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Regulation of human β_1-adrenergic receptors and their mRNA in neuroepithelioma SK-N-MC cells: Effects of agonist, forskolin, and protein kinase A

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Abstract

We determined the effect of long-term exposure to β-agonists on β_1-adrenergic receptors (β_1-AR) in human neuroepithelioma SK-N-MC cells because earlier studies have indicated that β_1-AR in this cell line are resistant to agonist-induced down-regulation. Exposing SK-N-MC cells to isoproterenol for 24 hr reduced the density of β_1-AR by 72%, whereas forskolin, an activator of all the isoforms of adenylyl cyclase, failed to affect the density of β_1-AR. Measurement of β_1-AR mRNA levels by the ribonuclease protection assay revealed that isoproterenol-induced down-regulation of β_1-AR was associated with a sharp decline in β_1-AR mRNA, while forskolin also failed to affect this parameter. The differences between the effects of isoproterenol and forskolin on β_1-AR were unrelated to cyclic AMP levels, since both agents increased cyclic AMP equally. Next, we determined the role of cyclic AMP-dependent protein kinase A (PKA) in this phenomenon. Inhibition of PKA by its specific inhibitor, H-89 {*N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 2HCl}, markedly reduced the magnitude of the isoproterenol-mediated down-regulation of the β_1-AR and its mRNA. Transient expression of the catalytic subunit of PKA in SK-N-MC cells down-regulated β_1-AR independently of isoproterenol. Therefore, PKA is central to the effect of β-agonists in down-regulating β_1-AR, and its spacial compartmentalization and access to the receptor appear to be essential components of its action.

Keywords: β_1-Adrenergic receptors; β_1-Adrenergic receptor mRNA; Protein kinase A; Isoproterenol; Ribonuclease protection assay; SK-N-MC cells

1. Introduction

Three subtypes of β-AR have been identified, each belonging to the superfamily of G protein-coupled receptors. All of this subgroup of receptors increase cyclic AMP production in response to the endogenous agonists norepinephrine and epinephrine. Prolonged stimulation of β_1- and β_2-AR (but not β_3-AR) subtypes results in desensitization of receptor-stimulated adenylyl cyclase, attenuation of receptor-mediated function, and receptor down-regulation [1]. The mechanisms underlying this adaptive process are characterized by an early phase that involves receptor phosphorylation by PKA and β-adrenergic receptor kinases, followed by receptor uncoupling and sequestration [2]. The late phase of desensitization involves receptor loss, i.e. agonist-promoted receptor down-regulation [3]. Although some studies suggest that β_1-AR are more resistant to early phase desensitization [4–7], recent studies suggest that both subtypes are regulated similarly by agonists [8]. Compared with early-phase receptor desensitization, less is known about agonist-promoted receptor down-regulation.

Agonist-induced down-regulation of the β_2-AR is thought to occur through cyclic AMP-dependent and -independent shortening in the half-life of β_2-AR mRNA [9,10], as well as enhanced receptor degradation [11]. In cells that endogenously express β_1-AR such as rat C6 glioma cells

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Abbreviations: β-AR, β-adrenergic receptor(s); PKA, cyclic AMP-dependent protein kinase A; DMEM, Dulbecco's modified Eagle's medium; ICYP, ^{125}I-iodocyanopindolol; PCR, polymerase chain reaction; and GPCR, G protein-coupled receptor.

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and in cells expressing recombinant β_1-AR, agonist-mediated down-regulation of the β_1-AR is associated with a parallel decline in its mRNA level [12]. Agonist-promoted destabilization of β_1-AR mRNA is thought to be due to a cyclic AMP-dependent reduction in the half-life of β_1-AR mRNA [12,13]. SK-N-MC cells are a human neuroepithelioma cell line that expresses β_1-AR and β_3-AR, which are both coupled to the GTP-binding regulatory stimulatory G protein, G_s [6,7,14]. Exposure of SK-N-MC cells for 24 hr to isoproterenol results in a 50−80% loss of β_1-AR, as determined by ligand-receptor binding [6]. However, if these cells were incubated with agents that elevate cyclic AMP by a nonreceptor pathway, such as forskolin which activates adenylyl cyclase directly, there was no effect on β_1-AR density, suggesting that agonist-promoted receptor down-regulation of β_1-AR in SK-N-MC cells is a cyclic AMP-independent process [6]. This apparently novel pathway of receptor regulation in this cell line is in marked contrast to agonist-mediated regulation of β_1-AR expressed in other cell lines, which are mediated, at least in part, through cyclic AMP. We therefore conducted studies to determine the mechanisms underlying chronic agonist-promoted β_1-AR down-regulation in the SK-N-MC cell line.

2. Materials and methods

2.1. Cell culture

SK-N-MC cells were grown in monolayer culture on 100-mm culture dishes containing DMEM supplemented with 10% fetal bovine serum in an atmosphere of 5% CO_2, 95% air at 37°. Cells were plated at a density of 10^5/plate on day 1, fed on day 2, and subcultured on day 3 with 0.1% trypsin in PBS, or harvested on day 5 for the preparation of membranes.

2.2. Receptor binding assays

To prepare membranes, the culture medium was aspirated, and the cells were washed twice with 5 mL of ice-cold PBS, followed by a 10-min incubation in ice-cold cell-release buffer (2 mM Na-HEPES, 2 mM EDTA, pH 7.4). Plates were scraped with a rubber policeman, and the cells were suspended in 5 mL of lysis buffer (cell-release buffer supplemented with protease inhibitors: 10 μg/mL of leupeptin, 10 μg/mL of aprotinin, and 1 mM benzamidine), and then homogenized with a Brinkmann polytron at setting 6 for 10 sec. The cell homogenate was centrifuged at 38,000 g_{av} for 20 min at 4°. This procedure was repeated twice, except that the cell pellet was resuspended in 5 mL of saline buffer (20 mM Tris, pH 7.4, and 154 mM NaCl with protease inhibitors) on the final wash. The membrane preparation was assayed for protein and stored at −80° until used. Before use, membranes were thawed and rehomogenized.

