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# $\beta_2$-Adrenergic Receptor Regulation after Transfection into a Cell Line Deficient in the cAMP-Dependent Protein Kinase

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**SUMMARY**

A mouse $\beta_2$-adrenergic receptor ($\beta_2$AR) DNA clone was transfected and expressed in a mouse adrenocortical tumor cell line (Kin8) that lacks both $\beta_2$AR and cAMP-dependent protein kinase (PKA). The receptor displayed a characteristic $\beta_2$AR agonist binding profile that was similar to that observed in $\beta_2$AR-transfected PKA$^+$ mouse adrenocortical tumor cells (Y1). Isoproterenol treatment of $\beta_2$AR-transfected Kin8 and Y1 cells resulted in a rapid loss of surface $\beta_2$AR, as determined by the binding of the hydrophilic $\beta_2$AR radioligand [$^3$H]CGP 12177 ([$^3$H]CGP), followed by a decrease in adenylate cyclase activity. Sequestration of $\beta_2$AR in Kin8 cells was $\beta_2$AR agonist specific, temperature dependent, and rapidly reversible. Repeated treatment and recovery from isoproterenol incubation resulted in a cycling of surface [$^3$H]CGP binding. The reappearance of [$^3$H]CGP binding following short isoproterenol treatment was not affected by cycloheximide treatment of the cells. Prolonged incubation of $\beta_2$AR-transfected Kin8 cells with isoproterenol resulted in the down-regulation of $\beta_2$AR protein without a change in $\beta_2$AR mRNA levels. Polysome profiles of control and down-regulated cells revealed that translation of $\beta_2$AR mRNA is inefficient and does not change upon prolonged agonist treatment. Protein synthesis was required to reverse the down-regulation of $\beta_2$AR. These results indicate that neither sequestration nor down-regulation of $\beta_2$AR depends on PKA.

The $\beta_2$AR is a member of a family of proteins that transduce hormonal and neurotransmitter signals through the cellular plasma membrane (1). The binding of agonists to $\beta_2$AR results in the activation of adenylate cyclase and a subsequent increase in intracellular cAMP levels. This activity is short-lived, however, resulting in a less responsive or "desensitized" receptor. $\beta_2$AR undergoes two types of desensitization, both resulting in a decrease of agonist-responsive adenylate cyclase activity. Heterologous desensitization refers to the attenuation of $\beta_2$AR, as well as other receptors coupled to adenylate cyclase via the stimulatory guanine regulatory protein, upon binding of the appropriate agonist to any one of these receptors. Homologous desensitization acts only to attenuate $\beta_2$AR after binding of its sepcific agonist (2). Homologous desensitization of $\beta_2$AR is rapidly reversible and has been associated with the sequestration of the receptor from the cell surface (3).

Phosphorylation of $\beta_2$AR has been implicated in both types of desensitization. Heterologous desensitization of adenylate cyclase in S49 lymphoma cells is dependent upon the activation of PKA (4). Benovic *et al.* (5) suggest that homologous desensitization requires a $\beta$ARK that is independent of cAMP.

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Prolonged incubation of cells that contain $\beta_2$AR with adrenergic agonists can result in the loss of both surface and internal $\beta_2$AR protein, in a process defined as down-regulation (6). Usually, protein synthesis is required to reverse $\beta_2$AR down-regulation, which supports the hypothesis that preexisting $\beta_2$AR was degraded rather than sequestered into an intracellular compartment (7).

The isolation of DNA clones for several adrenergic receptor subtypes, followed by their introduction into cells that do not normally express them, is providing insight into the mechanisms by which these proteins transduce signals presented to a cell. Recent studies have identified specific amino acid domains involved in ligand binding (8, 9), guanine nucleotide-binding protein coupling (10, 11), and receptor regulation (12, 13). In addition, specific radiolabeled agonists and antagonists for $\beta_2$AR, each with varying degrees of membrane permeability, have been used to characterize the events immediately following the binding of agonists to the receptor (14).

We have introduced the mouse $\beta_2$AR gene into a PKA-deficient cell line (Kin8) derived from the mouse adrenocortical tumor cell line Y1, a cell line that normally does not express $\beta_2$AR. We describe the sequestration and down-regulation of $\beta_2$AR protein and mRNA in this PKA$^-$ cell line after treatment

**ABBREVIATIONS:** $\beta_2$AR, $\beta_2$-adrenergic receptor; [$^3$H]CGP, [$^3$H]CGP 12177; [$^3$H]DHA, [$^3$H]dihydroalprenolol; PKA, cAMP-dependent protein kinase; BSA, bovine serum albumin; PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; cpt-cAMP, 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate; ACTH, adrenocorticotropin; $\beta$ARK, $\beta_2$-adrenergic receptor-specific kinase.

with a $\beta_2$AR agonist. Furthermore, we report the translational efficiency of $\beta_2$AR mRNA isolated from control or down-regulated cells. The results show that the mouse $\beta_2$AR protein is all that is required to confer catecholamine-inducible adenylate cyclase activity in Y1 and Kin8 adrenal cortex tumor cells. In addition, we demonstrate that PKA is not required for sequestration, homologous desensitization, or down-regulation of $\beta_2$AR but does appear to be required for heterologous desensitization.

## Materials and Methods

**Cell culture and transfections.** Kin8 and Y1 cells (15) were grown in F10 medium that was supplemented with 15% heat-inactivated horse serum, 2.5% heat-inactivated fetal calf serum, and antibiotics. They were transfected with the mouse genomic $\beta_2$AR clone as described elsewhere (16). Monoclonal cell lines were isolated using cloning cylinders, followed by serial dilution. All of the experiments presented in this manuscript were performed on representative, monoclonal, $\beta_2$AR-transfected Y1 (PKA$^+$$\beta$AR) or Kin8 (PKA$^-$$\beta$AR) lines.

