulation of the βAR. The first three occur within minutes, whereas down-regulation[1] occurs with a $t_{1/2}$ of 2–4 hr and has been most thoroughly studied after treatment of cells with high concentrations of agonists (3, 6).

In addition to these types of desensitization, it was found that prolonged treatment (2–24 hr) of astrocytoma cells with very low concentrations of isoproterenol (0.1–5 nM) (7) and of S49 lymphoma cells (8, 9) or L cells (10) with 0.3–3.0 nM epinephrine caused extensive desensitization of βAR stimulation of cAMP accumulation and adenylyl cyclase (7–9). This phenomenon may be quantitatively one of the most important forms of desensitization because the effects are large, and it may be one of the most physiologically relevant because the low epinephrine levels that elicit this effect are similar to those in the bloodstream. Nonetheless, it has received scant attention, and the mechanism and its relationship to the other pathways of desensitization that have been characterized after treatment of cells with relatively high concentrations of βAR agonists (10 nM to 100 μM) are not understood.

In terms of phenomenology, to date it has been shown that the prolonged treatment of cells with low concentrations of epinephrine (3 nM for 24 hr) induces an homologous desensitization of adenylyl cyclase and a down-regulation of βARs that is of sufficient magnitude to account for the decreased responsiveness of adenylyl cyclase to epinephrine (7–10). Effects on cAMP accumulation are more complex and include a partial heterologous component in S49 lymphoma cells, i.e., PGE_1 stimulation is slightly reduced, due at least in part to increased

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[1] Down-regulation specifically refers to the agonist-induced loss of βARs measured using relatively hydrophobic antagonists such as ICYP, to distinguish it from the sequestration/internalization of receptors that is detected by the loss of hydrophilic agonist and antagonist binding to surface receptors.

ABBREVIATIONS: β_2AR, β-adrenergic receptor; cAPK, cAMP-dependent protein kinase; PKC, protein kinase C; βARK, β-adrenergic receptor kinase; PDE, phosphodiesterase; D(259–262)βAR and D(343–348)βAR, mutant β-adrenergic receptors with amino acids 259–262 and 343–348, respectively, deleted; T(354)βAR, mutant β-adrenergic receptor with amino acids 354–418 deleted; ICYP, iodocyanopindolol; PGE_1, prostaglandin E_1.

PDE activity (8). In L cells transfected with βARs, elevations of cAMP in response to agonists of adenylyl cyclase are not readily observed because cAMP metabolism is largely dominated by the powerful, positively cooperative, cAMP PDE in these cells (10). However, the use of PDE inhibitors and low concentrations of forskolin made it possible to overcome the dampening effect of the PDE and to observe significant desensitization after growth in the presence of 3 nM epinephrine for 24 hr.

Recently we showed that the desensitization of adenylyl cyclase caused by treatment of S49 wild-type cells with 3 nM epinephrine for 24 hr did not resemble that caused by the rapid effects of either cAPK or PKC, because it was homologous (i.e., epinephrine but not PGE_1 stimulation was desensitized), was measurable in the presence of high levels of free Mg^{2+}, and was observed in *kin*$^-$ cells, which lack cAPK (9). In contrast, cAPK- and PKC-mediated desensitizations (1, 2, 11) are heterologous and substantially masked by high free Mg^{2+} in the adenylyl cyclase assay. The desensitization of epinephrine-stimulated adenylyl cyclase seen after 24 hr with 3 nM epinephrine resembled that caused by either βARK/β-arrestin (in cell-free preparations) or sequestration/internalization mechanisms, in that all are homologous. However, desensitization of βAR stimulation by βARK/β-arrestin or sequestration/internalization is rapid, requires incubation of cells with very high concentrations of epinephrine (EC_{50} = 100–200 nM), and does not result in significant down-regulation of βAR during the time period that gives maximal desensitization (2–20 min).