Binding assays were composed of 400 μL of binding buffer (20 mM Tris, pH 7.4, 154 mM NaCl, 0.025% ascorbic acid, 0.0008% bovine serum albumin, and 4 mM $MgCl_2$), without or with 100 μM (−)isoproterenol to define nonspecific binding. Various concentrations (9−120 pM) of ICYP (Dupont) were added to saturate the receptor binding sites. Binding was initiated by adding 100-μL aliquots containing 15–35 μg of protein and lasted for 3 hr at 37°. Binding was terminated by adding 10 mL of ice-cold wash buffer (10 mM Tris, pH 7.5, 154 mM NaCl) to each tube, followed by filtering the contents through Whatman GF/C filter paper (Whatman Paper Ltd.) on a Brandel Cell Harvester (Biomedical Research and Development Laboratories Inc.). The filters were washed twice with an additional 10 mL vol. of wash buffer. Radioactivity retained on the filters was counted using a Packard γ counter (Packard Instrument Co.). Maximal receptor density (B_{max}) and the equilibrium dissociation constant (K_d) were determined by Scatchard analysis of the binding data as previously described [15].

2.3. Cyclic AMP accumulation

Cells were grown to 90% confluence, washed twice with 5 mL of PBS, detached using 0.1% trypsin, suspended in 10 mL of PBS, and centrifuged at 1000 g_{av} for 2 min. The cell pellet was resuspended in DMEM supplemented with 50 mM HEPES and 1 mM isobutylmethylxanthine. Cells were incubated with vehicle or various concentrations of isoproterenol or 1 μM forskolin for 10 min at 37°. The reaction was stopped by centrifugation at 10,000 g_{av} for 15 sec. The medium was aspirated, and the cell pellet was resuspended in 0.5 mL of ice-cold 50 mM sodium acetate, pH 6.2, buffer. Cyclic AMP was quantified by radioimmunoassay (RIANEN Assay System, DuPont Co.) and expressed as picomoles of cyclic AMP accumulated per 10^6 cells using a standard curve that was run in parallel.

2.4. Measurement of β_1-AR mRNA levels in SK-N-MC cells by the ribonuclease protection assay

An antisense RNA probe for the measurement of β_1-AR mRNA levels in SK-N-MC RNA was generated as follows. Two primers, a forward primer 5′-ATCGAGACCCTGTGTGTCATTGCC and a reverse primer 5′-TTGGTGACGAAGTCGCAGCACTTG, were synthesized and used to amplify a 227-bp fragment from human β_1-AR cDNA (sequences between 522 and 748 relative to the initiator ATG) by PCR using the GC-Advantage PCR cloning kit (Clontech). The PCR product was gel-purified and cloned into the Topo 2.1 PCR cloning vector (Invitrogen). The insert was excised from the Topo vector by digestion with *Eco*RI and cloned into pGEM3ZF$^+$. The resulting plasmids were sequenced to verify the sequence and the orientation of the insert [16]. A plasmid with an insert in the reverse orientation relative to the T7 promoter was amplified and linearized by digestion with *Bam*HI. To control for the variability in RNA loading, a *Bam*HI linearized 103-bp antisense hu-

man cyclophilin cDNA probe (sequences between 38 and 140) was used (Ambion).

The antisense RNA probes were synthesized by *in vitro* transcription with T7 RNA polymerase (MAXIscript, Ambion). Briefly, 1 μg of DNA template and 0.5 mM ATP, CTP, and GTP were mixed in transcription buffer with 5 μL of 12.5 μM [α-^{32}P]UTP and T7 RNA polymerase supplemented with ribonuclease inhibitors for 1 hr at 37°. Template DNA was removed by digestion with RNase-free DNase I for 15 min, followed by inactivation with EDTA. Similarly, RNA size markers were synthesized by the *in vitro* transcription assay described earlier. The expected sizes of the synthetic riboprobes were 310 bp for the β_1-AR riboprobe and 165 bp for the cyclophilin riboprobe. The full-length labeled probes were separated from prematurely terminated transcription products by electrophoresis on denaturing gels comprised of 1X TBE (89 mM Tris-borate, 89 mM boric acid, 0.5 M EDTA, pH 8.0), 8.0 M urea, and 6% acrylamide, along with the RNA size markers. The area in the gel containing the full-length radiolabeled probes was excised and submerged in a microfuge tube with 350 μL of elution buffer (0.5 M ammonium acetate, 1 mM EDTA, 0.1% SDS) overnight at room temperature to extract the ^{32}P-labeled probes. These probes were used within 24 hr of synthesis.

At 80% confluency, SK-N-MC cells were exposed to 10 μM isoproterenol in 2.5% ascorbic acid without or with 20 μM H-89 89 {*N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 2HCl} in DMSO for 4, 8, and 12 hr followed by the extraction of total cellular RNA. The amounts of β_1-AR mRNA in SK-N-MC cells were determined by the ribonuclease protection assay. Briefly, 10 μg of total cellular RNA was mixed with 60,000 cpm of antisense human β_1-AR riboprobe and 10,000 cpm of antisense cyclophilin riboprobe. Control tubes contained the probes with 10 μg yeast tRNA. RNA and riboprobes were precipitated in 0.5 M sodium acetate and 2.5 vol. of ethanol, followed by centrifugation at 14,000 g_{av} for 10 min at 4° to pellet the RNA. To each RNA pellet, 20 μL of hybridization buffer composed of 80% deionized formamide, 1 mM EDTA, 10 mM sodium citrate, 300 mM sodium acetate, pH 6.4, was added followed by incubation at 90–95° for 3 min and at 42° overnight. The unhybridized RNA was degraded by adding 200 μL of a 1:100 dilution of Rnase A/RNase T mixture for 30 min at 37°. The diluted RNase solution was added to half of the yeast control RNA samples, while digestion buffer without RNase was added to the remaining yeast RNA control tubes. To terminate the reaction, 300 μL of RNase inactivation/precipitation solution containing 5 μg of Glycoblue™ was added for 15 min at −20°. RNA-RNA hybrids were precipitated by centrifugation at 14,000 g_{av} for 15 min at 4°, followed by removal of the supernatant. Each pellet was resuspended in 5 μL of gel loading buffer (95% formamide, 18 mM EDTA, 0.025% SDS, 0.025% xylene cyanol, and 0.025% bromophenol blue) and heated to 95° for 3 min. The samples were loaded on 6% acrylamide/8 M urea gels and separated by electrophoresis. The gels were dried and exposed to film at −80° for 2 days.