**Whole cell radioligand saturation binding assays.** Cells were grown until nearly confluent in 150-mm plates, treated with the indicated drugs for various times, washed three times with ice-cold PBS (0.14 M NaCl, 5 mM KCl, 8 mM $Na_2PO_4$, and 1.5 mM $KH_2PO_4$, pH 7.2), scraped, and suspended in serum-free F10 medium at ~3 × $10^6$ cells/ml. Surface $\beta_2$AR number was determined by incubating 200-$\mu$l aliquots of the suspended cells in F10 medium, 10% horse serum, and 0.1% BSA with increasing amounts of [$^3$H]CGP (Amersham), ranging from 0.05 to 10 nM, for 18 hr at 0°, in 0.5 ml (6). Total $\beta_2$AR number was determined by incubating duplicate cell aliquots with [$^3$H]DHA at 37° for 60 min (14). Binding was terminated by dilution with 10 ml of ice-cold PBS and rapid filtration through Whatman GF/C glass fiber filters. Nonspecific binding was determined by including 1 $\mu$M (−)-propranolol in duplicate tubes. The filters were then treated with 0.5 ml of Protosol (New England Nuclear, Boston, MA) and counted in a toluene/Omnifluor (New England Nuclear) scintillation cocktail. An aliquot was used to determine the protein concentration with the Bio-Rad protein assay kit, using BSA as a standard (Bio-Rad, Richmond, CA).

**Whole cell relative radioligand binding assays.** Cells were seeded into 24-well plates, grown until nearly confluent, and treated with various drugs at 37° for the times indicated in the figures. Drug treatment was terminated by placing the 24-well plate onto an ice-water slurry and adding ~4 volumes of ice-cold PBS, followed by three washings with PBS at 4°. Binding assays were performed essentially as described (17). Nonspecific binding, determined with 1 $\mu$M (−)-propranolol, was <10% of total bound ligand.

**Membrane preparation and adenylate cyclase assay.** Cells were grown until nearly confluent in 100-mm plates, incubated with drugs for indicated times, placed on an ice-water slurry, and washed three times with ice-cold PBS. Cells were then scraped into 5 ml of ice-cold PBS, pelleted at 200 × $g$ for 10 min at 4°, resuspended in 4 ml of 5 mM Tris·HCl, pH 7.4, 2 mM EDTA, and homogenized with a Tissumizer (Tekmar Co., Cincinnati, OH). The resultant membranes were centrifuged at 200 × $g$ for 5 min at 4° and the supernatant was transferred to a new tube and centrifuged at 48,000 × $g$ for 20 min at 4°. The pellet was resuspended in 4 ml of HAB (0.08 mM ascorbic acid, 18 mM $MgCl_2$, 50 mM HEPES, pH 7.4) and centrifuged at 48,000 × $g$ for an additional 20 min at 4°. The final pellet was brought up in 0.5 ml of HAB, homogenized with a Dounce homogenizer (Kontes Glass Co., Vineland, NJ), and used in the adenylate cyclase assay immediately. Adenylate cyclase activity was determined as described (18).

**$\beta_2$AR solution hybridization probe.** Ten picomoles of the 17-nucleotide oligomer (5′ATATTGACAATGAAGAA 3′), derived from position 1081–1098 of the mouse $\beta_2$AR sequence (16), were hybridized to 3 pmol of single-stranded M13 phage DNA that contained the mouse $\beta_2$AR mDNA sequence, in 500 mM NaCl, 50 mM $MgCl_2$, 50 mM Tris·HCl, pH 7.5. Then, 0.3 pmol of the M13-oligomer hybrid was extended in a buffer that contained 50 mM NaCl, 5 mM $MgCl_2$, 5 mM Tris·HCl (pH 7.5), 50–100 $\mu$Ci of $^{32}$P-labeled dCTP (3000 Ci/mmol), 0.3 mM dGTP, 0.3 mM dATP, 0.3 mM dideoxy-TTP, 10 mM dithiothreitol, 10 $\mu$g/ml BSA, and 1 unit of the Klenow fragment of DNA polymerase I. The reaction was incubated for 20 min and boiled, and the products were separated on a 10% polyacrylamide/8 M urea denaturing gel. The probe was visualized in the gel by autoradiography, excised, and eluted in 200 $\mu$l of 0.2 × SET butter (1× SET = 1% sodium dodecyl sulfate, 5 mM EDTA, 10 mM Tris·HCl, pH 7.5) (19). Under these conditions, 4 mol of $^{32}$P label would be incorporated per mol of probe, before the termination of the reaction by the incorporation of dideoxy-TTP opposite the adenosine residue at position 1063 of the $\beta_2$AR sequence. Up to 9 mol of $^{32}$P label may be incorporated if [$^{32}$P]dGTP is used instead of [$^{32}$P]dCTP.

**RNA isolation and analysis.** Total RNA was isolated essentially as described (20). Briefly, cells were washed twice with PBS at 4°, treated with proteinase K (100 $\mu$g/ml) in 1× SET buffer at 37° for 1 hr, extracted with phenol/chloroform, and precipitated with ethanol. Total nucleic acids were pelleted, resuspended in 2 mM $CaCl_2$, 10 mM $MgCl_2$, 50 mM Tris·HCl (pH 7.5), and digested with 14 units of DNAse I (Cooper Biomedical) for 30 min at 25°. The reaction was adjusted to 1× SET, 100 mM NaCl, and precipitated with ethanol. Solution hybridization was performed as described, using single-stranded M13 that contained the $\beta_2$AR mDNA sequence as a standard (20). The amount of $\beta_2$AR mRNA/$\mu$g of total RNA was determined taking the difference between the M13 standard and the $\beta_2$AR mRNA length into account.

