Phosphorylation Sites on Two Domains of the β2-Adrenergic Receptor Are Involved in Distinct Pathways of Receptor Desensitization*

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Continuous exposure of cells to neurotransmitter or hormone agonists often results in a rapid desensitization of the cellular response. For example, pretreatment of Chinese hamster fibroblasts (CHW cells) expressing β2-adrenergic receptors (β2AR) with low (nanomolar) concentrations of isoproterenol, a β-adrenergic agonist, causes decreases in the sensitivity of the cellular adenylyl cyclase response to the agonist, without changing the maximal responsiveness. In contrast, exposure of CHW cells to high (micromolar) concentrations of isoproterenol results in decreases in both sensitivity and the maximal responsiveness to agonist. To explore the role(s) of receptor phosphorylation in these processes, we expressed in CHW cells three mutants BzAR genes encoding receptors lacking putative phosphorylation sites for the cAMP-dependent protein kinase A and/or the cAMP-independent β2AR kinase. Using these mutants we found that exposure of cells to low concentrations of agonist appears to preferentially induce phosphorylation at protein kinase A sites. This phosphorylation correlates with the decreased sensitivity to agonist stimulation of the adenylyl cyclase response. At higher agonist concentrations phosphorylation on both the β2AR kinase and protein kinase A sites occurs, and only then is the maximal cyclase responsiveness elicited by agonist reduced. We conclude that low or high concentrations of agonist elicit phosphorylation of β2AR on distinct domains, with different implications for the functional coupling of the receptors with effector molecules.

Desensitization is a ubiquitous phenomenon whereby continuous exposure of cells to a stimulus results in a waning of the cellular response to that stimulus and, under some circumstances, to other stimuli as well. This process has been demonstrated in several neurotransmitter receptor systems (1, 2), but the molecular basis for desensitization in most of these systems is not well understood. One receptor for which this phenomenon has been extensively characterized is the β2-adrenergic receptor (β2AR).1 β2AR is a member of the large family of plasma membrane receptors whose actions are mediated via guanine nucleotide-binding regulatory proteins (G proteins). Exposure of cells to β-adrenergic agonists causes rapid activation of the stimulatory G protein Gs and thus adenylyl cyclase, with consequent rises in cellular cAMP levels. However, continuous exposure of cells to these agonists results, within minutes, in an attenuation of the enzymatic response.

What causes this functional desensitization? At least three agonist-triggered alterations in the properties of β2AR potentially play a role. First, prolonged treatment of cells with agonists results in a decrease in ligand binding to those cells. This process is termed "down-regulation" and reflects the internalization of receptors away from the plasma membrane. However, it occurs much too slowly (minutes to hours) to account for the rapid loss of the adenylyl cyclase response in most studies of β2AR regulation (3–7).2 Second, agonists trigger a faster (minutes) translocation or "sequestration" of β2AR into a cellular compartment distinct from the plasma membrane but where the receptors are still detectable by ligand binding. However, some experimental conditions which block β2AR sequestration do not prevent the rapid loss of the enzymatic response (5, 8–11). In addition, desensitization of the cyclase response has been detected prior to the onset of sequestration (5).

Finally, agonists also induce a rapid (minutes) modification(s) of β2AR such that even though the receptors remain located in the plasma membrane they are nonetheless functionally uncoupled from Gs (6, 13, 14). Two lines of evidence suggest that this modification is probably phosphorylation. First, phosphorylation of receptors in intact cells occurs rapidly and stoichiometrically when the cells are exposed to desensitizing concentrations of agonists (15–17). In addition, purified β2AR can be phosphorylated in vitro by either the cAMP-dependent protein kinase or by the novel β2AR kinase. Importantly, in both cases the phosphorylated β2AR exhibit impaired abilities to stimulate GTPase activity when reconstituted with Gs (18–20). In the case of β2AR kinase, the presence of additional cytosolic factors in the reconstitution assay is required for the full impairment to be seen (20).

Thus, both in vitro and in vivo (21) evidence suggests that receptor phosphorylation by at least two kinases is involved in desensitization. Examination of the proposed structure of β2AR, made possible by the recent cloning and sequencing of the genes encoding the hamster (22) and human (23) β2AR, reveals several potential phosphorylation sites. The sequence Arg-Arg-Ser-Ser appears twice in the receptor, representing consensus sequences for phosphorylation by protein kinase A

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1The abbreviations used are: β2AR, β2-adrenergic receptor; Gpp(NH)p, guanyl-5'-yl imidodiphosphate; DMEM, Dulbecco's modified Eagle's medium; CHW, Chinese hamster fibroblasts; PBS, phosphate-buffered saline; Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate.