To determine the cpm in each band, the dried gel was analyzed by electronic autoradiography for 12 hr using a Packard Instantimager. The gel was divided into 16–20 lanes, and the distribution of radioactivity in the 220-nt β_1-AR bands and in the 100-nt cyclophilin bands was plotted in each lane. The total counts under the peaks for binding of β_1-AR and cyclophilin riboprobes were determined after subtraction of background. In each experiment, all samples were run on the same gel along with the proper controls and RNA size markers. The β_1-AR mRNA counts were corrected for cyclophilin counts in each sample and expressed as percent of the zero time control. The effects of added agents were calculated as the percentage change from the incubation control in each experiment since this resulted in a more normal distribution of the data. Statistical comparisons for four independent measurements were made using Student's *t*-test on the paired differences.

2.5. Transfection of SK-N-MC cells

SK-N-MC cells were grown on 100-mm plates in DMEM supplemented with 10% FBS to 60–70% confluence. The expression vectors for the catalytic subunit of PKA under the control of the SRα promoter were provided by Dr. M. Muramatsu, DNAX [17]. The SRα promoter, composed of the SV40 early promoter and the RU5 segment of human T-cell leukemia virus type I long terminal repeat, directs the efficient expression of the open reading frame of the catalytic subunit of PKA. For transient transfections, SK-N-MC cells were switched to DMEM and transfected with 4 μg of the expression vector for the C-subunit of PKA, or the empty vector (pCDNA3.1, Invitrogen) and 0.5 μg of RSV-β-galactosidase (to correct for transfection efficiency) using LipofectAMINE™ PLUS as described by the manufacturer. After 6 hr, the medium was aspirated and replaced with DMEM + 10% FBS for 24 hr. The percentile of the cells that were transfected was determined by β-galactosidase staining followed by light microscopy [18]. These determinations indicated that the transfection efficiency within each experimental set was similar.

3. Results

3.1. β_1-AR density and responsiveness in SK-N-MC cells

SK-N-MC cells express β_1- and β_3-AR [14]. The affinity of ICYP to β_1-AR is at least 100-fold higher than its affinity to β_3-AR, suggesting that the concentrations of ICYP used in our binding assay would label the β_1-AR subpopulation only [14,19]. We tested this assumption by incubating a 50 nM concentration of the selective β_3-AR agonist CL 316,243 in our binding assay [14,19]. At this concentration, CL 316,243 did not compete with ICYP binding, indicating

Table 1
Effect of drug treatment on β_1-AR binding parameters in SK-N-MC cells

Treatment	Isoproterenol 10 μM		Forskolin (1 μM)	
	B_{max} (fmol/mg protein)	K_d (pM)	B_{max} (fmol/mg protein)	K_d (pM)
Control (vehicle)	234 ± 26	7.6 ± 1	200 ± 56	10 ± 4
Treatment	66 ± 18*	7.5 ± 0.5**	183 ± 37**	10 ± 4

SK-N-MC cells were grown to 80% confluence, then pretreated for 24 hr with vehicle (2.5% ascorbic acid) or 10 μM isoproterenol in ascorbic acid. A parallel set of cells was treated with vehicle (DMSO) or 1 μM forskolin for 24 hr. Membranes were prepared and incubated with ICYP as described in "Materials and methods." Specific binding of ICYP was measured by Scatchard analysis to determine maximal receptor binding (B_{max}) and affinity (K_d) of ICYP. The data represent means ± SEM of three experiments, each in triplicate.

* $P = 0.00005$, control vs isoproterenol.

** Not significant ($P > 0.05$) vs control.

that ICYP was binding to the β_1-AR subpopulation. The B_{max} and K_d values (mean ± SEM) of β_1-AR expressed in SK-N-MC cells were 234 ± 26 fmol/mg protein and 7.6 ± 1 pM, respectively (Table 1). These values are similar to the binding characteristics of ICYP to β_1-AR heterologously expressed in Cos-7 or HEK-293 cells [8,20].

3.2. Effect of isoproterenol and forskolin on β_1-AR density

We determined the effect of long-term exposure (24 hr) of isoproterenol, which activates the β-AR pathway, and forskolin on β_1-AR density in SK-N-MC cells (Table 1). Exposure of cells for 24 hr to isoproterenol caused a 72% decrease in β_1-AR density, whereas forskolin had no statistically significant effect on this parameter

To determine the time course of isoproterenol-induced β_1-AR loss, we exposed cells to 10 μM isoproterenol for 0–24 hr (Fig. 1A). Little, if any, change in receptor density was observed within the first hour of exposure, but thereafter β_1-AR density was reduced by 50% within 4 hr and by 75% in 12 or 24 hr. The dependency of the isoproterenol-induced receptor loss upon exposing the cells to the agonist for 24 hr was also determined (Fig. 1B). The concentration of isoproterenol that resulted in a 50% loss of β_1-AR was 0.35 ± 0.5 nM, and maximal β_1-AR down-regulation was achieved by concentrations of 0.1 μM and higher. β_1-AR loss was blocked by co-incubating isoproterenol with a 10 nM concentration of the hydrophilic, nonselective β-adrenergic receptor antagonist CGP-12177 or with a 0.5 μM concentration of the selective β_1-AR antagonist CGP-20712A (data not shown).