**Preparation of cytoplasmic extracts.** PKA$^-$$\beta$AR cells were treated with 1 $\mu$M isoproterenol for 24 hr, to down-regulate $\beta_2$AR, or were untreated. The cells were then incubated with cycloheximide (100 $\mu$g/ml) in growth medium, at 37° for 5 min to freeze the polysomes. The medium was removed and the monolayers were washed with 1× PBS that contained 100 $\mu$g/ml cycloheximide, at 37°. The cells were detached from the plate in 2 ml of 1× PBS that contained 0.5 g/ml trypsin, 100 $\mu$g/ml cycloheximide, and 0.5 mM EDTA, for 3 min at 37°. Next, 5 ml of 1× PBS that contained 1 mM phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO) and 100 $\mu$g/ml cycloheximide were added, and the cells were transferred to a 50-ml polypropylene tube at 4°. The plates were rinsed in the same buffer and pooled. The cells were then pelleted at 1000 × $g$ for 10 min at 4°, resuspended in 10 ml 1× PBS that contained 100 $\mu$g/ml cycloheximide at 4°, and repelleted at 1000 × $g$ for 5 min at 4°. The resultant cell pellet was then suspended in 0.75 ml of 1× LSB (10× LSB = 200 mM Tris·HCl, pH 7.4, 100 mM NaCl, 30 mM $MgCl_2$) and swelled on ice for 3 min. The cells were then lysed by addition of 0.25 ml of detergent buffer (1.2% Triton N-101, 0.2 M sucrose, 1× LSB), were immediately transferred to a 7-ml Dounce homogenizer on ice, and were homogenized with eight strokes. The lysate was centrifuged in a 15-ml Corex (Corning) tube at 10,000 rpm, at 4°, for 30 sec, to remove the nuclei and 0.1 ml of 1× LSB containing 10 mg/ml heparin and 1.5 M NaCl was added to the supernatant. The cytoplasmic extract was then layered onto a 15–35% linear sucrose gradient containing a 1-ml 2 M sucrose pad (in 1× LSB) and was centrifuged in a Beckman SW40.1 rotor for 110 min, at 4°, essentially as described by White *et al.* (21).

**RNA purification from sucrose gradient fractions, electrophoresis, and hybridization.** RNA from the sucrose gradient was prepared and Northern analysis was performed using a $\alpha$-$^{32}$P-labeled bovine actin probe, as described by White *et al.* (21). $\beta_2$AR mRNA levels were determined by solution hybridization, as already described.

## Results

**Expression of $\beta_2$AR in PKA$^-$ cells.** The mouse $\beta_2$AR gene was cotransfected with the bacterial neomycin resistance gene (16) into a PKA$^-$ cell line, Kin8 (15). Monoclonal cell lines were isolated using cloning cylinders, followed by serial dilution. All of the experiments presented in this manuscript were

performed on representative, monoclonal, $\beta_2$AR-transfected Y1 (PKA$^+$$\beta$AR) or Kin8 (PKA$^-$$\beta$AR) lines. Membranes were prepared and assayed for radioligand binding to demonstrate the expression of functional $\beta_2$AR protein in several monoclonal cell lines that express the neomycin resistance gene. There was no significant difference in the affinity (≈26 pM) for [$^{125}$I] iodocyanopindolol between the clonal PKA$^-$$\beta$AR cell lines or PKA$^+$$\beta$AR cell lines (data not shown).

**Titration of isoproterenol-induced sequestration of $\beta_2$AR.** PKA$^-$$\beta$AR cells were treated with increasing amounts of isoproterenol, from 1 nM to 100 $\mu$M, at 37° for 15 min. Fig. 1 shows the specific [$^3$H]CGP bound at each concentration, relative to that bound to untreated cells. Nearly maximal sequestration was observed in cells that had been treated with 1 $\mu$M isoproterenol under the conditions described. No loss of [$^3$H] CGP binding was observed at any isoproterenol concentrations tested (up to 100 $\mu$M) if the isoproterenol incubation was carried out at 4° (data not shown).

PKA$^-$$\beta$AR cells were treated with 1 $\mu$M isoproterenol for 15 min or 24 hr or were untreated, were suspended, and were incubated with increasing amounts of [$^3$H]DHA or [$^3$H]CGP at 37° or 0°, respectively. Fig. 2 depicts a representative Scatchard analysis of radioligand equilibrium binding. Single plates were treated with isoproterenol, cells were harvested, and duplicate samples were incubated with each of the two radioligands. Control PKA$^-$$\beta$AR cells possessed 910 fmol/mg of protein $\beta_2$AR, using the hydrophobic radioligand [$^3$H]DHA (Fig. 2A), and 774 fmol/mg (85%), using the hydrophilic radioligand [$^3$H] CGP (Fig. 2B). PKA$^-$$\beta$AR cells treated with 1 $\mu$M isoproterenol for 15 min possessed 1026 fmol/mg, using [$^3$H]DHA as the radioligand (Fig. 2A); however, the same cells displayed only 382 fmol/mg using [$^3$H]CGP, a decrease of 63% (Fig. 2B). Prolonged incubation (24 hr) of PKA$^-$$\beta$AR cells with 1 $\mu$M isoproterenol resulted in a decrease in [$^3$H]DHA binding to 478 fmol/mg of approximately 50% that of control cells (Fig. 2A).

Similar experiments with the PKA$^+$$\beta$AR cell line revealed 349 fmol/mg in control cells and 364 fmol/mg in isoproterenol-treated cells (15 min), using [$^3$H]DHA (Fig. 3A). Interestingly,

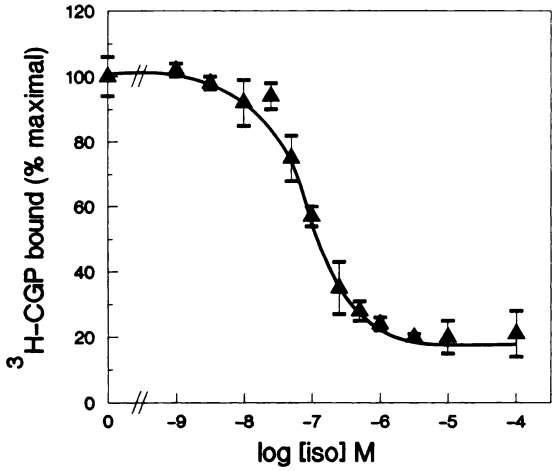


**Fig. 1.** Titration of isoproterenol-induced loss of [$^3$H]CGP binding to PKA$^-$$\beta$AR cells. Cells were treated for 15 min with increasing amounts of isoproterenol at 37°, washed, and incubated with 20 nM [$^3$H]CGP for 4 hr at 4°. Nonspecific binding was monitered by including 1 $\mu$M propranolol and was always less than 10% of total counts bound. Values reported are the specific counts bound at each isoproterenol concentration, relative to that bound to untreated cells, times 100. Each *point* is the average of three determinations, plus or minus standard deviation.

