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A constitutively active mutant β_2-adrenergic receptor is constitutively desensitized and phosphorylated

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ABSTRACT **The β_2-adrenergic receptor (β_2AR) can be constitutively activated by mutations in the third intracellular loop. Whereas the wild-type receptor exists predominantly in an inactive conformation (R) in the absence of agonist, the mutant receptor appears to spontaneously adopt an active conformation (R*). We now demonstrate that not only is the mutant β_2AR constitutively active, it is also constitutively desensitized and down-regulated. To assess whether the mutant receptor can constitutively engage a known element of the cellular desensitization machinery, the receptor was purified and reconstituted into phospholipid vesicles. These preparations retained the essential properties of the constitutively active mutant receptor: agonist-independent activity [to stimulate guanine nucleotide-binding protein (G_s)-GTPase] and agonist-specific increase in binding affinity. Moreover, the purified mutant receptor, in the absence of agonist, was phosphorylated by recombinant βAR-specific kinase (βARK) in a fashion comparable to the agonist-occupied wild-type receptor. Thus, the conformation of the mutated receptor is equivalent to the active conformation (R*), which stimulates G_s protein and is identical to the βARK substrate.**

Receptor activation by agonists is the first step in signal transduction. It is generally considered that receptors exist in an equilibrium between an inactive (R) and an active (R*) conformation. The binding of agonists to receptors stabilizes, and therefore shifts, the equilibrium toward the active conformation R* (1, 2). Adrenergic receptors (ARs) provide a model for the family of guanine nucleotide-binding protein (G protein)-coupled receptors (3), and some recent studies have provided evidence supporting this two-state model of receptor activation: mutations in the C-terminal portion of the third intracellular loop of the α_{1B}- (4, 5), β_2- (6), and α_2AR subtypes (7) constitutively activate these receptors, leading to an agonist-independent activation of the corresponding effector enzymes. The properties of such constitutively active mutant (CAM) receptors have been rationalized by assuming that they preferentially adopt the active rather than the inactive conformation and therefore spontaneously mimic an agonist-occupied form of the receptor (6).

In addition to cellular activation, the binding of agonists to cell surface receptors often results in a waning of cellular responsiveness (desensitization). In the well-studied β_2-adrenergic system, such attenuation operates mainly at the level of the receptor itself. Indeed, besides the activation of adenylyl cyclase (AC) through the G_s protein, the binding of an agonist results in both receptor loss (down-regulation) and receptor uncoupling from the G protein (8). Uncoupling from the G protein results mainly from the receptor being phosphorylated by two kinases, cAMP-dependent protein kinase and β-adrenergic receptor-specific kinase (βARK) (9).

Inasmuch as a CAM receptor mimics an agonist-occupied form of the receptor with respect to cellular activation mechanisms, it might therefore also be expected to constitutively engage the attenuation mechanisms that are normally activated only by occupation of the wild-type (WT) receptor by an agonist. To test this hypothesis, we have taken two complementary approaches: (*i*) the signal transduction properties of the WT and CAM β_2-adrenergic receptor (β_2AR) were assessed in stably transfected mammalian cells, and (*ii*) the molecular mechanisms underlying these properties were studied with purified and reconstituted preparations of these receptors.

MATERIALS AND METHODS

Permanent Cell Lines. Establishment and maintenance of clones permanently expressing the WT and CAM β_2AR in the Chinese hamster ovary (CHO) cell line, cell membrane preparation, determination of receptor density, and AC assays were performed and analyzed as described (6).