3.3. Role of cyclic AMP in isoproterenol-mediated down-regulation of β_1-AR

Exposure of SK-N-MC cells to isoproterenol resulted in a concentration-dependent increase in cyclic AMP accumulation (Fig. 2). Isoproterenol and forskolin concentrations of 10 and 1

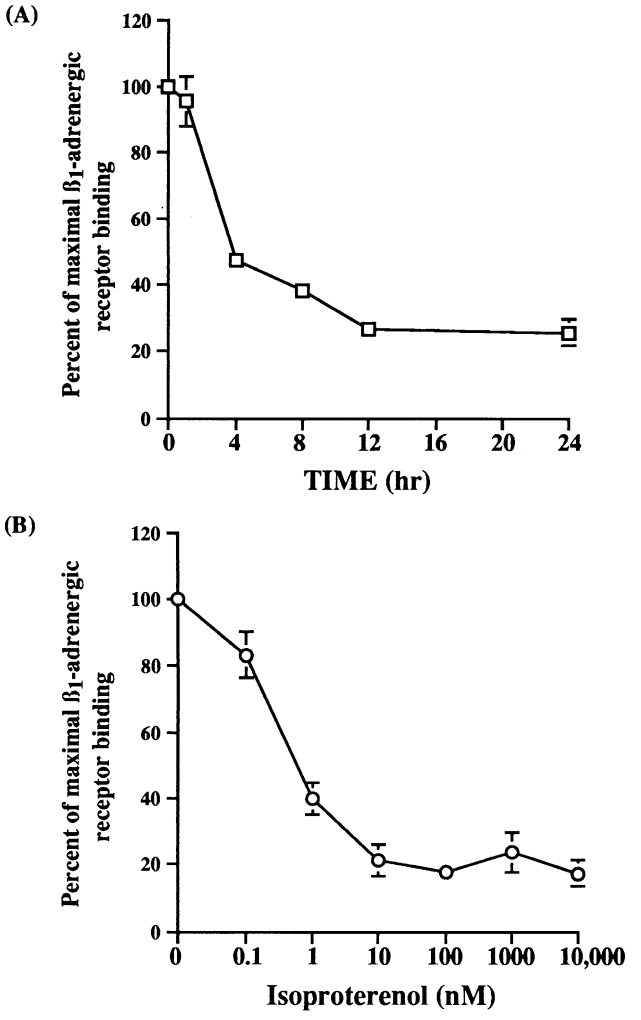

Fig. 1. Time course and concentration–effect relationships of isoproterenol on β_1-AR density in SK-N-MC cells. Panel A: SK-N-MC cells were grown to 80% confluence and then were treated for 24 hr with vehicle (2.5% ascorbic acid) or 10 μM isoproterenol in 2.5% ascorbic acid. Panel B: SK-N-MC cells were exposed to the indicated concentrations of isoproterenol, shown on the abscissa, in 2.5% ascorbic acid for 24 hr. Membranes were prepared from these cells and incubated with ICYP as described in "Materials and methods." Specific binding of ICYP was determined by Scatchard analysis, and the results are reported as the means ± SEM of four determinations, each from triplicate samples.

μM, respectively, stimulated cyclic AMP accumulation by 30 ± 9- and 33 ± 12-fold, respectively, over basal accumulation, which was 2.8 ± 1.5 pmol/10^6 cells. Therefore, the failure of forskolin to down-regulate the density of β_1-AR in SK-N-MC cells was not due to insufficient production of cyclic AMP, since isoproterenol induced maximal β_1-AR loss at concentrations below 0.1 μM, which are associated with significantly less cyclic AMP production than 1 μM forskolin.

3.4. Effect of the PKA inhibitor H-89 on isoproterenol-mediated down-regulation of β_1-AR in SK-N-MC cells

Activation of the β-AR signaling pathway by isoproterenol or activation of adenylyl cyclase by forskolin results, in

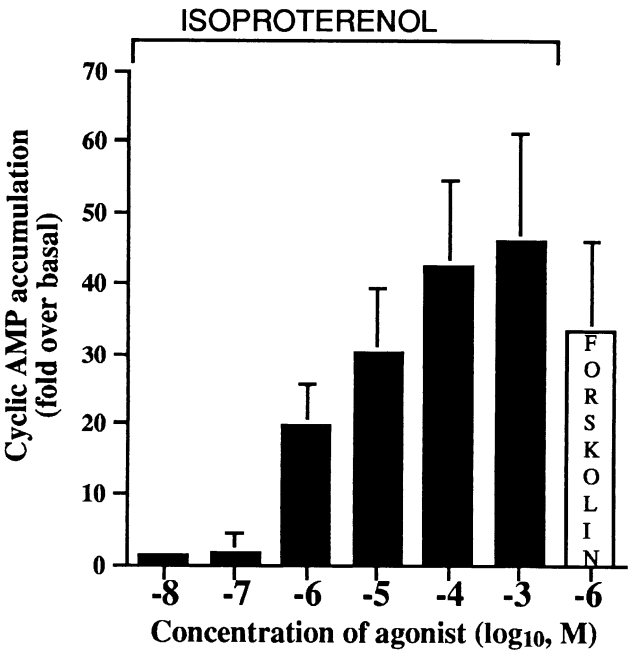

Fig. 2. Cyclic AMP accumulation in SK-N-MC cells in response to isoproterenol and forskolin. SK-N-MC cells grown to 90% confluence were harvested, washed, and suspended in DMEM supplemented with 50 mM HEPES and 1 mM isobutylmethylxanthine. Cells were aliquoted to contain 0.5 to 1 $\times$ 10^6 cells/mL and incubated for 20 min at 37° with various concentrations of isoproterenol or 1 μM forskolin, followed by the determination of cyclic AMP levels by radioimmunoassay. The data represent the means $\pm$ SEM of two determinations, each from triplicate samples.

both instances, in elevation of cyclic AMP. If cyclic AMP or its mediators are involved in the down-regulation of β_1-AR expression, then the pools of cyclic AMP generated by forskolin are not engaging the appropriate mediator(s), because forskolin has no effect on β_1-AR density in SK-N-MC cells. A plausible mediator of the downstream effects of cyclic AMP is PKA. Therefore, we determined whether PKA is involved in the isoproterenol-mediated β_1-AR down-regulation by inhibiting PKA with the cell-permeant specific PKA inhibitor H-89 and then measuring the effect of this intervention on β_1-AR density in SK-N-MC cells (Table 2). H-89 alone had no effect on β_1-AR density, even at the relatively high concentration that we used. Pretreatment of the cells with H-89 reduced the down-regulation of β_1-AR by isoproterenol by 50% over 24 hr.