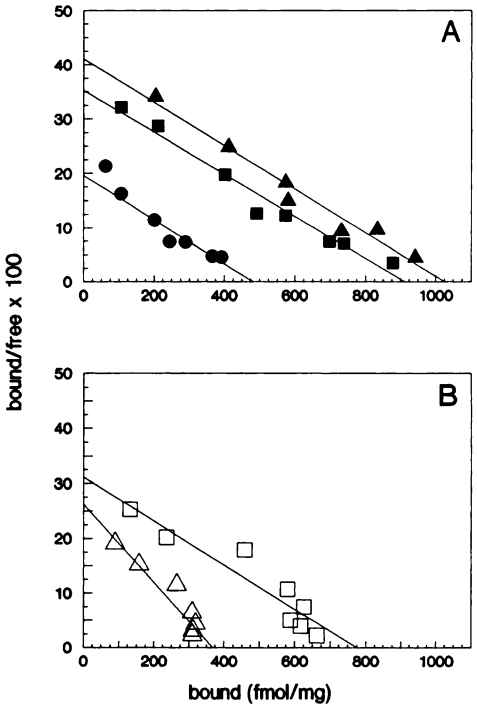


**Fig. 2.** Scatchard analysis of $\beta_2$AR on intact PKA$^-$$\beta$AR cells after treatment with 1 $\mu$M isoproterenol for 15 min or 24 hr. A, [$^3$H]DHA saturation binding isotherms (shown as Scatchard analysis) for PKA$^-$$\beta$AR cells treated with 1 $\mu$M isoproterenol for 15 min (▲) or 24 hr (●) or untreated (■). [$^3$H]DHA incubations were carried out at 37° for 1 hr, as described in Materials and Methods. Similar data were obtained in duplicate experiments. Values for the equilibrium dissociation constant ($K_d$) and maximal binding ($B_{max}$) in control cells were 7.5 × 10$^{-10}$ M and 910 fmol/mg of protein; in cells treated with 1 $\mu$M isoproterenol for 15 min and 24 hr, 8.5 × 10$^{-10}$ M and 1026 fmol/mg and 8.9 × 10$^{-10}$ M and 478 fmol/mg, respectively. B, [$^3$H]CGP saturation binding isotherms for duplicate samples of the same cell preparation described above. Cells were treated with 1 $\mu$M isoproterenol for 15 min (△) or were untreated (□). [$^3$H]CGP incubations were carried out at 0° for 18 hr, as described in Materials and Methods. $K_d$ and $B_{max}$ values for control and isoproterenol-treated cells were 7.2 × 10$^{-10}$ M and 774 fmol/mg and 5.1 × 10$^{-10}$ M and 382 fmol/mg, respectively.

the same cell preparations, when incubated with [$^3$H]CGP, displayed only 33 fmol/mg in untreated cells and 16 fmol/mg in the isoproterenol-treated cells (Fig. 3B).

**Rate of sequestration in response to various drugs.** PKA$^-$$\beta$AR cells were treated with 1 $\mu$M isoproterenol, 1$\mu$M ACTH, or 150 $\mu$M cpt-cAMP for up to 15 min, in replicate assays of 24-well plates. Treatment of PKA$^-$$\beta$AR cells with isoproterenol for as little as 3 min resulted in a ≈70% decrease in specific binding, as measured by [$^3$H]CGP, whereas treatment with ACTH or cpt-cAMP had little or no effect (Fig. 4). By 15 min of exposure to isoproterenol, the relative surface $\beta_2$AR number had decreased to ≈20% that of untreated cells.

**Recovery of surface binding in isoproterenol-treated PKA$^-$$\beta$AR cells.** Treatment of PKA$^-$$\beta$AR cells with isoproterenol followed by removal of the drug resulted in the rapid reappearance of [$^3$H]CGP binding (Fig. 5). Replicate 24-well plates were treated with isoproterenol for 15 min at 37°, washed three times, and incubated with drug-free medium at 37° for the times indicated. Specific binding rapidly recovered to ≈80% of the initial binding within 15 min of removal of the isoproterenol and to nearly 100% by 60 min. The ability of these receptors to undergo a second round of isoproterenol-induced

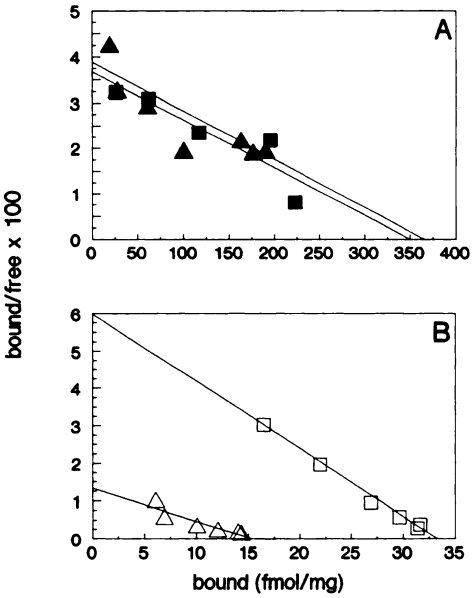


**Fig. 3.** Scatchard analysis of $\beta_2$AR on intact PKA$^+$$\beta$AR cells after treatment with 1 $\mu$M isoproterenol for 15 min. A, [$^3$H]DHA saturation binding isotherms for PKA$^+$$\beta$AR cells treated with 1 $\mu$M isoproterenol for 15 min (▲) or untreated (■). [$^3$H]DHA incubations were carried out at 37° for 1 hr, as described in Materials and Methods. $K_d$ and $B_{max}$ values for control and isoproterenol-treated cells were $4.7 \times 10^{-9}$ M and 349 fmol/mg and $4.2 \times 10^{-9}$ M and 364 fmol/mg, respectively. B, [$^3$H]CGP saturation binding isotherms for duplicate samples of the same cell preparations described above. Cells were treated with 1 $\mu$M isoproterenol for 15 min (△) or were untreated (□). [$^3$H]CGP incubations were carried out at 0° for 18 hr, as described in Materials and Methods. $K_d$ and $B_{max}$ values for control and isoproterenol-treated cells were $2.9 \times 10^{-10}$ M and 33 fmol/mg and $8.1 \times 10^{-10}$ M and 16 fmol/mg, respectively.