AC Data Analysis. Sensitization was quantitated as the gain of signal transduction efficacy, as detailed earlier (10). In brief, after subtraction of basal activity, the concentration–response curves of control and treated membranes were fitted and compared by using the following algorithm (11):

$$E = E_m\{\tau^n A^n/(K_a + A)^n + \tau^n A^n\},$$

where E is the effect, E_m is the maximal possible effect, A is the agonist concentration, K_a is the association constant of the agonist–receptor complex, and n is a slope factor, which was never significantly different from 1 in all experiments presented here. τ is a parameter describing the signal transduction efficacy of the system and is estimated individually for each curve, whereas all the other parameters are shared for each pair of treated/control curves. When $\tau_{treated} > \tau_{control}$, $\tau_{treated}/\tau_{control}$ is taken as a measure of sensitization (-fold). Intuitively, this parameter corresponds to the increase of the receptor population that would give an equivalent enhancement of receptor-modulated AC activity (sensitization). Thus, a discrepancy between the variation of τ (determined by curve fitting) and the variation of receptor density (determined through direct radioligand binding) reflects a modulation of receptor–effector coupling.

Purification and Reconstitution of the WT and Mutant β_2AR. The cDNAs encoding the WT and CAM β_2AR (6, 12) were subcloned into vector pAcC4 and cotransfected in Sf9

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Abbreviations: β_2AR, β_2-adrenergic receptor; CAM, constitutively active mutant; WT, wild type; AC, adenylyl cyclase; G protein, GTP-binding regulatory protein; G_s, AC-stimulatory G protein; βARK, βAR-specific kinase.

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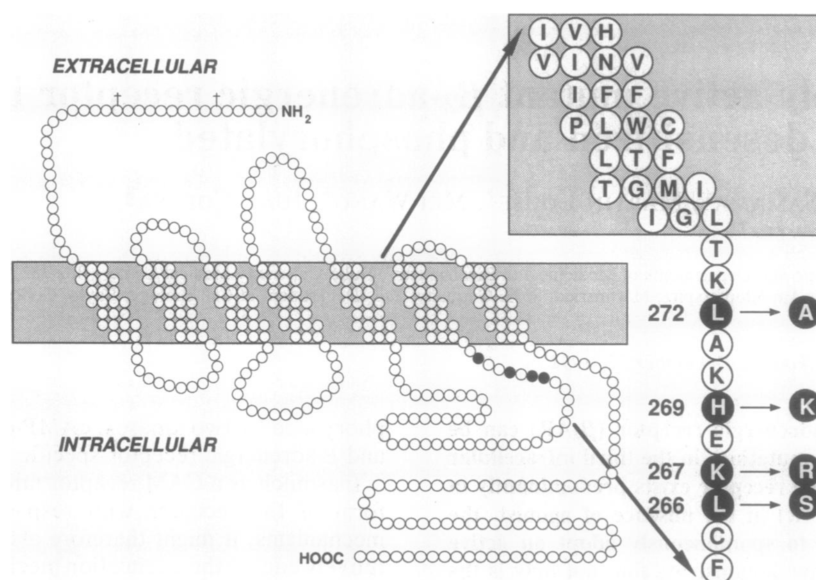

FIG. 1. Constitutively active mutant β_2AR. The C-terminal portion of the β_2AR was substituted with the homologous region of the α_{1B} subtype, generating the indicated point mutations (6). Standard one-letter code for amino acids was used.

cells with linearized viral DNA (Baculogold transfection kit; PharMingen) to produce recombinant baculovirus. Positive viral clones were isolated by plaque assay, and the receptor expression was measured by the binding of the βAR-specific ligand [^{125}I]iodocyanopindolol (NEN). WT and CAM β_2AR were prepared from Sf9 cells as described (13). Briefly, receptors were solubilized in 20 mM Tris·HCl, pH 7.5/1.5% digitonin/100 mM NaCl/2 mM EDTA, purified on an alprenolol-Sepharose column, reconstituted in phosphatidylcholine vesicles as described (14), and stored at −70°C.

Radioligand Binding Assay. Binding of [^{125}I]iodocyanopindolol to β_2AR on cell membranes was carried out as described (6). Binding assays on reconstituted receptors were performed at 30°C for 60 min in 20 mM Tris·HCl, pH 7.5/100 mM NaCl/0.05% digitonin. Bound and free radioligand were separated by G-50 gel-filtration columns. All binding data were subjected to nonlinear least-squares regression analysis (15).