In this study we examined potential molecular mechanisms and receptor domains involved in the desensitization observed with long term exposure to low concentrations of epinephrine (3 nM epinephrine for 24 hr), by using several βAR mutants expressed in L cells. They included two deletion mutants, D(259–262)βAR and D(343–348)βAR, that lack the consensus sites for cAPK and PKC and a truncation mutant, T(354)βAR, that lacks amino acid residues 354 to 418 in the carboxyl-terminal tail. The deletion mutants allowed us to assess the potential role of phosphorylation of cAPK/PKC consensus sites in the desensitization process (9). The truncation mutant T(354)βAR, lacking the carboxyl-terminal domain, allowed us to assess the potential role of βARK in the desensitization observed with 3 nM epinephrine, because considerable data suggest that the βAR is desensitized after βARK phosphorylation of the serine- and threonine-rich carboxyl-terminal domain of the agonist-occupied βAR and β-arrestin binding (5, 12, 13).

Materials and Methods

Cell culture. Mouse L cells transfected with wild-type and mutant hamster βARs (1, 2) were generously provided by Dr. Catherine D. Strader and Dr. Richard A. F. Dixon (Merck, Sharp, and Dohme Research Laboratories). The cells were grown at 37° in Dulbecco's modified Eagle's medium with 10% fetal calf serum, 2 mM L-glutamine, and 0.4 mg/ml geneticin. For the experimental protocols, epinephrine or its vehicle (1.0 mM thiourea and 0.1 mM ascorbate) was added to the growth medium for 24 hr unless otherwise indicated.

Adenylyl cyclase assays. Adenylyl cyclase activity was assayed in L cell membranes prepared using previously described techniques (1, 2); the assay was a modification (11) of the procedure of Salomon *et al.* (14). The incubation period was 10 min, and experimental values are the mean ± standard error of triplicate determinations. Unless otherwise noted, free Mg^{2+} concentrations were either 0.3 or 1.0 mM. In this range, Mg^{2+} concentration had no significant effects on the extent of the desensitization of adenylyl cyclase caused by the 3 nM epinephrine treatment for 24 hr (see Fig. 3). Additionally, the experiments were conducted at two different times, and the standard assay conditions varied in that, with the earlier experiments, lower concentrations of GTP (1 versus 10 μM) were typically used for the assay and the incubation was at 37° (versus 30° used in latter experiments). Although absolute adenylyl cyclase activities were lower using 10 μM GTP and 30° incubation, these conditions did not alter the relative changes observed with the 3 nM epinephrine treatment.

To determine the EC_{50} and the V_{max} for epinephrine stimulation of adenylyl cyclase, the data were analyzed with a linear regression analysis of Eadie-Hofstee plots. Basal activity was subtracted in calculations of epinephrine and PGE_1 stimulation of adenylyl cyclase, to eliminate the nonhormonal component of adenylyl cyclase activity. Basal activity was not subtracted from the forskolin-stimulated activity because it was used to assess G_s stimulation of the catalytic subunit. Basal adenylyl cyclase levels ranged from 1.1 to 4.4 pmol of cAMP/min/mg of protein, and basal activity was 16–50% lower in the cells treated with 3 nM epinephrine for 24 hr. The difference was statistically significant only in cells with D(343–348)βAR ($p < 0.025$). A paired *t* test was used to analyze the differences between the control group and the group treated with 3 nM epinephrine, and differences between the four cell lines were assessed with Student's *t* test.

Determination of βAR levels. Levels of βARs were measured in membranes with [^{125}I]ICYP, as described previously (15). A paired *t* test was used to analyze the differences between the control group and the group treated with 3 nM epinephrine, and differences between the four cell lines were assessed with Student's *t* test.

Results

Effect of 24-hr treatment with 3 nM epinephrine on epinephrine stimulation of adenylyl cyclase. When L cells expressing the wild-type βAR were grown in the presence of 3 nM epinephrine for 24 hr, subsequent epinephrine stimulation of adenylyl cyclase in membrane preparations was attenuated (Fig. 1). The desensitization was characterized by a significant increase in EC_{50} for epinephrine stimulation of adenylyl cyclase and a significant decrease in V_{max} ($p < 0.005$ for both). In seven experiments, the mean increase in EC_{50} was 296 ± 79% and the mean decrease in V_{max} was 44 ± 4%. A time course study in which the 3 nM epinephrine treatment was varied from 4 to 48 hr suggested a $t_{1/2}$ for the desensitization of 3–6 hr (data not shown).

