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ACCELERATED COMMUNICATION

Separation of the Structural Requirements for Agonist-Promoted Activation and Sequestration of the β-Adrenergic Receptor

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SUMMARY

The deletion of residues 222–229 from the hamster β_2-adrenergic receptor (βAR) resulted in an inability of the mutant receptor to couple to the guanine nucleotide-binding protein (G protein) G_s and to undergo the agonist-mediated sequestration response that is associated with desensitization [*Mol. Pharmacol.* **34**:132–138 (1989)]. Replacement of this region of the βAR with the analogous region of the M_1-muscarinic acetylcholine receptor restored the sequestration response but not the G protein activation. These data suggest that there is a structural, rather than a functional, relationship between these two processes and demonstrate that G protein coupling is not a prerequisite for receptor sequestration.

Many hormones and neurotransmitters mediate their intracellular effects through signal transduction pathways that are coupled via G proteins to various effector systems. The cloning of several of these G protein-coupled receptors has revealed them to constitute a family of structurally related proteins consisting of seven conserved stretches of 20–25 hydrophobic amino acid residues connecting eight more divergent, hydrophilic loops (1–7). By analogy with the model that has been proposed for rhodopsin (8), the seven hydrophobic domains of these receptors are postulated to form transmembrane α-helices, with the hydrophilic loop regions exposed alternatingly extracellularly and cytoplasmically. Currently, genetic and biochemical determinations of the structure-function relationships of G protein-coupled receptors are being used to develop an understanding of the mechanisms of transmembrane signaling by these proteins.

Mutagenesis studies of the βAR, which couples to the G protein G_s to activate adenylyl cyclase, suggest that the ligand binding domain of the receptor involves amino acid residues within the hydrophobic core of the protein (9, 10), similar to the binding of retinal to opsin. In contrast, G protein activation requires residues within the intracellular hydrophilic domains of the receptor (9, 11–13). Deletion mutagenesis of the βAR has implicated the regions at the N- and C-termini of the third intracellular loop of the receptor in its coupling to G_s. This assignment agrees with the results of proteolysis studies on rhodopsin, which also argue for a role for the third intracellular loop in the interaction with transducin (8). Furthermore, molecular replacement of the third intracellular loop of the M_1AR with the analogous region of the M_2AR suggested that this region of the MAR is critical for determining the specificity of G protein coupling (14).

Prolonged exposure of cells to agonists results in a desensitization of the activation response, characterized by several interrelated processes. During desensitization, the βAR undergoes rapid uncoupling from the G_s-adenylyl cyclase system, sequestration away from the cell surface, and phosphorylation on Ser and Thr residues (15). The individual contributions of these various processes to the functional inactivation of the receptor during desensitization have not been fully defined. Exposure to normal physiological levels of agonist results in a rapid uncoupling of the receptor from G_s in response to cAMP-dependent protein kinase (16). The site of action of the kinase has been identified as Ser^{262} in the C-terminal region of the third intracellular loop of the βAR, because deletion of this Ser residue prevents this rapid cAMP-dependent protein kinase-mediated form of desensitization (17). Exposure to higher concentrations of agonists causes a second form of desensitization, associated with sequestration of the βAR away from the cell surface and with phosphorylation of the βAR by the BAR-

ABBREVIATIONS: G protein, guanine nucleotide-binding regulatory protein; βAR, β_2-adrenergic receptor; βARK, β-adrenergic receptor-specific kinase; M_1AR, M_1-muscarinic acetylcholine receptor; M_2AR, M_2-muscarinic acetylcholine receptor; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate; PBS, phosphate-buffered saline.

specific kinase (βARK) (18). Phosphorylation by βARK is greatly attenuated by deletion of the C-terminal tail of the receptor or by replacement of several Ser residues within this domain with Ala residues (19, 20). Deletion of this Ser-rich C-terminal region also delays the onset of the uncoupling and sequestration processes, suggesting a relationship between βARK-mediated phosphorylation and the rapid initial phase of desensitization (13, 19, 20).

We have recently observed that mutant βARs that are unable to couple to G_s and activate adenylyl cyclase also fail to undergo the agonist-mediated sequestration response that is associated with desensitization (13, 21). This correlation between G protein coupling and receptor sequestration might reflect a functional relationship between these two processes, such that G protein activation is a prerequisite for the subsequent inactivation that accompanies sequestration. Alternatively, the activation and inactivation processes could require similar structural features of the receptor without sharing a common mechanistic basis. In the present study, we have replaced a region of the β_2AR that is critical for G protein coupling with the analogous region of the M_1AR. The resulting hybrid receptor is unable to stimulate G proteins, although it undergoes normal agonist-mediated receptor sequestration, demonstrating that these two processes are structurally, rather than functionally, related.