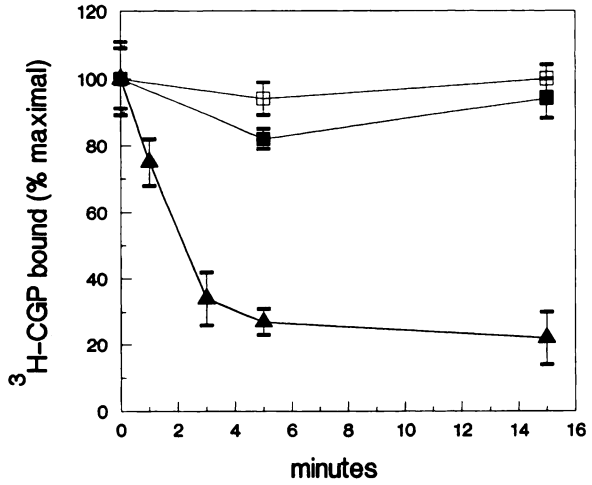


**Fig. 4.** [$^3$H]CGP binding after treatment of PKA$^-$$\beta$AR cells with various drugs. Cells were treated with 1 $\mu$M isoproterenol (▲), 1 $\mu$M ACTH (■), and 150 $\mu$M cpt-cAMP (□) for the indicated times at 37°, washed, and assayed for [$^3$H]CGP binding. Each point is the average of four to eight determinations, plus or minus standard deviation.

sequestration of $\beta_2$AR was demonstrated in cells that had recovered for as little as 5 min (Fig. 5).

**Role of translation in the reappearance of [$^3$H]CGP binding in recovering PKA$^-$$\beta$AR cells.** PKA$^-$$\beta$AR cells treated with either 1 $\mu$M isoproterenol or 1 $\mu$M isoproterenol and 100 $\mu$g/ml cycloheximide for 15 min at 37° specifically bound ≈20% of [$^3$H]CGP, relative to untreated cells. Within 1 hr after isoproterenol was removed from the cells, the amount

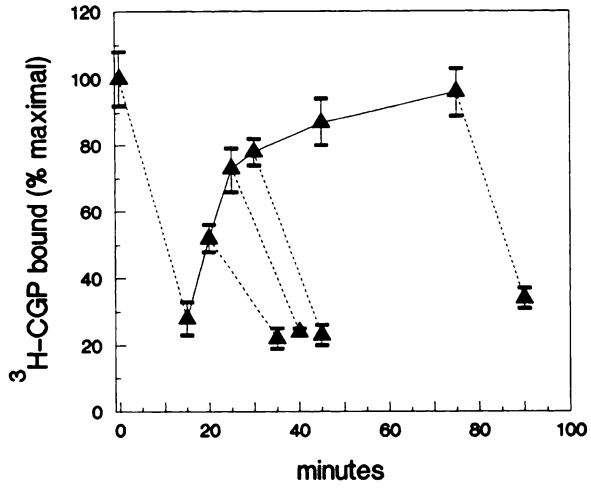


**Fig. 5.** Recycling of [$^3$H]CGP binding after isoproterenol treatment of PKA$^-$$\beta$AR cells. Cells were treated with 1 $\mu$M isoproterenol for the times indicated (*dashed lines*), washed with drug-free medium at 37°, incubated in the same medium (*solid lines*), and assayed for [$^3$H]CGP binding. Each *point* is the average of three to seven determinations, plus or minus standard deviations.

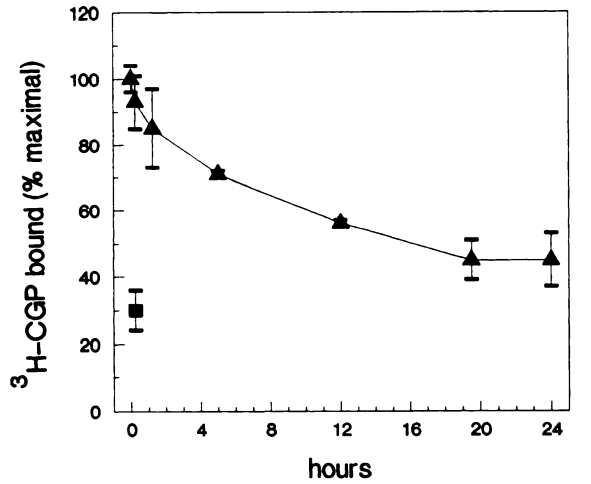


**Fig. 6.** [$^3$H]CGP binding after prolonged incubation of PKA$^-$$\beta$AR cells with isoproterenol. Cells were treated with 1 $\mu$M isoproterenol for the times indicated, washed with drug-free medium at 37°, incubated in the same medium for an additional 15 min (▲), and assayed for [$^3$H]CGP binding. ■, Cells treated with 1 $\mu$M isoproterenol for 15 min without recovery. Each *point* represents four determinations, plus or minus standard deviations.

of [$^3$H]CGP binding in cycloheximide-treated and control cells had returned to greater than 90% that of untreated cells. The level of protein synthesis was monitored by [$^3$H]leucine incorporation and was shown to be less than 1% in cells treated with cycloheximide, compared with untreated cells. Longer incubations of the cells with cycloheximide resulted in a gradual decrease in the amount of specific binding, to ≈40% after 24 hr, which probably reflects the turnover of $\beta_2$AR in these cells in the absence of protein synthesis (data not shown).

**Down-regulation of $\beta_2$AR in PKA$^-$$\beta$AR cells.** Prolonged incubation of PKA$^-$$\beta$AR cells with 1 $\mu$M isoproterenol resulted in a gradual loss of receptors (Fig. 6). PKA$^-$$\beta$AR cells were treated with isoproterenol for up to 24 hr, washed three times with warm culture medium, and allowed to recover for 15 min. Short incubation periods with isoproterenol followed by recovery resulted in the restoration of greater than 80% of the initial

specific binding. As the duration of the isoproterenol treatment was increased, however, the recoverable levels of [$^3$H]CGP binding diminished to ≈50% after 24 hr. A solution hybridization assay, using an oligonucleotide probe derived from the mouse $\beta_2$AR sequence (see Materials and Methods), revealed no significant change in $\beta_2$AR mRNA levels after prolonged incubation of PKA$^-$$\beta$AR cells with isoproterenol. Untreated cells contained 0.36 ± 0.05 pg of $\beta_2$AR mRNA/$\mu$g of total RNA, whereas PKA$^-$$\beta$AR cells exposed to 1 $\mu$M isoproterenol for 24 hr retained 0.39 ± 0.06 pg/$\mu$g of total RNA (six determinations).