The D(259–262)βAR, D(343–348)βAR, and T(354)βAR mutants also exhibited desensitization to epinephrine stimulation

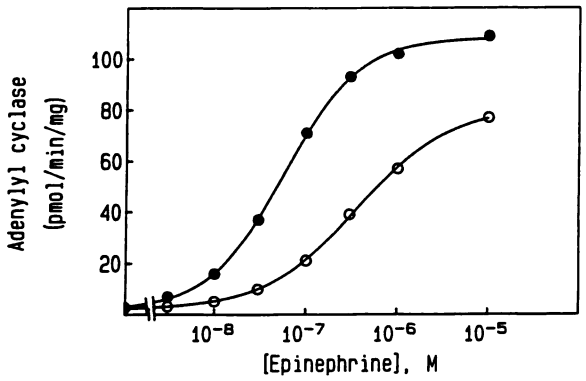

Fig. 1. Epinephrine stimulation of adenylyl cyclase in membranes from L cells grown in the presence of 3 nM epinephrine for 24 hr. Epinephrine stimulation of adenylyl cyclase was measured in membranes prepared from L cells (expressing the wild-type βAR) grown in the presence of vehicle (●) or 3 nM epinephrine (○) for 24 hr.

after 24-hr treatment with 3 nM epinephrine (Table 1). The EC_{50} was significantly increased for all three mutants, with $p < 0.005$ for the D(259–262)βAR and T(354)βAR mutants and $p < 0.025$ for the D(343–348)βAR mutant. The percentage increases varied from 199% for the D(259–262)βAR mutant to 397% for the D(343–348)βAR mutant and were not significantly different from the increase in EC_{50} observed in cells with the wild-type βAR. V_{max} was significantly reduced by the 3 nM treatment of the D(343–348)βAR mutant (44 ± 4%, $p < 0.05$). The D(259–262)βAR and T(354)βAR mutants had reductions in V_{max} of 8 ± 5% and 21 ± 14%, respectively, both of which were significantly less than the decrease observed in cells with the wild-type βAR ($p < 0.0005$ and $p < 0.05$, respectively).

Effect of 24-hr treatment with 3 nM epinephrine on PGE_1 and forskolin stimulation of adenylyl cyclase. The 3 nM epinephrine treatment of cells expressing the wild-type βAR also resulted in a small but significant decrease in PGE_1 stimulation of adenylyl cyclase (11 ± 4%, $p < 0.05$) and a significant decrease in forskolin stimulation of adenylyl cyclase (25 ± 4%, $p < 0.01$) when submaximal concentrations of agonist were used (Table 2). The decrease, however, was substantially less than the 75 ± 3% decrease in adenylyl cyclase activity that was observed with epinephrine (epinephrine concentration at or near the EC_{50}). Comparable experiments with the mutants revealed no significant effect of the 3 nM epinephrine treatment on PGE_1 stimulation of adenylyl cyclase, but there was a significant reduction in forskolin stimulation for the D(343–348)βAR mutant (36 ± 12%, $p < 0.025$) and T(354)βAR mutant (33 ± 12%, $p < 0.025$).

Magnesium sensitivity of desensitization observed after 24-hr treatment with 3 nM epinephrine. We previously showed that the presence of high concentrations of free Mg^{2+} in the adenylyl cyclase assay masks both the cAPK- and PKC-mediated desensitizations (1, 2, 15). Such was not the case when L cells expressing the wild-type βAR were grown in the presence of 3 nM epinephrine for 24 hr. That is, the expression of the desensitization was not altered by high concentrations of Mg^{2+} in the adenylyl cyclase assay (Fig. 2). Likewise, high Mg^{2+} did not mask the desensitization observed with the deletion and truncation βAR mutants, although there was a slight reduction at concentrations of 10 mM free Mg^{2+}, compared with 0.25 mM Mg^{2+}, with the D(259–262)βAR and T(354)βAR mutants.

Changes in βARs after 24-hr treatment with 3 nM epinephrine. As was found in S49 cells (9), the 3 nM epinephrine treatment of L cells expressing wild-type βARs caused a significant loss of binding sites available for [^{125}I]ICYP ($p < 0.0005$) (Table 3). The mean decrease in six experiments was 54 ± 4%, and this was of sufficient magnitude to account for the desensitization of adenylyl cyclase.