GTPase Assay. Heterotrimeric G_s protein was purified as described (16). Purified β_2AR (100 fmol) and G_s protein (50 fmol) were co-reconstituted in phospholipid vesicles, and receptor-mediated GTPase activity was determined as described (14).

Receptor Phosphorylation. Recombinant βARK was purified from baculovirus-infected Sf9 cells as described (17). The reconstituted receptors were phosphorylated by βARK as described (18) in the absence or presence of 100 mM isoproterenol (Sigma). At appropriate time intervals, reactions were

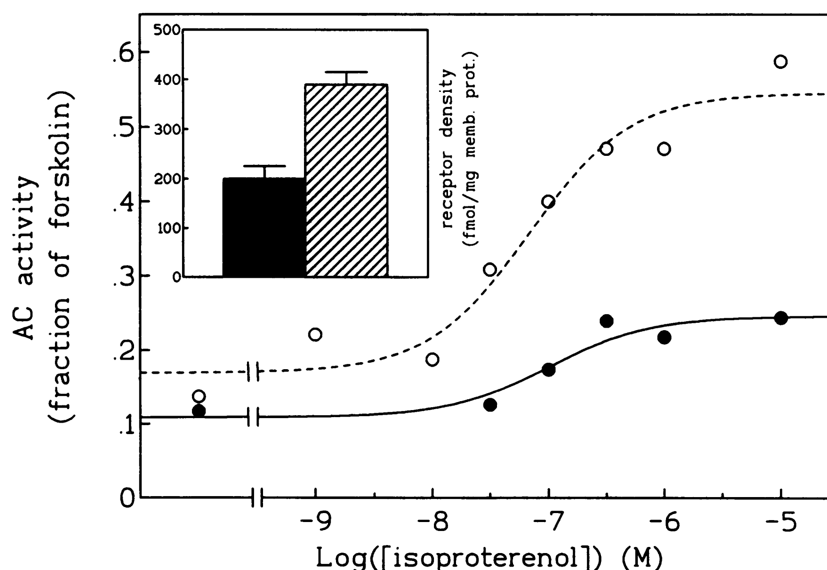

FIG. 2. Sensitization of CAM β_2AR-coupled AC by betaxolol. Cells harboring the CAM β_2AR at 200 fmol per mg of membrane protein were incubated for 17 hr in ITS (Collaborative Research) supplemented serum-free F12 medium (GIBCO) containing 10 μM betaxolol (dashed curve) or vehicle only (solid curve). Cell membranes were prepared as described (6) with the following modifications: lysis buffer (5 mM Tris·HCl, pH 7.5/2 mM EDTA) was supplemented with 10 μM betaxolol for cell lysis and the first rinse of the membrane fraction. Samples were then subjected to three more sedimentation–resuspension cycles in lysis buffer devoid of betaxolol and subsequently assayed for AC activation by isoproterenol. Data were normalized to the activity evoked by 100 μM forskolin (≈25 pmol per min per mg of membrane protein). Solid curve, EC_{50} = 100 nM; dashed curve, EC_{50} = 63 nM. Control and betaxolol-treated set of data were also compared to yield the increase of τ that gauges the enhancement of cyclase responsiveness (see *Materials and Methods*). In the experiment shown, $\tau_{sensitized}/\tau_{control}$ = 6.4. (*Inset*) Receptor densities corresponding to the curves shown. Shown is a representative of three similar experiments done in triplicate.

stopped with an equal volume of 2× SDS sample loading buffer and electrophoresed on SDS/10% polyacrylamide gels. Phosphorylation stoichiometries were determined either by excising and counting the receptor bands or by using a PhosphorImager (Molecular Dynamics) as described (19).