Experimental Procedures

Mutagenesis and expression. The nucleotide sequence of the hamster βAR and the human M_1AR have been published (2, 4). Expression of the βAR gene in mouse L cells and oligonucleotide-directed mutagenesis were performed as previously described (9). The identities of the mutations were confirmed by dideoxy sequencing of the mutant plasmids. Purity was determined by retransformation of each mutant into *Escherichia coli* and hybridization at high stringency with the oligonucleotide used to create the mutation. All mutant receptors used were >99% pure (i.e., 100 of 100 colonies examined contained the mutation). Cell were grown in Dulbecco's modified Eagle's medium in an atmosphere of 5% CO_2 at 37°.

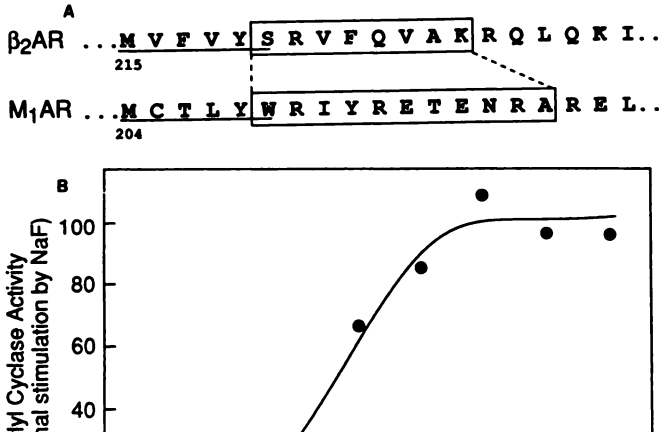

Fig. 1. A, Construction of M_1[220–230]βAR. The sequence of the βAR indicated with the *box* (residues 220–227) was deleted and replaced with residues 209–219 (shown in the *box*) from the M_1AR to make the hybrid M_1[220–230]βAR, as described in Experimental Procedures. For both receptors, the *underlined* regions represent residues within the fifth transmembrane helix. B, Adenylyl cyclase stimulation by wild-type and mutant βAR. Adenylyl cyclase stimulation was measured in the presence of increasing concentrations of isoproterenol, as described in Experimental Procedures, and is expressed as a percentage of the maximal stimulation by 10 mM NaF. For the experiment shown, the adenylyl cyclase activity for the various receptors expressed as basal/10^{-5} M isoproterenol-stimulated/10 mM NaF-stimulated, in pmol of [^{32}P]cAMP/mg of protein/min, was as follows: wild-type βAR, 6/39/37 (●); D(222–229)βAR, 9/9/102 (□); and M_1[220–230]βAR, 22/21/108 (○). The data shown are representative of two to six separate experiments.