The 3 nM epinephrine treatment also resulted in a loss of binding sites for [^{125}I]ICYP in cells with mutant βARs (Table 3). The decrease was significant for the D(259–262)βAR mutant ($p < 0.005$) and the D(343–348)βAR mutant ($p < 0.0005$) but was not significant for the T(354)βAR mutant (57 ± 9%, $p < 0.1$). The 38 ± 6% receptor loss observed with the D(259–262)βAR mutant was significantly less than both the 54% loss observed with the wild-type cells ($p < 0.05$) and the 71 ± 6% loss observed with the D(343–348)βAR mutant ($p < 0.005$). The 71% decrease observed with the D(343–348)βAR mutant was slightly but significantly greater than the loss observed with the wild-type cells ($p < 0.025$).

Discussion

The present results provide evidence that prolonged treatment of the fibroblast-like L cells with very low concentrations of epinephrine causes a 60–80% desensitization of βAR stimulation of adenylyl cyclase, which closely resembles the desensitization/down-regulation previously reported for astrocytoma (7) and lymphoma (8, 9) cells under similar conditions. The desensitization of L cells can be explained in large part by the loss of βARs, as measured by the binding of the hydrophobic antagonist [^{125}I]ICYP. Although there is a small heterologous component in the L cells, as indicated by the slight loss of PGE_1 stimulation of adenylyl cyclase, it does not appear to be a significant component. A significant loss of forskolin stimulation in cells expressing the wild-type and mutant βARs was also observed, and this indicated an effect of the 3 nM/24-hr pretreatment on components downstream of the receptor. However, the relationship of this to the loss of βAR stimulation of adenylyl cyclase is unknown, because forskolin activates all adenylyl cyclases and neither their number nor their relationship to the βAR in L cells is known.

Little is understood about either the domains of the βAR or the mechanisms involved in the desensitization of adenylyl cyclase caused by prolonged treatment with low concentrations

TABLE 1

Effect of 3 nM epinephrine treatment on epinephrine stimulation of adenylyl cyclase in membranes from L cells expressing wild-type and mutant βARs

Epinephrine stimulation of adenylyl cyclase was measured in membranes prepared from L cells grown in the presence of thiourea-ascorbate (control) or 3 mM epinephrine for 24 hr. The number of experiments (n) is indicated, and the values are the mean ± standard error. The percentage increase is the mean increase in EC_{50} for the number of experiments indicated, and the percentage decrease is the mean decrease in V_{max} for the number of experiments indicated. A paired t test was used to compare the control and 3 nM epinephrine groups. Comparison of the control groups revealed that the EC_{50} and V_{max} were significantly greater in the cells with the wild-type βAR than with the T(354)βAR mutant ($p < 0.05$).

Cell line	EC_{50}		EC_{50} increase	V_{max}		V_{max} decrease
	Control	3 nM Epinephrine		Control	3 nM Epinephrine	
	nM		%	pmol of cAMP/min/mg		%
Wild-type βAR (n = 7)	61 ± 18	194 ± 39[a]	296 ± 79	73 ± 18	42 ± 12[a]	44 ± 4
D(259–262)βAR (n = 6)	41 ± 13	111 ± 27[a]	199 ± 45	49 ± 11	43 ± 9	8 ± 5
D(343–348)βAR (n = 5)	27 ± 10	106 ± 33[b]	397 ± 129	56 ± 18	29 ± 7[c]	44 ± 4
T(354)βAR (n = 5)	14 ± 5	42 ± 10[c]	308 ± 73	30 ± 6	23 ± 4	21 ± 14

[a] $p < 0.005$.
[b] $p < 0.025$.
[c] $p < 0.05$.