**Polysome profiles of $\beta_2$AR mRNA from control and down-regulated PKA$^-$$\beta$AR cells.** To examine the question of whether the loss of $\beta_2$AR protein was due to the attenuation of $\beta_2$AR mRNA translation, we isolated polysomes from control and down-regulated PKA$^-$$\beta$AR cells. Quantitation of $\beta_2$AR mRNA in fractions collected from sucrose gradients revealed no change in the number of ribosomes actively translating the transcript after incubation of the cells with isoproterenol for 24 hr (Fig. 7A), indicating that the translational efficiency of $\beta_2$AR mRNA does not mediate the observed decrease in receptor number after down-regulation. However, the average number of ribosomes translating the $\beta_2$AR mRNA was lower than expected, considering its size. To rule out the possibility of mRNA degradation or ribosome run-off, we examined the polysome profile of actin mRNA in these gradients as well (Fig. 7, B and C). The difference in the absolute amount of $\beta_2$AR or actin mRNA between control and down-regulated cells is due to differences in the amount of material loaded onto each sucrose gradient.

**Recovery of $\beta_2$AR after down-regulation in PKA$^-$$\beta$AR cells.** The recovery of [$^3$H]CGP binding in PKA$^-$$\beta$AR cells after down-regulation was monitored with or without cycloheximide. PKA$^-$$\beta$AR cells were incubated with 1 $\mu$M isoproterenol for 24 hr, washed three times with or without 5 $\mu$g/ml cycloheximide, and incubated for up to 20 hr at 37°. The cells were then washed three times with PBS at 4° and were incubated with [$^3$H]CGP as outlined previously. Fig. 8A shows that the recovery of [$^3$H]CGP binding occurred only in the absence of cycloheximide and was complete after 20 hr. To determine whether the recovery of $\beta_2$AR was dependent on cell proliferation, PKA$^-$$\beta$AR cells were serum arrested by incubation in 1% horse serum for 48 hr before the isoproterenol treatment (Fig. 8B).

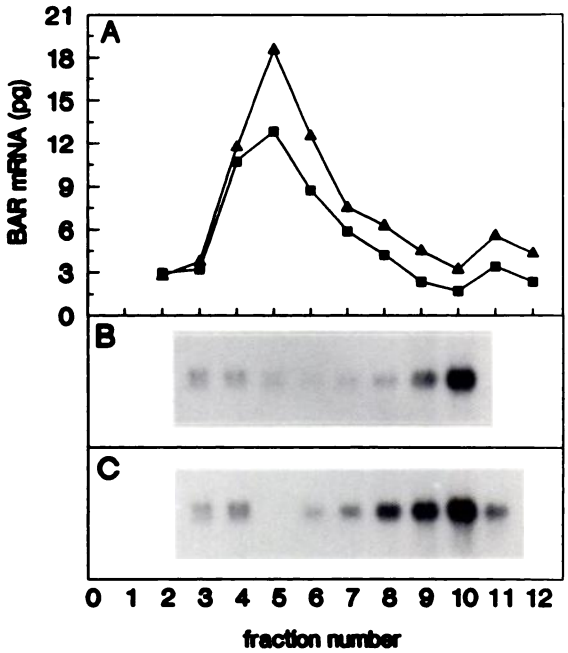


**Fig. 7.** Distribution of $\beta_2$AR and actin mRNA across polysome fractions from control and down-regulated PKA$^-$$\beta$AR cells. Polysomes in sucrose gradients were fractionated and solution hybridization (A) or Northern analysis (B and C) was performed as described in Materials and Methods. A, $\beta_2$AR solution hybridization of RNA isolated from control (■) and down-regulated (▲) cells. B and C, Autoradiographs from Northern analysis of RNA from control (B) or down-regulated (C) cells, hybridized with an $^{32}$P-labeled actin probe. Sample 5 in C was lost during mRNA isolation.

**Adenylate cyclase activity of PKA$^-$$\beta$AR and PKA$^+$$\beta$AR cell membranes.** Membranes were prepared from PKA$^-$$\beta$AR and PKA$^+$$\beta$AR cells that were treated with various drugs for 2 hr and they were assayed immediately for adenylate cyclase activity with or without isoproterenol. The observed, isoproterenol-stimulated, adenylate cyclase activity after drug treatment is depicted in Fig. 9, relative to that of control cells. Preincubation of both PKA$^-$$\beta$AR and PKA$^+$$\beta$AR cells with isoproterenol resulted in a decrease of adenylate cyclase activity associated with purified membranes. ACTH treatment of PKA$^+$$\beta$AR cells, but not PKA$^-$$\beta$AR cells, also attenuated adenylate cyclase activity.

## Discussion

Previously, we demonstrated that Y1 cells transfected with the mouse $\beta_2$AR gene expressed $\beta_2$AR mRNA and protein (16); isoproterenol treatment of these cells resulted in an increase in steroid synthesis and secretion as well as a rapid change in cell morphology. Preliminary observations reported in that study suggested that the transfected receptor was subject to cellular controls governing its ability to activate adenylate cyclase.

The identical mouse $\beta_2$AR clone was transfected into Kin8 cells, a cell line derived from Y1 cells, which are deficient in PKA activity (PKA$^-$) (22). Kin8 cells, like Y1 cells, respond to ACTH treatment by increasing adenylate cyclase activity, leading to an increase in cAMP accumulation (23). Unlike Y1 cells, however, there is no change in steroid synthesis or secretion and the cells do not undergo any change in morphology, presumably due to the lack of PKA activity (15). Neomycin-resistant Kin8 lines transfected with the mouse $\beta_2$AR genomic clone (PKA$^-$$\beta$AR) expressed an mRNA that was indistinguishable from $\beta_2$AR mRNA that was isolated from $\beta_2$AR-transfected Y1 cells (PKA$^+$$\beta$AR) or day 17 fetal mouse liver (data not shown).