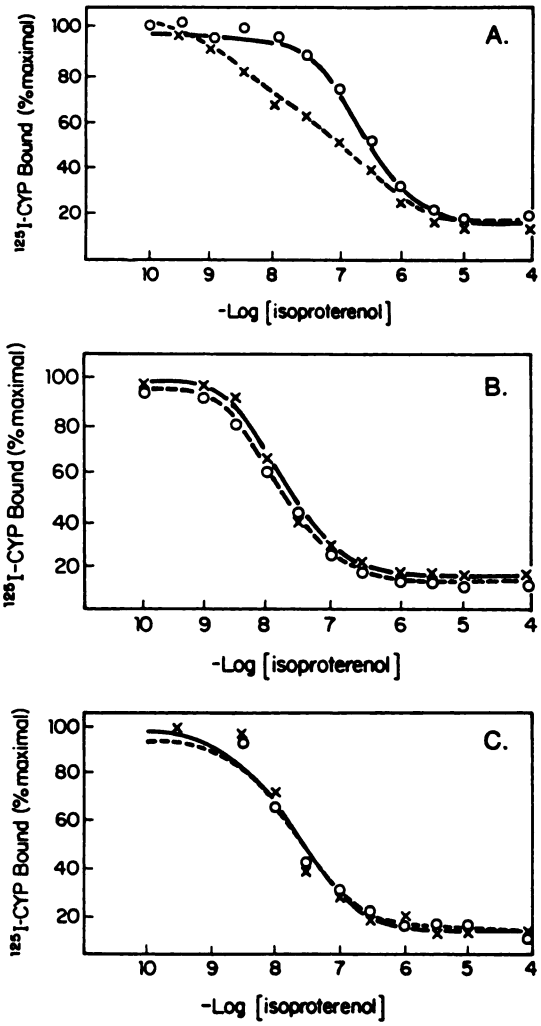

Fig. 2. Agonist competition binding to wild-type and mutant βAR. Isoproterenol binding to membrane preparations from L cells was performed in competition with [^{125}I]iodocyanopindolol, as described in Experimental Procedures. Data were analyzed using the LIGAND program of Munson and Rodbard (24). Data shown are means of duplicate determinations, with each curve representative of two or three separate experiments. The symbols represent competition of [^{125}I]iodocyanopindolol binding to membranes from wild-type βAR (A), D(222–229)βAR (B), and M_1[220–230]βAR, (C), by isoproterenol alone (×) and isoproterenol in the presence of 100 μM Gpp(NH)p (○). B_{max} values determined for [^{125}I]iodocyanopindolol binding for the wild-type βAR, D(222–229)βAR, and M_1[220–230]βAR were 100, 150, and 67 fmol/mg, respectively.

Membrane preparation and assays. Cell membranes were prepared by hypotonic lysis of cell monolayers with 1 mM Tris, pH 7.5, as previously described (9). Membranes were resuspended at a protein concentration of 1–2 mg/ml in TME buffer (75 mM Tris, pH 7.5, 12.5 mM $MgCl_2$, 1.5 mM EDTA). Adenylyl cyclase assays were performed as previously described (9, 22). Isoproterenol competition binding to membranes was performed in a volume of 0.25 ml of TME buffer containing 5–7 pM βAR, 35 pM [^{125}I]iodocyanopindolol, and (−)-isopro-

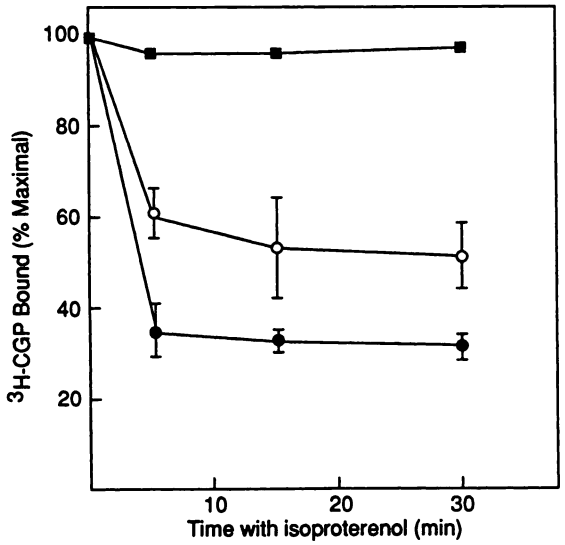

Fig. 3. Sequestration of cell surface wild-type and mutant βAR. Monolayers of L cells expressing wild-type βAR (●), D(222–229)βAR (■), or M_1[220–230]βAR (○) were incubated at 37° in the presence of 10 μM isoproterenol for the times indicated and washed with PBS, and [^{3}H]CGP 12177 binding to the cell surface receptors was determined at 4°, as described in Experimental Procedures. The B_{max} values determined for ^{3}H-CGP12177 binding to the wild-type βAR, D(222–229)βAR, and M_1[220–230]βAR were 84, 81, and 55 fmol/mg, respectively. The data shown are the means of three to five separate experiments, with each determination done in duplicate.

terenol at the concentrations described in the legend to Fig. 2. At the end of the incubation period, the membranes were collected on GF/C glass fiber filters, and the bound radioactivity was determined using a γ counter. The data were analyzed using the iterative program LIGAND (23).

Receptor sequestration assay. L cells grown in monolayer culture in 24-well plates were incubated in their growth medium with 10^{-5} M (−)-isoproterenol. At the end of the incubation time, the plates were transferred to an ice bath to stop the reaction and washed three times with ice-cold PBS. The binding of [^{3}H]CGP 12177 (Amersham, Arlington Heights, IL) was measured directly on the monolayers with 6 nM [^{3}H]CGP 12177, in a total volume of 0.25 ml of PBS containing 0.1% bovine serum albumin, for 4 hr at 4°. The monolayers were then washed three times with cold PBS and removed from the plates in 1% sodium dodecyl sulfate, and the radioactivity bound was measured in a liquid scintillation counter. Nonspecific binding was determined in the presence of 10^{-5} M alprenolol.