3.5. Effect of isoproterenol on β_1-AR mRNA levels in SK-N-MC cells

Isoproterenol-mediated down-regulation of β_1-AR may be caused by transcriptional, post-transcriptional, or translational mechanisms. The effect of isoproterenol on β_1-AR mRNA levels was determined by the ribonuclease protection technique because this technique is quantitative as opposed to the comparative reverse transcriptase–PCR technique [21]. β_1-AR mRNA levels in SK-N-MC cells were

Table 2
Effect of H-89 on isoproterenol-mediated β_1-AR loss in SK-N-MC cells

	K_d (pM)	% Change in β_1-adrenergic receptor density
Control (vehicle)	9 $\pm$ 1	
Isoproterenol (10 μM)	7 $\pm$ 1	−56*
H-89 (30 μM)	10 $\pm$ 2	+18
H-89 + isoproterenol	10 $\pm$ 2	−26

SK-N-MC cells were exposed to vehicle (1% DMSO) or the indicated concentration of isoproterenol or H-89 alone and together for 6 hr at 37°. ^{125}I-ICYP binding to membranes prepared from these cells was measured as described in "Materials and methods." Each binding assay was performed in triplicate and repeated twice. The data represent the means $\pm$ SD of three determinations, each in triplicate, (N = 9).

*$P < 0.05$.

determined using a 310-bp *in vitro* transcribed riboprobe that encoded a 227-bp antisense human β_1-AR mRNA fragment. Incubation of this probe with SK-N-MC RNA generated the expected 227-bp RNase-resistant fragment, indicating that the protected sequence did not contain significant secondary structures (Fig. 3). The number of cpm of ^{32}P-incorporated per band was determined by electronic counting. Isoproterenol caused a gradual decline in β_1-AR mRNA in SK-N-MC cells (Fig. 4A). β_1-AR mRNA declined by 40 $\pm$ 6% in 8 hr, and the loss of β_1-AR mRNA was sustained for at least 24 hr (Fig. 4A). The time courses of isoproterenol-mediated down-regulation of membranous β_1-AR in Fig. 1A and β_1-AR mRNA were plotted together in Fig. 4A. These data reveal that the agonist-induced loss of β_1-AR binding and mRNA occurred in similar time frames. β_1-AR mRNA levels in cells that were exposed to forskolin (1 μM) for 24 hr were no different from those in control cells exposed to vehicle.

To determine whether PKA is involved, SK-N-MC cells were exposed to H-89 and isoproterenol, followed by β_1-AR mRNA measurement (Fig. 4B). H-89 blocked isoproterenol-mediated loss of β_1-AR mRNA that was seen at 4 and 8 hr. SK-N-MC cells that were exposed to isoproterenol and H-89 concurrently for 24 hr had significantly higher levels of β_1-AR mRNA than those cells exposed to the β-agonist only. These data suggest that PKA activation appears to be the primary signal for β_1-AR mRNA down-regulation in SK-N-MC cells because cyclic AMP levels were equally elevated in cells exposed to β-agonist in the absence or presence of H-89.

To further characterize the involvement of PKA in regulating β_1-AR mRNA levels, SK-N-MC cells were transiently transfected with the constitutively active catalytic subunit of PKA. Transfection of SK-N-MC cells either with the empty mammalian expression vector or that harboring the catalytic subunit of PKA did not raise cyclic AMP levels in these cells (data not shown). The transfection efficiency of this technique as determined by β-galactosidase staining was 28 $\pm$ 6% (N = 4). As shown in Fig. 5, β_1-AR mRNA

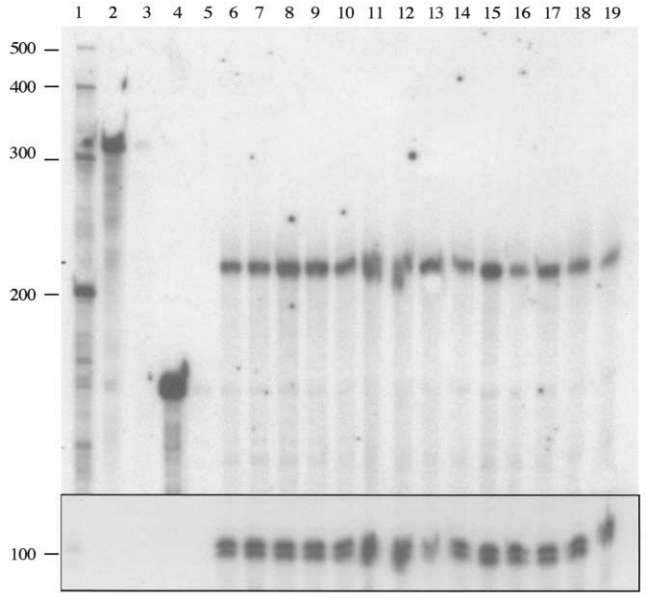

Fig. 3. Determination of β_1-AR and cyclophilin mRNA levels by the ribonuclease protection assay. ^{32}P-Labeled antisense human β_1-AR riboprobe (60,000 cpm) and ^{32}P-labeled antisense cyclophilin riboprobe (10,000 cpm) were mixed with 10 μg of SK-N-MC RNA and subjected to ribonuclease protection assay as described in "Materials and methods." In lane 1, labeled RNA markers (7000 cpm) were electrophoresed to determine the size of the protected RNA-RNA hybrids. ^{32}P-Labeled antisense human β_1-AR riboprobe (5000 cpm) was electrophoresed with 10 μg of yeast tRNA in the absence (lane 2) or presence of RNase (lane 3). Similarly, lanes 4 and 5 represent ^{32}P-labeled antisense human cyclophilin riboprobe (5000 cpm) in the absence or presence of RNase, respectively. RNA from untreated control SK-N-MC cells (lanes 6 and 7) and from SK-N-MC cells that were exposed to 10 μM (–)isoproterenol for 1, 4, 8, and 24 hr were loaded in lanes 8–11, respectively. Lanes 12–15 contain RNA from SK-N-MC cells that were exposed to DMSO (lane 12), 20 μM H-89 for 2 hr (lane 13), DMSO + isoproterenol (lane 14), or isoproterenol + H-89 for 2 hr (lane 15). Lanes 16 and 18 contain RNA from cells exposed to isoproterenol + DMSO for 8 and 24 hr, respectively. Lanes 17 and 19 contain RNA from cells exposed to isoproterenol + H-89 for 8 and 24 hr, respectively. The figure represents an autoradiogram of a gel that was exposed to film at –70° for 2 days for measurement of β_1-AR mRNA and for 18 hr for measurement of cyclophilin mRNA. These experiments were replicated four times on different RNA samples.