2One exception may be the L cell system in which Cheung et al. (12) report that 40% of β2AR are down regulated following only a 5-min exposure to agonist.
Sequestration was defined as the percentage of radioligand binding not displaced by CGP 12177. Exposure of wild type or mutant cells for up to 15 min to 2 μM isoproterenol resulted in a loss in [3H]iodopindolol binding, or down-regulation, of less than 50%. Membrane Preparation—Following exposure to agonist, cells were treated as described in the binding assay, then scraped into 10 ml of 5 mM Tris, pH 7.4, 2 mM EDTA and lysed with a Polytron homogenizer (4 bursts for 5 s at maximum setting). The lysate was first centrifuged at 200 × g to remove nuclei and unbroken cells, and then the supernatant was centrifuged at 40,000 × g for 20 min at 4 °C and the pellet washed once in the same buffer. 

The pelleted membranes were resuspended in 0.75 ml of 75 mM Tris, pH 7.4, 2 mM EDTA, and either 12 mM MgCl2 (Fig. 1) or 5 mM MgCl2 (Figs. 4 and 6) and assayed immediately. Both concentrations of magnesium ion gave qualitatively identical results with regard to the altered patterns of desensitization seen with the mutants.

ADENYLYL CYCLASE ASSAY—Adenyl cyclase activities of membrane preparations from untreated and desensitized cells were measured by the method of Solomon et al. (29), as modified (30). Stimulation of adenylyl cyclase by isoproterenol in all four cell lines examined in this study was essentially identical. Basal and maximally stimulated cyclase activities ranged from 3.4 to 5.4 and 19 to 24 pmol of cAMP/min/mg, respectively, and EC50 values for isoproterenol ranged from 77 to 92 nM. Treatment of the data was as follows. In every experiment the maximal adenyl cyclase response to agonist in membranes from untreated cells was first determined by computer fit (see "Statistical Analysis") and the basal cyclase values subtracted. The resulting data were set equal to 100% for each cell type and experiment, and all other adenyl cyclase activities for membranes from the same cell type in that experiment were then expressed as a percentage of this value. The corresponding values from several experiments were then averaged. In some experiments (those depicted in Figs. 5 and 8), the cyclase responses were first expressed as percentages of the stimulation seen in the same membrane preparation with 100 μM forskolin and then treated as described above. Expressing these values as percentages of forskolin-stimulated activity does not affect the pattern or magnitude of the desensitization seen (compare Fig. 1A with C). All other results are expressed as percentages of the untreated cells.

Whole Cell Phosphorylation Experiments—These were performed essentially as described previously (30). Briefly, cells were detached from their flasks by collagenase treatment, washed twice with phosphate-free DMEM, and incubated for 90 min in 40 ml of DMEM containing approximately 3 mM of carrier-free [32P]Pi to allow [32P] incorporation into the cells. Cells were then treated with 0.1 mM acetic acid and either 10 nM or 2 μM (-)-isoproterenol for ~10 min in DMEM, after which the membranes were prepared as described previously (31). The lysates were then centrifuged at 200,000 × g, the cells were washed twice with ice-cold PBS, and then disrupted by sonication (3 bursts of 15 s each) in ice-cold 20 mM Tris-HCl, pH 7.4, 5 mM EDTA, and 10 mM Na2PO4 containing a mixture of benzamidine, leupeptin, soybean trypsin inhibitor, and pepstatin (all from Sigma). The lysates were centrifuged at 40,000 × g, washed once in the same buffer, and the resulting membranes solubilized in 100 mM NaCl, 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, and 2% digitonin. The βAR was purified by alprenolol-Sepharose affinity chromatography as described (30) and examined by gel electrophoresis and autoradiography. Laser densitometric scanning of the autoradiograms was used to assess the incorporation of [32P] into βAR.

Photoaffinity Labeling—Membranes were incubated in the dark with [125I]idoxopindolol diazirane (250 pm) with or without 10 μM alprenolol in PBS containing 5 mM EDTA for 3 h at 25°C. Membranes were then washed twice with ice-cold PBS/EDTA containing 0.5% bovine serum albumin and, once without bovine serum albumin, and resuspended in 1 ml of the same buffer. Each sample was layered in a well of a 6-well culture dish on ice and UV-irradiated with a Hanovia 450 medium pressure mercury lamp filtered with 5 mm of Pyrex glass for 5 min. The samples were then washed with 90 ml of ice-cold PBS and the membranes solubilized in 0.5% sodium deoxycholate in 10 mM Tris-EDTA, 0.1% SDS, and 0.1% polyacrylamide gel electrophoresis sample buffer.

SDS-Polyacrylamide Gel Electrophoresis—Gel electrophoresis was performed with 10% slab gels as described (31). After electrophoresis, the gels were dried and exposed at ~90°C with Cronex video imaging film (Dupont, Boston, Mass.), with an intensifying screen.

Statistical Analysis—Assessment of the statistical significance of differences between groups was measured using the Newman-Keuls multiple range test. In general, a p value less than 0.05 was used as
the criterion of significance, and for simplicity all significant p values throughout the text are expressed as p < 0.05. Adenyl cyclase curves were compared as described previously (32). Each curve was formed by averaging individual experiments, and the mean curves were fitted independently by a computer algorithm to a four-parameter logistic equation and parameters (minimum, maximum, EC50, and slope factors) determined. Then the curves were analyzed simultaneously with parameters constrained to a common value, and significant alterations in the goodness of fit (p < 0.05) were used to determine if the parameters (and thus the curves) were indistinguishable.