RESULTS AND DISCUSSION

To test the hypothesis that constitutively active receptors might also be constitutively desensitized, we sought means of reversing the constitutive activity of the β_2-CAM receptor (Fig. 1). Betaxolol (a gift from Synthelabo, Bagneux, France), a β_1-adrenergic antagonist, was found to repress this basal activity (20). Incubation of cells expressing the CAM receptor with betaxolol at the saturating concentration of 10 μM for 17 hr led to both up-regulation of the receptor (Fig. 2 *Inset*) and sensitization of βAR-coupled AC, as indicated by a simultaneous increase in basal and maximal activities (Fig. 2). No such effects were observed for the WT receptor exposed to similar treatment (data not shown).

We next examined the respective contributions of the increase in receptor density and the increase of receptor coupling efficiency to the increase of AC responsiveness. A numerical analysis was applied to the AC dose–response curves, yielding estimates for a parameter (τ) that gauges variations of receptor-mediated cyclase activity (see *Materials and Methods*). The betaxolol treatment resulted in a 6.2 ± 0.2-fold sensitization of AC, while the binding site density increased concomitantly by only 2.0 ± 0.2-fold (mean ± SEM; $n = 3$; Fig. 2). Thus, AC sensitization in the presence of the negative antagonist reflects an increase of receptor/AC coupling in addition to receptor up-regulation. Moreover, the treatment of cells with betaxolol, which increases the sensitivity of AC to an adrenergic agonist, had no effect on the sensitivity of AC to NaF and forskolin (direct activators of the G protein and the catalytic moiety of cyclase, respectively; data not shown). Thus, the CAM receptor in its native state is both tonically down-regulated and partially uncoupled from the G protein.

These findings suggest that the CAM β_2AR is able to tonically engage not only G_s protein but also various components of the cellular desensitization machinery. To test this hypothesis, we examined the role of a well-defined element of this machinery: βARK-mediated phosphorylation of the receptor (9). We first sought to obtain purified preparations of both WT and CAM receptors for the purpose of comparative studies.

The receptors were expressed in Sf9 cells by infection with recombinant baculoviruses. Expression levels for the mutant were ≈3 pmol per mg of membrane protein, as compared to 5–10 pmol per mg of membrane protein for the WT β_2AR. Both receptors were purified and reconstituted into phospholipid vesicles (see *Materials and Methods*). The reconstituted mutant β_2AR had a 20-fold higher affinity than the WT receptor for the agonist isoproterenol, whereas both receptors had identical affinities for the antagonist alprenolol (Fig. 3*A*), in keeping with our previous observations in membranes (6). Reconstitution of both receptors with purified G_s protein yielded agonist-stimulated GTPase activities (Fig. 3*B*). However, the agonist-independent GTPase-stimulating activity of the CAM β_2AR was comparable to the agonist-dependent activity of the WT receptor. This finding reproduces what we previously observed at the level of AC stimulation for receptors in membranes (6). Controls with receptors alone indicated the absence of contaminating GTPase activity in the receptor preparations (data not shown). Thus, the purified CAM β_2AR exhibits the two major properties of CAM receptors in membranes: (*i*) agonist-specific, G-protein-independent affinity increase (Fig. 3*A*) and (*ii*) elevated agonist-independent signaling activity (Fig. 3*B*). This indicates that purified preparations of the CAM β_2AR can be used to investigate the molecular basis of constitutive desensitization.