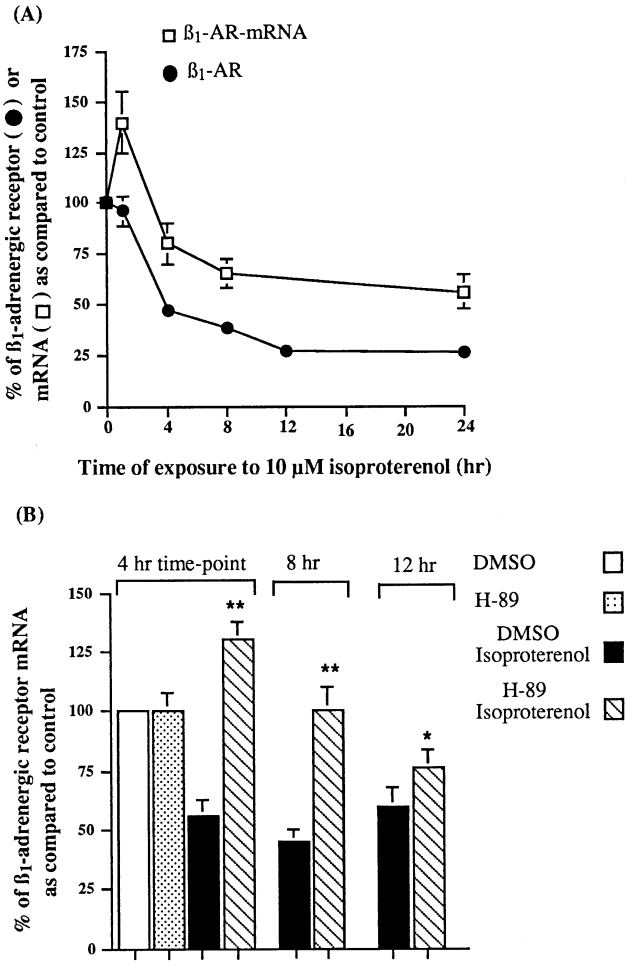

Fig. 4. Reversal of isoproterenol-mediated down-regulation of β_1-AR mRNA levels in SK-N-MC cells by H-89. Panel A: time course for the effect of isoproterenol on β_1-AR mRNA levels in SK-N-MC cells. SK-N-MC cells were exposed to 10 μM isoproterenol for the indicated time periods, followed by the extraction of RNA. β_1-AR and cyclophilin mRNA levels were measured, and the corrected data are presented as the percentage of the zero time control. The values are the means ± SEM of four separate determinations. The data for the time course of the effect of isoproterenol on β_1-AR density in Fig. 1A were superimposed on the mRNA data. Panel B: effect of H-89 on isoproterenol-mediated down-regulation of β_1-AR mRNA. SK-N-MC cells were exposed to vehicle (DMSO) or 20 μM H-89 in the absence or presence of 10 μM isoproterenol for 4, 8, and 12 hr. After each time period, RNA was extracted. The levels of β_1-AR and cyclophilin mRNAs were measured, and the corrected data are presented as the percentage of the 4-hr vehicle control. The values are the means ± SEM of four separate determinations. The values of β_1-AR mRNA remaining after 4 hr were 100% in DMSO, 100 ± 7% in H-89, 53 ± 6% in DMSO + isoproterenol, and 140 ± 6% in H-89 + isoproterenol. At the 8-hr time point, these values were 45 ± 4% for DMSO + isoproterenol vs 100 ± 9% for H-89 + isoproterenol. At the 12-hr time point, these values were 49 ± 7% for DMSO + isoproterenol vs 75 ± 7% for H-89 + isoproterenol. Statistically significant effects of H-89 on isoproterenol-mediated effects on β_1-AR mRNA are indicated by one or two asterisks (**$P < 0.01$, *$P < 0.05$).

levels in untransfected cells or in cells transfected with the empty mammalian expression vector were comparable, indicating that this technique did not affect the cells adversely. Exposing untransfected SK-N-MC cells or cells transiently transfected with the empty mammalian expression vector to isoproterenol for 24 hr produced 43 ± 7 and 40 ± 5% loss of β_1-AR mRNA, respectively. In SK-N-MC cells that were transiently transfected with the catalytic subunit of PKA, there was a 38 ± 5% decline in β_1-AR mRNA within 24 hr after transfection. Exposing SK-N-MC cells that were transfected with the catalytic subunit of PKA to 0.1 μM isoproterenol for 24 hr reduced β_1-AR mRNA by 52 ± 8%. These data indicate that activated PKA is capable of reducing β_1-AR mRNA levels in SK-N-MC cells. Isoproterenol further decreased β_1-AR mRNA either because not all the cells were efficiently transfected with the PKA expression vector or because β_1-AR down-regulation involves PKA-dependent and -independent mechanisms that are addressed in the "Discussion."