Results

As previously demonstrated, deletion of amino acid residues 222–229 from the hamster βAR results in a mutant receptor [D(222–229)βAR] that, when expressed in mouse L cells, fails to mediate adenylyl cyclase activation by the agonist isoproterenol (Fig. 1B). Replacement of residues 220–230 of the hamster βAR with the analogous residues from the human M_1AR, forming M_1[220–230]βAR (as shown in Fig. 1A), does not restore the ability of the receptor to stimulate adenylyl cyclase activity (Fig. 1B), even at high concentrations of agonist. This mutant receptor also fails to activate adenylyl cyclase when expressed in Chinese hamster ovary (data not shown). Because the M_1AR normally couples to the G protein G_p to activate phospholipase C, the ability of M_1[220–230]βAR to activate phospholipase C was also determined. Whereas the wild-type M_1AR expressed in these cells causes a 10–15-fold stimulation of inositol phosphate formation in response to the muscarinic agonist carbachol, M_1[220–230]βAR does not activate phospholipase C in response to either adrenergic or cholinergic agonists (data not shown). Both D(222–229)βAR and M_1[220–230]βAR bind agonists and antagonists normally (Ref. 13, Fig. 2, and data not shown).

In order to directly assess the coupling of the mutant receptors to G proteins, the sensitivity of agonist binding to the nonhydrolyzable GTP analog Gpp(NH)p was determined. As shown in Fig. 2A, the wild-type βAR binds the agonist isoproterenol with two classes of affinity sites, with the high affinity state ($K_d = 1 \times 10^{-9}$ M) being characteristic of a receptor-G protein complex and the low affinity state ($K_d = 1 \times 10^{-7}$ M) representing the uncoupled form of the receptor. The addition of Gpp(NH)p results in the conversion of all of the receptor into the low affinity uncoupled state. As previously reported (13), D(222–229)βAR, which does not activate adenylyl cyclase, binds isoproterenol with a single class of sites with intermediate affinity ($K_d = 1 \times 10^{-8}$ M), which are insensitive to Gpp(NH)p (Fig. 2B). The occurrence of a single class of GTP-insensitive binding sites with intermediate affinity for agonists has been observed to be characteristic of mutant βARs that do not interact with G_s and has been interpreted in terms of a stable conformation of the uncoupled receptor-ligand complex (11). As shown in Fig. 2C, M_1[220–230]βAR also binds the agonist isoproterenol with a single class of intermediate affinity sites ($K_d = 1 \times 10^{-8}$ M). The affinity of the receptor for the agonist is not affected by the addition of Gpp(NH) (Fig. 2C), indicating an absence of G protein coupling to this mutant receptor.

The ability of mutant βARs to undergo agonist-promoted sequestration away from the cell surface was measured after treatment with isoproterenol, using the hydrophilic antagonist [^{3}H]CGP 12177, which does not penetrate the intact cell. As previously demonstrated (13), the wild-type βAR responds to exposure to isoproterenol with a rapid sequestration of approximately 70% of the cell surface receptors ($t_{1/2} < 5$ min), whereas D(222–229)βAR is not sequestered at times of up to 1 hr (Fig. 3). In contrast, M_1[220–230]βAR undergoes rapid sequestration in response to agonist exposure. The initial rate of sequestration of this mutant receptor appeared normal ($t_{1/2} < 5$ min), with a maximal extent of sequestration of approximately 50% (Fig. 3). The EC_{50} for the isoproterenol-mediated sequestration response of the mutant receptor (3×10^{-8} M) is slightly lower than that of the wild-type βAR (2×10^{-7} M), probably reflecting the increased affinity of isoproterenol for the mutant receptor, as described above. That the loss of [^{3}H]CGP 12177 binding sites upon exposure to isoproterenol corresponds to sequestration of cell surface receptors rather than to the irreversible binding of the agonist to the receptor was shown by performing the isoproterenol incubation at 4°, a temperature that is permissive for ligand binding but not for receptor sequestration. In that experiment, no significant loss of [^{3}H]CGP 12177 binding was observed after a 30-min incubation with 10^{-5} M isoproterenol.