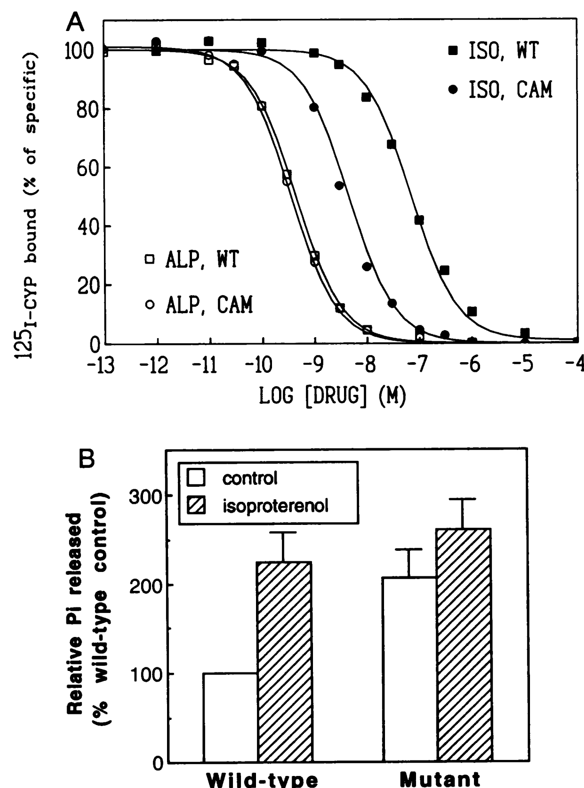

FIG. 3. Properties of purified CAM and WT β_2AR. (*A*) Ligand binding. [^{125}I]Iodocyanopindolol (CYP) binding to reconstituted WT or mutant β_2AR was determined as described. Results are representative of three independent experiments. Dissociation constants (K_i) of isoproterenol (ISO) binding were 6.5 ± 1.8 and 0.33 ± 0.11 nM, and those of alprenolol (ALP) binding were 49 ± 7.1 and 37 ± 3.2 pM, respectively, for WT or mutant β_2AR. (*B*) Activation of G_s protein. β_2AR (100 fmol) was reconstituted with 50 fmol of G_s protein as described (14) and preincubated on ice for 20 min in the absence or presence of 100 μM isoproterenol. Activation was conducted at 30°C for 20 min. Receptor-independent activity of the G protein, determined after reconstitution with heat-inactivated receptors, was subtracted from all measurements. The agonist-independent (basal) activity of the WT receptor was then taken as 100%. Results are means ± SE of three independent experiments. A typical experiment yielded the following results: receptor-independent activity, 0.10 mol of Pi per mol of G_s per min; basal WT, 0.16 mol of P_i per mol of G_s per min; isoproterenol-stimulated WT, 0.25 mol of Pi per mol of G_s per min; basal CAM, 0.24 mol of Pi per mol of G_s per min; isoproterenol-stimulated CAM, 0.26 mol of P_i per mol of G_s per min.

We proceeded to assess βARK-mediated phosphorylation of the purified receptors. As was previously observed (21), little if any phosphorylation of the WT receptor occurs in the absence of the agonist isoproterenol (Fig. 4*A*). However, the CAM receptor shows considerable phosphorylation under the same conditions (Fig. 4*A*). The rate and extent of the agonist-independent phosphorylation of the CAM β_2AR were comparable to the agonist-dependent phosphorylation of the WT β_2AR (Fig. 4*B*). The maximal phosphorylation of the CAM β_2AR in the presence of isoproterenol was about twice that of the WT β_2AR (Fig. 4*B*). However, in the presence of purified G-protein $\beta\gamma$ subunits, which greatly enhance β_2AR phosphorylation by βARK (17), both receptors were maximally phosphorylated to ≈8 mol of Pi per mol (data not shown). These results clearly demonstrate an agonist-independent, βARK-mediated phosphorylation of the CAM β_2AR. Such spontaneous susceptibility to the βARK could in