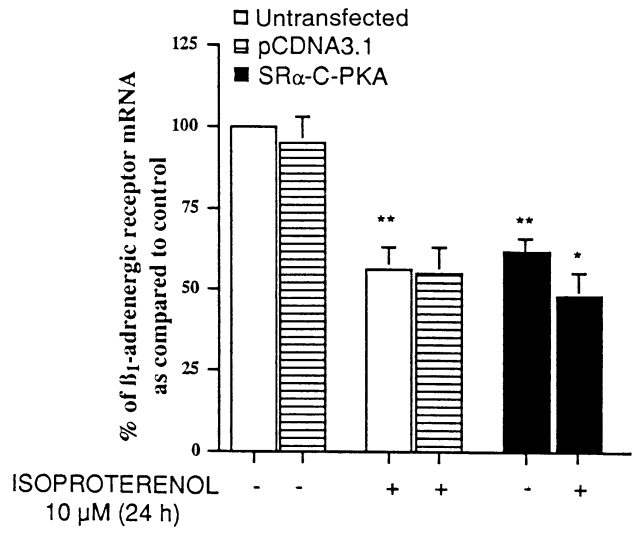

Fig. 5. Effect of transient expression of the catalytic subunit of protein kinase A on β_1-AR mRNA levels in SK-N-MC cells. SK-N-MC cells in DMEM were transiently transfected with either 10 μg of a mammalian expression vector harboring the catalytic subunit of human protein kinase A or the empty mammalian expression vector pCDNA3.1 with 2 μg of SV40-*βgal* by the LipofectAMINE™ PLUS method. After 6 hr, the medium was aspirated, and the cells were cultured in DMEM + 10% FBS in the absence or presence of 10 μM isoproterenol. After 24 hr, RNA was extracted, and β_1-AR mRNA levels were determined. In each separate experiment, β-galactosidase staining was used to determine the percent of the cells that were transfected. The values of β_1-AR mRNA are the means ± SEM of three separate determinations. The percents of β_1-AR mRNA in untransfected without or with isoproterenol were: 100 vs 56 ± 6; in pCDNA3.1 without or with isoproterenol, 96 ± 8 vs 55 ± 6; and in SRα-C-PKA without or with isoproterenol, 62 ± 4 vs 48 ± 6. Key: (**) $P < 0.01$ for β_1-AR mRNA in untreated vs isoproterenol or SRα-C-PKA; and (*) $P < 0.05$ for β_1-AR mRNA in SRα-C-PKA without vs SRα-C-PKA with isoproterenol.

4. Discussion

The processes of agonist-mediated GPCR regulation may be divided into rapid and slow processes [3]. Rapid processes of receptor regulation are those that occur in early time frames after agonist-induced receptor activation. This process is initiated by agonist-dependent phosphorylation of serine/threonine residues in the carboxy terminal tail of the GPCR [2]. Phosphorylated receptors interact with cytoplasmic arrestins that facilitate the translocation of the receptor to clathrin-coated pits where they are sequestered from the plasma membrane to intracellular endosomes. If the agonist is withdrawn within minutes, the GPCR is dephosphorylated within the endosome by endosomal phosphatases and recycled back to the plasma membrane to promote full receptor recovery [2,3].

Slower processes of agonist-mediated GPCR regulation operate over a much longer time scale (hours or days) and are associated with receptor down-regulation manifested by the reduced total number of receptors in either cells or tissues [3,22]. The processes involved in long-term receptor regulation are less defined and appear to involve more than one mechanism and operate on both the GPCR protein and its mRNA. Many of these mechanisms were derived from studies involving heterologous expression of GPCR into established cell lines. Some studies reveal that the initial steps of agonist-promoted receptor down-regulation are shared with the aforementioned pathway implicated in recycling. In this pathway, agonist-occupied receptors translocate via clathrin-coated pits to endosomes where an unspecified sorting mechanism diverts the receptor-containing endosomes to lysozomes in which the receptor is proteolyzed. There are also data to suggest that the mechanisms mediating the processes of sequestration and down-regulation are distinguishable. Recent studies have shown that GPCR can also undergo ligand-dependent proteolysis by a clathrin-independent non-lysosomal mechanism [23].

Unlike the studies described above, our study involved the effects of isoproterenol and forskolin on β_1-AR that are expressed from the intact gene and possess a functional 3′-untranslated region. In these cells, continuous exposure (hours) to isoproterenol, but not forskolin, caused a time- and concentration-dependent reduction in the density of β_1-AR and its mRNA. The difference between isoproterenol- and forskolin-mediated desensitization of the β_1-AR was previously observed also after short-term (minutes) exposure to these agents [8]. The pattern of agonist-induced β_1-AR down-regulation in SK-N-MC cells reported here is in sharp contrast with the results of Zhou and Fishman [4], which revealed that β_1-AR are not desensitized in SK-N-MC cells after short-term agonist exposure. However, this same group later found that β-agonists indeed down-regulate the β_1-AR complement of SK-N-MC cells after short- or long-term exposures [24]. Also, in agreement with our data, Curran and Fishman [24] found that 10 μM forskolin did not affect the density of β_1-AR in SK-N-MC cells.

There are other data that examined the regulation of β_1- and β_2-AR in rat C6 glioma cells [12]. What is unusual in this study was that isoproterenol and forskolin did not destabilize β_1-AR mRNA and apparently inhibited the transcription of the β_1-AR gene [12]. We have determined the responsiveness of the rat β_1-AR promoter-luciferase chimera to isoproterenol and forskolin in several cell lines, including SK-N-MC and C6, and did not observe an effect of these agents on reporter gene activity [25].

The novel finding reported here concerns the unexpected role of PKA in β_1-AR down-regulation after long-term exposure to agonists. We investigated this hypothesis with H-89, a specific inhibitor of PKA that significantly inhibited isoproterenol-induced β_1-AR mRNA down-regulation. Moreover overexpression of the catalytic subunit of PKA resulted in a decline in β_1-AR mRNA similar to that induced by isoproterenol, indicating that PKA is obligate for isoproterenol-promoted down-regulation of β_1-AR mRNA in these cells. PKA is also obligate for β-AR recycling and MAP kinase activation following short-term agonist exposure because mutagenesis of the PKA phosphorylation site

in the β_2-AR or pretreatment of the cells with H-89 prevents agonist-mediated activation of mitogen-activated protein (MAP) kinases [26]. The effects of PKA in long-term agonist-mediated receptor down-regulation are complex. In addition to its role in β-AR trafficking, PKA promoted the degradation of β_1-AR mRNA after prolonged exposure of the cells to β-agonists. Our data show that β_1-AR mRNA rapidly increased by about 40% above control after a 1-hr exposure to isoproterenol, followed by declines of 25 and 40% compared with controls after 4 and 8 hr of isoproterenol exposure, respectively (Fig. 4A). In contrast, β_1-AR mRNA increased to about the same extent after exposure to either H-89 or isoproterenol for 4 hr, suggesting that H-89 has no effect on the rapid isoproterenol-promoted up-regulation of β_1-AR mRNA. Instead, H-89 maintained those elevated levels for 4 hr, whereas in its absence β_1-AR mRNA declined by 20% of control. The effect of H-89 on β_1-AR mRNA regulation following prolonged exposure waned, so that after 12 hr of co-exposure H-89 only partially prevented β_1-AR mRNA down-regulation. Waning of the effect of H-89 could be due to the instability of H-89, which may increase with time causing a diminution of its effect. In addition, there may be additional mechanisms that are involved in agonist-mediated long-term receptor regulation that manifest their effect in later time frames, causing a waning in the effect of H-89. Consequently, our data with H-89 imply that down-regulation of GPCR after long-term agonist exposure involves PKA-dependent and -independent mechanisms. The PKA-dependent mechanism operates at the level of receptor down-regulation as well as mRNA down-regulation since H-89 prevented the loss of the β_1-AR protein and its mRNA (Table 2 and Fig. 4). The preference of the PKA-independent mechanism(s) towards β_1-AR protein or mRNA down-regulation could not be ascertained in this study because the status of H-89 at the end of the 24 hr of incubation is not known. However, this study demonstrates that a meaningful understanding of the processes involved in long-term GPCR regulation by agonists must be carried out on the complete receptor transcript in order to analyze the processes involved in regulating post-transcriptional (such as mRNA stability) and post-translational (receptor phosphorylation and trafficking) regulation of the receptor under study.