Discussion

We have previously demonstrated that deletion of amino acid residues 222–229 from the third intracellular loop of the hamster βAR results in a complete loss of the ability of the receptor to couple to G_s and stimulate adenylyl cyclase (11). In addition, it was observed that mutant βARs that fail to couple to G_s also fail to undergo agonist-promoted sequestration upon exposure

to high concentrations of isoproterenol (13, 21). We suggested that either this correlation could signify a functional relationship between receptor activation and inactivation, such that activation is a prerequisite for the sequestration process, or these processes could be functionally independent but both involve amino acid residues within the same region of the receptor protein (13).

In the present study, we have replaced this region of the βAR with the analogous sequence from the M_1AR, a receptor that activates the G_p-phospholipase C pathway. M_1[220–230]βAR binds agonists and antagonists with normal affinity, indicating that the tertiary structure of the receptor is not grossly perturbed by this molecular replacement. However, in this hybrid receptor, agonist binding does not result in receptor activation. Like the deletion mutant, this chimeric receptor shows no detectable coupling to G proteins, as assessed by the insensitivity of agonist affinity to GTP analogs and by the failure of the mutant receptor to activate either adenylyl cyclase or phospholipase C. In contrast, this hybrid receptor is sequestered normally in response to isoproterenol, whereas the deletion mutant is not. Thus, the region at the N-terminus of the third intracellular loop of the M_1AR can substitute for the analogous region of the βAR in mediating receptor sequestration but not in stimulating coupling to G proteins.

This dissociation of agonist-promoted G_s coupling from agonist-mediated receptor sequestration in M_1[220–230]βAR argues against a requirement for functional coupling of the receptor to a G protein in signaling of the sequestration process. In agreement with this observation, the βAR in the cyc^- variant of S49 lymphoma cells, which lack active G_s, shows an intact sequestration response to high concentrations of isoproterenol (24). In addition, the kin^- variant of S49 cells, lacking the catalytic subunit of cAMP-dependent protein kinase, can also support this form of receptor desensitization, suggesting that the functional signal transduction pathway does not need to be intact for sequestration to occur (24).

The nature of the structural requirements for βAR signaling of the sequestration response is not known. The defective sequestration response observed upon deletion of amino acid residues 222–229 from the βAR, like the defective G_s coupling of this mutant βAR, is specific for the removal of those particular residues from the third intracellular loop, because three other deletions of similar length in this region [D(250–259)βAR, D(238–251)βAR, and D(229–236)βAR] showed normal G protein coupling (13) and normal sequestration responses to isoproterenol (data not shown). The ability to delete sequences in the middle portion of the third intracellular loop without affecting G_s coupling or receptor sequestration demonstrates that these regions cannot be directly involved in the sequestration or coupling responses. Likewise, the majority of the C-terminal tail of the βAR could be deleted without impairing the level of receptor sequestration (21). However, other regions of the receptor besides residues 220–230, which are implicated here, including residues outside the third intracellular loop and the C-terminal tail of the βAR, may also be involved in receptor sequestration.

One explanation that is consistent with the pleiotropic effects of the deletion mutation would be that the region of the βAR containing residues 222–229 interacts directly with both G_s and an arrestin-like protein involved in desensitization. In the visual transduction system, arrestin potentiates the uncoupling of phosphorylated rhodopsin from transducin by a mechanism that is thought to involve competitive inhibition of the rhodopsin-transducin interaction (25). High concentrations of arrestin are also capable of stimulating the uncoupling of βARK-phosphorylated βAR from G_s, suggesting the existence of an arrestin homolog in the βAR-G_s system (26). Sequence homology between the C-terminal regions of arrestin and transducin has been noted, suggesting that these regions may bind to the same site on the rhodopsin molecule (27, 28). A similar homology between a βAR-specific arrestin homolog and G_s might, therefore, exist, suggesting that there might be overlapping binding sites for G_s and this postulated arrestin homolog on the βAR. The results of the present study would be consistent with a role for the residues at the N-terminus of the third intracellular loop of the βAR in forming a binding site for both G_s and an arrestin homolog. Inasmuch as the substitution of the analogous region from the M_1AR into this region of the βAR reinstates the normal sequestration response of the receptor but does not restore G protein coupling, structural requirements for binding to G_s may be more stringent or more specific than those for receptor sequestration. Determination of the molecular basis for the independent involvement of this region of the receptor in G protein coupling and in receptor sequestration awaits further biochemical characterization of these signaling pathways.

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