Our results regarding the sequestration of $\beta_2$AR [as determined by the binding of [$^3$H]CGP (24–26)] in both PKA$^+$$\beta$AR and PKA$^-$$\beta$AR cells are similar to published reports that describe $\beta$AR sequestration in wild-type and PKA$^-$ S49 cells (6). Both studies indicate that the rapid decrease in [$^3$H]CGP binding upon incubation of intact cells with isoproterenol is not dependent on PKA activity. The requirement for agonist binding to the receptor and the rate of receptor sequestration and recovery, as well as the ability of "recovered" receptors to bind isoproterenol, suggest that PKA$^-$$\beta$AR cells possess the ability to regulate catecholamine-dependent activation of adenylate cyclase. De Blasi *et al.* (27) showed that incubation of human mononuclear leukocytes with isoproterenol also resulted in a redistribution of $\beta$AR and was accompanied by desensitization of the receptor. Using the hydrophobic agonist zinterol,

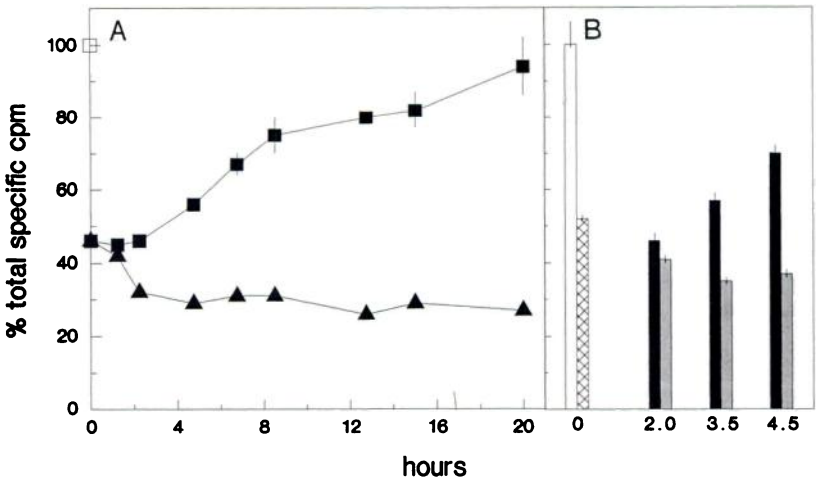


**Fig. 8.** Recovery of [$^3$H]CGP binding in down-regulated PKA$^-$$\beta$AR cells with or without cycloheximide. A, PKA$^-$$\beta$AR cells were treated with 1 $\mu$M isoproterenol for 24 hr (■, ▲) or were untreated (□), at 37°. The cells were washed three times with (▲) or without (■) 5 $\mu$g/ml cycloheximide and were incubated for up to 20 hr at 37°. At the times indicated, the cells were washed three times with PBS at 4° and incubated in 20 nM [$^3$H]CGP, as described in Materials and Methods. The results are presented as the percentage of total [$^3$H]CGP cpm present in untreated cells (□). B, Same as above except that PKA$^-$$\beta$AR cells were switched to medium containing only 1% horse serum 48 hr before the isoproterenol treatment. □, Total [$^3$H]CGP binding; ⊠, binding after isoproterenol treatment; ■, recovery of [$^3$H]CGP binding in the presence of cycloheximide; ■, recovery in the absence of cycloheximide. The values reported represent the average of three determinations, plus or minus standard deviations.

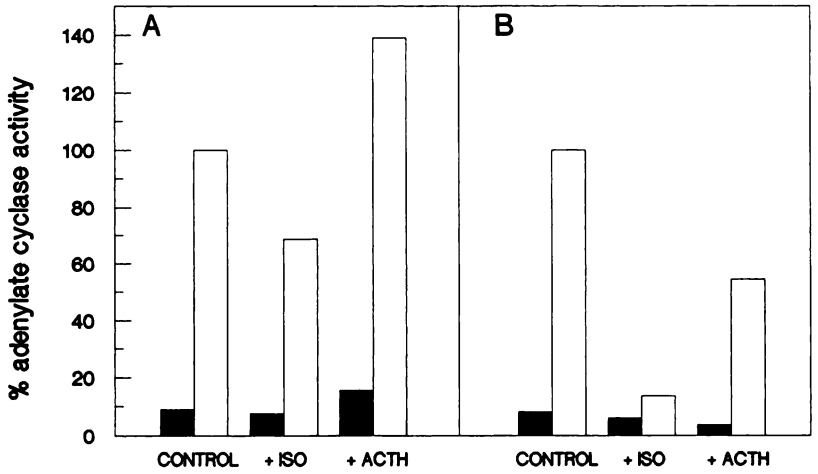


**Fig. 9.** Adenylate cyclase activity of PKA$^-$$\beta$AR and PKA$^+$$\beta$AR membrane preparations after preincubation with various drugs. Cells were treated with 1 $\mu$M isoproterenol or 1 $\mu$M ACTH, or were untreated, for 2 hr at 37°. Membranes were prepared and immediately assayed for adenylate cyclase activity, as described (18). ■, Adenylate cyclase activity induced in membranes with GTP; □, adenylate cyclase activity with GTP and isoproterenol (*ISO*) stimulation. Values are reported as the percentage of activity/mg of protein of that present in isoproterenol-induced control membranes. Similar results were obtained in duplicate experiments. A, PKA$^-$$\beta$AR cell membranes; B, PKA$^+$$\beta$AR cell membranes.

they demonstrated that the sequestered receptors were not functionally coupled to adenylate cyclase (27).

To determine whether the decrease in [$^3$H]CGP binding that we observed was correlated with the desensitization of the receptor, we assayed adenylate cyclase activity after pretreatment of transfected cells with agonist. The maximal isoproterenol-induced adenylate cyclase activity that was associated with isolated PKA$^-$$\beta$AR and PKA$^+$$\beta$AR membranes decreased with preincubation of the cells with isoproterenol. Preincubation with ACTH had no effect on PKA$^-$$\beta$AR isoproterenol-induced adenylate cyclase activity, indicating the requirement for PKA in heterologous desensitization, but did appear to modify the ability of isoproterenol to stimulate adenylate cyclase in PKA$^+$$\beta$AR membranes. The magnitude of desensitization observed in the PKA$^-$$\beta$AR cells is somewhat smaller than expected but time course experiments indicate that the decrease was maximal by 30 min, consistent with the kinetics of homologous desensitization in other cell lines that normally express $\beta_2$AR (data not shown) (12). This observation may be due to the number of receptors expressed on the surface of the PKA$^-$$\beta$AR cell line. The observation that the ratio of surface to intracellular receptors in the PKA$^+$$\beta$AR cell line (in addition to two other independent, monoclonal, $\beta_2$AR-transfected Y1 lines) is a fraction of that observed in PKA$^-$$\beta$AR cells supports this hypothesis. We are in the process of determining the role, if any, that PKA plays in regulating the cellular distribution of $\beta_2$AR protein in these two cell lines. The sensitivity of PKA$^+$ Y1 cells to intracellular cAMP, however, may have resulted in the selection for this unusual receptor distribution.