TABLE 2

Effect of 3 nM epinephrine treatment on agonist stimulation of adenylyl cyclase in membranes from L cells expressing wild-type and mutant βARs

Epinephrine (concentration at or near the EC_{80}), PGE_1, and forskolin stimulations of adenylyl cyclase were measured in membranes prepared from L cells grown in the presence of thiourea-ascorbate (control) or 3 nM epinephrine for 24 hr. The number of experiments (*n*) is indicated, and the values are the mean ± standard error. Basal activity was subtracted for the epinephrine and PGE_1 groups but not for forskolin, and the mean ± standard error values for the control and 3 nM epinephrine groups were 2.2 ± 0.55 and 1.09 ± 0.33 (wild-type), 2.19 ± 0.52 and 1.83 ± 0.49 [D(259–262)βAR], 3.7 ± 0.4 and 1.95 ± 0.46 [D(343–348)βAR], and 4.2 ± 0.6 and 2.27 ± 0.49 pmol of cAMP/mg/min [T(354)βAR], respectively. The percentage change is the mean increase or decrease in adenylyl cyclase activity in the 3 nM epinephrine group for the number of experiments indicated. A paired *t* test was used to compare the control and 3 nM epinephrine groups.

Cell line	Epinephrine			500 nM PGE_1			10 μM Forskolin		
	Adenylyl cyclase		Change	Adenylyl cyclase		Change	Adenylyl cyclase		Change
	Control	3 nM Epinephrine		Control	3 nM Epinephrine		Control	3 nM Epinephrine	
	pmol of cAMP/mg/min		%	*pmol of cAMP/mg/min*		%	*pmol of cAMP/mg/min*		%
Wild-type βAR (*n* = 6 or 7)	37 ± 9	11 ± 4[a]	−75 ± 3	16 ± 2	14 ± 1[b]	−11 ± 4	38 ± 7	29 ± 6	−25 ± 4
D(259–262)βAR (*n* = 6)	24 ± 6	12 ± 3[c]	−50 ± 7	33 ± 5	29 ± 6	−14 ± 10	38 ± 12	36 ± 14	−15 ± 6
D(343–348)βAR (*n* = 4 or 5)	26 ± 8	6 ± 2[d]	−79 ± 2	38 ± 14	35 ± 12	−7 ± 4	44 ± 8	30 ± 10[d]	−36 ± 12
T(354)βAR (*n* = 4 or 5)	14 ± 4	5 ± 2[c]	−65 ± 4	33 ± 7	36 ± 5	17 ± 20	57 ± 9	40 ± 13[d]	−33 ± 12

[a] $p < 0.005$.
[b] $p < 0.05$.
[c] $p < 0.01$.
[d] $p < 0.025$.

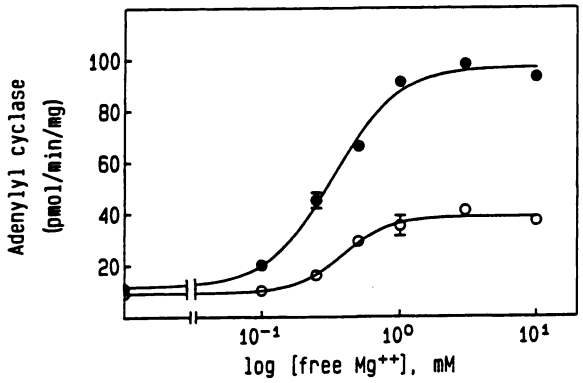

Fig. 2. Effect of free Mg^{2+} concentration on the desensitization measured in L cells grown in the presence of 3 nM epinephrine for 24 hr. Stimulation of adenylyl cyclase activity by 100 nM epinephrine was assayed at various concentrations of Mg^{2+} in membranes prepared from L cells (expressing the wild-type βAR) grown in the presence of vehicle (●) or 3 nM epinephrine (○) for 24 hr.

TABLE 3

Effect of 3 nM epinephrine treatment on [^{125}I]ICYP binding to membranes from L cells expressing wild-type and mutant βARs

[^{125}I]ICYP binding to membranes from L cells grown in the presence of thiourea-ascorbate (control) or 3 nM epinephrine for 24 hr was measured. The number of experiments (*n*) is indicated, and the values are the mean ± standard error. The percentage decrease is the mean decrease in binding for the number of experiments indicated. A paired *t* test was used to compare the control and 3 nM epinephrine groups. Comparing the control groups revealed that the B_{max} for the D(259–262)βAR mutant was significantly greater than the B_{max} for cells with the wild-type βAR ($p < 0.025$).