Why does continuous β_1-AR stimulation in these cells cause a profound down-regulation of mRNA and receptor density while continuous exposure to forskolin does not, despite the fact that intracellular cyclic AMP levels over time are similar during both treatments? A simple answer is that agonist-promoted β_1-AR down-regulation in SK-N-MC cells is cyclic AMP-independent. This notion, however, is refuted by the fact that cyclic AMP is the sole activator of PKA, and PKA activation down-regulated the density of β_1-AR in this cell line independently from cyclic AMP (Fig. 5). Therefore, the efficacy of PKA that is generated by β-agonists in phosphorylating critical sites involved in down-regulation of β_1-AR is higher than the efficacy of PKA that is generated by forskolin in phosphorylating these sites. Compared with agonist-promoted down-regulation of β_2-AR, little is known about β_1-AR down-regulation. Although early studies suggested that β_1- and β_2-AR are regulated differently [4–6], more recent studies indicate that both subtypes are regulated similarly [8]. The PKA phosphorylation sites in the third intracellular loop and in the carboxy-terminal domain of the β_2-AR are critical for receptor down-regulation [27]. If that is also true for β_1-AR, then substantial differences in the phosphorylation of the PKA site by PKA and β-agonists should be evident. Two studies have examined this point directly and found that the magnitude of forskolin-mediated phosphorylation of the β_1-AR is about 15% of that mediated by isoproterenol [8,28]. On the other hand, the efficacy of forskolin and isoproterenol in phosphorylating the β_2-AR is similar, and the magnitude of their down-regulation of the β_2-AR is comparable [5,29]. The discrepancy between PKA and β-agonists in phosphorylating the β_1- and β_2-AR most probably lies in the structure of their respective third intracellular loops. Hydrophilic residues surround the PKA site in the third intracellular loop of the β_2-AR, which facilitates the access of PKA to this site. On the other hand, the PKA site in the third intracellular loop of the β_1-AR is adjacent to a proline-rich domain, which imparts significant hydrophobicity and rigidity that hinders the access of PKA to this site [16,28]. In addition, the proline-rich region of the β_1-AR binds SH3 containing proteins related to the endophilin family, which may further block the access of PKA, particularly in endophilin-expressing neuronal cells [30]. Another related hypothesis proposes that the efficacy of PKA generated by forskolin is less than the efficacy of PKA generated by β-agonist in down-regulating the β_1-AR because transduction of signals across cell membranes is known to involve anchors, scaffolds, and adaptor proteins [31]. The A kinase anchoring proteins are a diverse family of proteins that assist in assembling the appropriate enzymes into specific signaling pathways. Consequently, within the recycling endosome, the pools of PKA that are activated via β-agonists may be more intimately linked to the process of β_1-AR down-regulation than the pools of PKA that are activated by forskolin, particularly if those pools are inaccessible to the cargo within the trafficking endosomes.

In addition to the role of the PKA site in internalization, different residues in GPCR appear to be involved in the processes of short- and long-term receptor regulation [22]. Hausdorff *et al.* [32] determined that serine/threonine residues within a short region in the carboxy-terminal domain of the β_2-AR between residues 355 and 364 are selectively involved in rapid receptor regulation. In addition, Cao *et al.* [33] have reported that Ser^{411} in the carboxy-terminal domain of the β_2-AR is a substrate for G protein-coupled receptor-kinase-5 phosphorylation and is required for proper recycling of the receptor. Therefore, if differences exist in the types of adenylyl cyclase or receptor kinases that are activated by β-agonists versus forskolin, that might

explain their different effects on β_1-AR down-regulation in SK-N-MC cells.

A third cause for the observed differences between β-agonists and forskolin may be related to cell-specific mechanisms. It is well known that the sequelae of GPCR regulation depend on the cell type in which they are studied. For example, the β_2-AR is proteolyzed by an endocytosis-independent mechanism when expressed in A431 cells, but by an endocytosis-dependent and lysosomal-dependent mechanism when expressed in HEK-293 cells [22]. Similarly, distinct mechanisms unique to SK-N-MC cells may play a role in promoting agonist-dependent down-regulation of β_1-AR in this cell line. SK-N-MC cells express β_3-AR, which may increase the efficacy of PKA generated by isoproterenol versus that generated by forskolin [14]. The β_3-AR is activated by isoproterenol, but unlike the β_1- or the β_2-AR does not undergo significant agonist-mediated desensitization [34]. Consequently, β-agonists could maintain a high level of PKA activation if they significantly stimulate the β_3-AR subpopulation. In our study, we could not measure the complement of β_3-AR in SK-N-MC cells by the radioligand assay that we used, because the labeling of β_3-AR occurs at a concentration 10-fold higher than the maximal concentration of ICYP that we used [14]. Instead, we used CL 316,243 to show that the β_3-AR subpopulation was not markedly involved in isoproterenol-mediated β_1-AR down-regulation. However, there are reports indicating that CL 316,243 is more selective towards the rodent than the human β_3-AR [35]. Therefore, our results must be interpreted with caution until a more selective human β_3-AR agonist becomes available to us. In summary, while the phenomena involved in long-term receptor regulation are complex, SK-N-MC cells offer a unique model to dissect the various avenues of GPCR down-regulation.

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