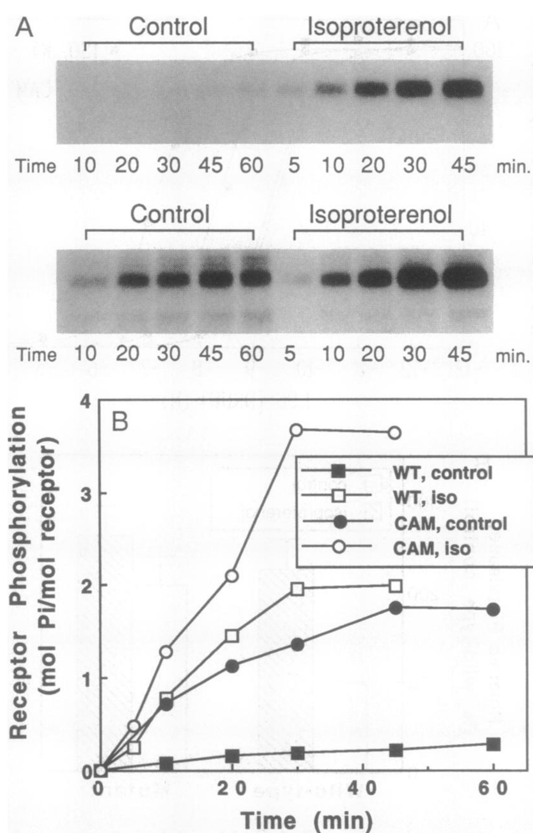

FIG. 4. Phosphorylation of β_2AR by βARK in the absence or presence of isoproterenol. (*A*) Reconstituted WT (*Upper*) or mutant (*Lower*) β_2AR (20 nM) was incubated at 30°C with βARK (30 nM) in 20 nM Tris·HCl, pH 8.0/2 mM EDTA/10 mM $MgCl_2$/1 mM dithiothreitol/100 mM ATP (≈2000 cpm/pmol) in the absence or presence of isoproterenol (iso). Stoichiometries of P_i incorporation in *A* were plotted against incubation time in *B*. Experiments were repeated three times with similar results.

part account for the tonic uncoupling of the CAM β_2AR from the G protein indicated by the whole-cell data (see above) and further demonstrates the equivalence of the unliganded CAM β_2AR and the agonist-occupied WT β_2AR with respect to recognition by βARK.

Recent reports have revealed that point mutations are sufficient to constitutively activate G-protein-coupled receptors, some of which are involved in human diseases (reviewed in ref. 22). We previously investigated the effects of these mutations in AR on the modulation of effector enzymes, such as AC and phospholipase C. However, another important aspect of cell signaling is the ability of receptors to mediate a loss of cellular responsiveness. In the present report, we show that a mutant β_2AR that constitutively activates the AC also engages cellular attenuation mechanisms in an agonist-independent fashion. These include down-regulation, receptor uncoupling, and βARK-mediated phosphorylation. Interestingly, this suggests that the activity of CAM receptors, both basal and agonist-stimulated, might have been previously underestimated (6).

The properties of these mutants have been rationalized within a theoretical model of receptor activation that involves an allosteric transition between two putative conformations of the receptor: one inactive (R), and the other (R*) capable of activating the G protein (6). Constitutively activating mutations were assumed to make the R to R* transition agonist independent. However, the molecular nature of the hypothetical active conformer R* has remained to be defined. We confirm here that the activation properties of the mutant receptor are conserved throughout purification and reconstitution procedures. Furthermore, we demonstrate that the ligand-free CAM β_2AR has molecular properties close to those of an agonist-occupied WT receptor. One such property is the susceptibility to βARK-mediated phosphorylation (19). The CAM receptor indeed undergoes βARK-mediated phosphorylation even in the absence of agonist. The finding that the agonist-independent phosphorylation of the CAM receptor closely matches the agonist-induced phosphorylation of the WT receptor parallels our observation of the corresponding G_s-activating (this work) and AC-stimulating (6) activities. In addition, we have previously shown that when transfected in COS-7 cells, various CAM α_2ARs are subject to βARK-mediated, agonist-independent phosphorylation in proportion to their constitutive activity (7). Taken together, these findings suggest that constitutive activation and susceptibility to βARK-mediated phosphorylation have the same molecular basis. Thus, the results with the purified CAM β_2AR strengthen the idea that the postulated R* conformer is equivalent to the βARK substrate.

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