Cell line	B_{max}		
	Control	3 nM Epinephrine	Decrease
	fmol/mg		%
Wild-type βAR (*n* = 6)	260 ± 24	122 ± 16[a]	54 ± 4
D(259–262)βAR (*n* = 4)	557 ± 145	370 ± 129[b]	38 ± 6
D(343–348)βAR (*n* = 4)	333 ± 43	105 ± 31[a]	71 ± 6
T(354)βAR (*n* = 4)	241 ± 87	84 ± 19	57 ± 9

[a] $p < 0.0005$.
[b] $p < 0.005$.

of epinephrine. Therefore, in this study we addressed these problems through the use of several mutant βARs expressed in L cells. We demonstrated that there is not a requirement for either of the two domains known to be consensus sites for both cAPK and PKC, namely $RRSSK_{263}$ and $RRSSSK_{348}$ (1, 2), because the 3 nM/24-hr treatment caused a highly significant desensitization of epinephrine stimulation of adenylyl cyclase with our deletion mutants. This was measured by both the increase in the EC_{50} (Table 1) and the desensitization at the EC_{50} concentration (Table 2), as well as by the significant loss of βARs.

It should be noted that there were significant differences in the desensitizations of the wild-type βAR and the D(259–262)βAR; namely, there was no decrease in the V_{max} for epinephrine stimulation with the D(259–262)βAR, and the decrease in the B_{max} for this mutant was less than that for the wild-type βAR. The latter result may reflect the fact that on average there were twice as many βARs in the D(259–262)βAR-expressing cells, compared with the wild-type βAR-expressing cells. Thus, there are actually similar losses of receptors in the two cell lines. Theoretical considerations[2] also lead to the prediction that changes in V_{max} will be more pronounced with desensitization as the number of βARs/cell decreases. Alternatively, the slightly diminished desensitization of this mutant βAR may indicate some minor role of the 259–262 domain in the down-regulation and desensitization of adenylyl cyclase. This interpretation would be consistent with our previous demonstration that 3 nM/24-hr treatment of S49 *kin*$^-$ lymphoma cells, which lack cAPK activity, produced desensitization that was slightly (but not significantly) less than that which occurred in the S49 wild-type cell line (9).

By all comparisons the profile of desensitization and down-regulation for the T(354)βAR mutant, which lacks the carboxyl-terminal domain amino acids 354–418, was very similar to that for the wild-type βAR. This suggests that the βARK/β-arrestin pathway, which has been proposed to involve phosphorylation of the serine- and threonine-rich carboxyl-terminal

[2] B. Whaley, N. Yuan, R. B. Clark, and R. Barber. Relation of β_2AR number to activation of adenylyl cyclase. Manuscript in preparation.

domain by βARK and subsequent binding of β-arrestin, was not involved in the desensitization. Also, the repeated observations that much higher concentrations (20–100-fold greater) of epinephrine are required for the rapid homologous desensitization, at least a part of which is attributed to βARK (5, 12, 13), are also consistent with this conclusion.

Our studies also suggest that internalization is not involved in the 3 nM/24-hr desensitization because, as with the βARK pathway, high agonist concentrations appear to be required to trigger internalization of the βAR. Also, the lack of any effect of the T(354)βAR mutation on the 3 nM/24-hr desensitization may suggest that internalization of the βAR is not involved, because Lefkowitz and co-workers (16) recently demonstrated that βAR with the substitutions S356→G and S364→G is not internalized (internalization was measured by the loss of CGP12177 binding). If, as that study concluded, these residues are required for internalization, then the T(354)βAR, which lacks these serines, could not be internalized, suggesting that the 3 nM/24-hr desensitization does not require internalization. Unfortunately, previous studies by Lefkowitz's group (17) introduce some ambiguity. Those authors found that substitution of 11 of the serines and threonines in the carboxyl-terminal tail (from Ser-355 to Ser-411) with alanine or glycine (11 total substitutions including S355→A and S364→G) did not alter internalization of the βAR. Further confounding the issue, Hausdorff *et al.* (18) found that the substitutions S355→A, S356→G, T360→A, and S364→G eliminated all forms of rapid desensitization and internalization but allowed down-regulation in response to 10 μM isoproterenol to proceed normally. These results strongly suggest that alteration of the 355–364 domain produces delocalized varied effects. Furthermore, Cheung *et al.* (19) have shown that there is some internalization of the T(354)βAR, although interpretation of this result was hampered because internalization was not clearly distinguished from down-regulation. Additional studies will be required to conclusively resolve whether there is a role for internalization in the 3 nM epinephrine-induced desensitization, although it certainly appears that there is not.