Prolonged incubation of PKA$^-$$\beta$AR cells with isoproterenol resulted in the down-regulation of $\beta_2$AR protein. Scatchard analysis, using [$^3$H]DHA on whole cells treated with isoproterenol for 24 hr, revealed that the number of receptors present in the cell was 50% that of untreated cells. The amount of [$^3$H]CGP binding present after 24 hr of isoproterenol treatment and 15 min of recovery, the time required to reverse receptor sequestration, was less than 50% that of untreated cells (Fig. 6). $\beta$AR down-regulation in S49 wild-type and PKA$^-$ cells occured with a $t_{1/2}$ of ≈4 hr (6), similar to our results in PKA$^-$$\beta$AR cells.

Down-regulation of $\beta_2$AR protein in DDT$_1$ MF-2 hamster vas deferens cells was accompanied by a decrease in $\beta_2$AR mRNA (28). In that study, $\beta_2$AR protein levels decreased 45% after 1 hr of exposure of the cells to isoproterenol. The decrease of $\beta_2$AR mRNA was of a similar magnitude but occurred after a 4-hr lag from the time the isoproterenol incubation began. These results are in contrast to observations in PKA$^-$$\beta$AR cells, where down-regulation resulted in a ≈50% decrease in $\beta_2$AR protein after 20 hr of isoproterenol treatment, independent of a change in steady state $\beta_2$AR mRNA levels. Similar results were obtained for PKA$^+$$\beta$AR cells (data not shown). Perhaps the sequences responsible for regulating the transcription of the $\beta_2$AR gene after down-regulation are not included within the transfected mouse $\beta_2$AR clone. Alternatively, the

proteins required for this regulation may not be present in Y1 or Kin8 cells. However, the observation that $\beta_2$AR protein decreases well in advance of any changes in $\beta_2$AR mRNA levels in the hamster vas deferens cells and that down-regulation occurs in PKA$^-$$\beta$AR cells without a change in $\beta_2$AR mRNA levels indicate that this process is not dependent upon a decrease in $\beta_2$AR mRNA levels.

Our results demonstrate that protein synthesis is required to reverse down-regulation in PKA$^-$$\beta$AR cells, suggesting that preexisting $\beta_2$AR protein is degraded after prolonged isoproterenol treatment. The decrease in the total number of receptors with down-regulation could be the result of a decrease in receptor synthesis or an increase in receptor clearance. Because the steady state $\beta_2$AR mRNA levels are insensitive to prolonged agonist treatment, we analyzed the polysome profile of the $\beta_2$AR transcript during down-regulation, to determine whether changes in the translation of the receptor mRNA could account for the decrease in receptor protein. However, polysome profiles of $\beta_2$AR mRNA isolated from control or down-regulated PKA$^-$$\beta$AR cells were indistinguishable, suggesting that the translational efficiency of $\beta_2$AR mRNA remains constant.

These observations imply that down-regulated receptors have a shorter $t_{1/2}$ (2–4-fold) than untreated receptors. We measured receptor $t_{1/2}$ with or without isoproterenol in the presence of cycloheximide but observed no difference; however, if a protein with a short $t_{1/2}$ were necessary for $\beta_2$AR down-regulation, this approach would be inadequate.

The size of the polysomes translating $\beta_2$AR mRNA was smaller than expected for a transcript encoding a protein of approximately 46 kDa. Northern analysis demonstrated that actin mRNA, which is found on polysomes of ≈15 ribosomes (21), was located on considerably larger polysomes than the $\beta_2$AR transcript. These results indicate that the translation of $\beta_2$AR mRNA is significantly less efficient than that of actin in PKA$^-$$\beta$AR cells. The mouse $\beta_2$AR sequence near the site of translational initiation, however, agrees well with the consensus eukaryotic translational initiation sequence (29). A possible explanation for the relatively low number of ribosomes associated with the $\beta_2$AR transcript may be the presence of a short, 19-amino acid, open reading frame in the 5′ untranslated region of the transcript, beginning at +107 and terminating at +164 (16). Previous studies have shown that initiation of translation in eukaryotic mRNA occurs at internal AUG codons, if upstream reading frames terminate before reaching the internal AUG (30). Although the upstream AUG in $\beta_2$AR is surrounded by sequences that are not ideal for efficient translational initiation (29) (GCUGAAUGA compared with CCACCAUGG), the initiation of translation at this site may be responsible for masking translation of the $\beta_2$AR transcript beginning at +218. Translation of human $\beta_2$AR mRNA that lacks the 5′ AUG codon, in an *in vitro* rabbit reticulocyte system or after injection into *Xenopus* oocytes, resulted in ≈10-fold more protein than when control mRNA was tested (31). The presence of small open reading frames in the 5′ untranslated regions of several members of the $\beta_2$AR gene family, including the $M_2$ muscarinic receptor (32), hamster and human $\beta_2$AR (33, 34), STE2 receptor (35), rat 5-hydroxytryptamine$_{1c}$ receptor (36), and the rat $D_2$ dopamine receptor (37), supports the hypothesis that translational masking may be important in regulating the number of receptors present in a cell.

Sequestration of $\beta_2$AR has been associated with homologous desensitization, which in turn is thought to be mediated via a specific receptor kinase, $\beta$ARK (38). In this study, we characterize the sequestration, desensitization, and down-regulation of $\beta_2$AR in the PKA-deficient Kin8 cell line. The attenuation of adenylate cyclase activity in these cells demonstrates that Kin8 cells can regulate the coupling of $\beta_2$AR stimulation to adenylate cyclase, independent of PKA activity. These observations suggest that Kin8 cells possess $\beta$ARK activity even though they do not normally express $\beta_2$AR. A plausable explanation for this observation might be that $\beta$ARK activity mediates the sequestration and desensitization of a family of receptors including $\beta_2$AR (39).

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