To the extent that at least the cAMP-independent component of down-regulation in response to high epinephrine concentrations relates to the 3 nM epinephrine-induced down-regulation/desensitization, it is useful to consider several other studies. Campbell *et al.* (20) examined down-regulation in a series of deletion and substitution mutants and concluded that down-regulation was correlated with the ability of the mutant human receptors to couple to adenylyl cyclase. This conclusion was largely based on the properties of three mutant βARs, i.e., C341→G, in which the cysteine thought to be palmitoylated was converted to glycine, and S(263–274)βAR and S(216–237;263–274;327–339)βAR, in which the indicated domains of the βAR were substituted with corresponding residues of the α_{2a}-adrenergic receptor. These mutants displayed impaired coupling to G_s and decreased down-regulation (approximately 50, 50, and 75%, respectively). However, those investigators also found that a deletion mutant, D(267–273)βAR, with impaired coupling demonstrated normal down-regulation. This observation and the likelihood that extensive substitutions cause delocalized disruptions of the βAR suggest that the efficiency of βAR coupling to adenylyl cyclase was not consistently correlated with down-regulation.

The involvement of Tyr-350 and/or -354 in down-regulation of the human βAR was suggested by the studies of Valiquette *et al.* (21), in which substitution of both of these tyrosines by alanine appeared to cause a 1–2-hr delay in the initial rate of receptor loss and about a 30% decrease in the maximal extent of down-regulation in response to 1.0 μM isoproterenol. However, the effects were partial and complicated by the reduced coupling of the mutant receptor to adenylyl cyclase. In the present study, we found that the hamster T(354)βAR, which lacks the homologous Tyr-354, displayed normal down-regulation and desensitization in response to 3 nM epinephrine, demonstrating that at least Tyr-354 is not required.

To summarize our results, we have demonstrated that the 60–80% desensitization of βAR stimulation of adenylyl cyclase and the accompanying comparable decreases in βAR levels caused by prolonged treatment of L cells or S49 lymphoma cells with 3 nM epinephrine do not involve the consensus sites for cAPK, PKC, and βARK, nor do they appear to require sequestration/internalization. cAPK effects on transcription, translation, or mRNA stability also appear to be unlikely, because the down-regulation occurs in S49 *kin*$^-$ cells (9) and cAMP levels in L cells are only very transiently (if at all) elevated because of a powerful, positively cooperative, cAMP-dependent PDE (10). Rather, it appears that the mechanism involves another process (or processes) that leads to the down-regulation of the receptor. The lack of involvement of cAPK is perhaps the most enigmatic conclusion from our studies. A similar conclusion was reached by Hadcock *et al.* (22, 23), based on studies of the down-regulation of βAR mRNA in S49 *kin*$^-$ and H21a cells in response to 10 μM isoproterenol, which appeared to be equivalent to that in the parental S49 wild-type cells.

If cAPK is not involved, what is the second messenger or signal that induces the slow down-regulation? Several recent results suggest that the βAR may stimulate a cAMP-independent pathway. Barber *et al.* (24) reported that mutant βARs that are expressed in L cells [with the most effective being the deletion mutant D(258–270)βAR] and are uncoupled from adenylyl cyclase retain activation of the Na^+/H^+ exchanger. Khac *et al.* (25) have found that isoproterenol (with an EC_{50} of 0.5 nM) inhibits Ca^{2+} entry mediated by carbachol and oxytocin in the guinea pig myometrium. This effect of isoproterenol appears to be cAMP independent and is blocked by pertussis toxin. Maguire and Erdos (26) have shown that βAR agonists inhibit Mg^{2+} transport in a cAMP-independent manner in S49 wild-type and *kin*$^-$ lymphoma cells. Those studies, along with the present work, collectively suggest that the desensitization/down-regulation of the βAR caused by prolonged treatment with 3 nM epinephrine could involve another, cAMP-independent, pathway.

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