RESULTS

Agonist Concentration Dependence of Patterns of Desensitization—We first examined the abilities of different concentrations of the β-adrenergic agonist isoproterenol to elicit desensitization of the adenyl cyclase response in CHW cells expressing the transfected wild type human β2AR gene. As shown in Fig. 1A, pretreatment of cells for 15 min with a high concentration of agonist (2 μM) alters both the sensitivity to agonist (manifested as a rightward shift in the concentration-response curves) and the maximum response to subsequent stimulation by the agonist. In contrast, preexposure of the cells to relatively low concentrations of agonist (0.2 and 20 nM) decreases only the sensitivity of the response to agonist without diminishing the maximal response. In fact, even a 60-min preincubation of the cells with a low concentration of agonist (10 nM) failed to attenuate the maximal enzyme responsiveness (data not shown). Importantly, however, the decrease in sensitivity which is seen with 20 nM isoproterenol is comparable to that seen with 2 μM isoproterenol. These results are reflected in the changes in the EC50 values for isoproterenol for the cyclase dose-response curves (control: 129 ± 9 nM; exposed to 0.2 nM isoproterenol: 163 ± 27 nM; exposed to 20 nM isoproterenol: 267 ± 51 nM; exposed to 2 μM isoproterenol: 221 ± 21 nM (n = 4–10)). The EC50 values for 20 nM and 2 μM isoproterenol are both significantly different (p < 0.05) from the control value.

Potential Role of Sequestration—The finding that low and high concentrations of agonist have more pronounced effects on the sensitivity and the maximal enzyme responsiveness, respectively, suggested the possibility that distinct molecular mechanisms are responsible for each aspect of desensitization. To assess the possible contribution of one potential mechanism, receptor sequestration, to the changes in the enzyme response seen with agonist, we determined the amount of sequestration of β2AR that takes place upon exposure of the cells to low and high concentrations of agonist. As shown in Fig. 1B, little agonist-induced sequestration occurs upon exposure of cells to the low concentration of agonist. Therefore, receptor sequestration cannot be responsible for the changes in sensitivity of the adenyl cyclase response which occur under these conditions. On the other hand, exposure of cells to the high concentration of agonist results in sequestration of up to 25% of the receptors. Thus, we cannot exclude the possibility that sequestration is involved in the functional desensitization seen following cellular exposure to the high concentration of agonist.

Mutant β-Adrenergic Receptors—Mutations involving large deletions or truncations of the β2AR gene can yield receptors whose abilities to transmit agonist-induced signals are apparently nonspecifically altered (12, 33); this can complicate biochemical analyses of receptor function. We therefore designed substitution mutants of potential phosphorylation sites of the human β2AR gene (Fig. 2) in an attempt to minimize nonspecific conformational effects. One mutated receptor was created by replacing the serines within the consensus sequences for protein kinase A phosphorylation with alanines (mutant A); a second involved substitution of the 11 serine

![Fig. 1. Effects of various concentrations of isoproterenol (ISO) on desensitization and sequestration of β2AR. A. desensitization: CHW cells expressing the wild-type human β2AR were pretreated with only 0.1 mM ascorbic acid (●) or with ascorbic acid and 0.2 mM (○), 20 mM (▲), or 2 μM isoproterenol (□) for 10 min. Particulate fractions were then prepared and the adenyl cyclase activities measured in response to various concentrations of isoproterenol. In these experiments the basal adenyl cyclase activity for control cells was 5.8 ± 0.7 pmol/min/mg, and the maximal cyclase response 30.2 ± 3.0 pmol/min/mg (n = 10). For each experiment the maximal isoproterenol response minus the basal response of untreated cells was set equal to 100%, and all other cyclase activities in that experiment normalized to that. Adenyl cyclase activities are presented as the mean of 4–10 experiments, depending on condition. B, sequestration: wild type cells (○), mutant A cells (▲), mutant B cells (●), and mutant AB cells (●), were pretreated with 0.1 mM ascorbic acid and either 10 nM or 2 μM isoproterenol (ISO) for the indicated period of time. Cells were then washed extensively and sequestration measured as described under “Experimental Procedures.”]
and threonine residues in the carboxyl-terminal segment (putative substrates for \( \beta_2 \)AR kinase) by alanine or glycine residues (mutant B); and the third mutant comprised both sets of mutations (mutant AB). These mutant genes were then transfected into mammalian CHW cells. Cell lines expressing tentative substrates for P2AR kinase) by alanine or glycine residues (putative phosphorylation sites) were selected for further study.

In initial experiments we determined that the mutant receptors do not differ in their intrinsic abilities to bind agonist (data not shown) or to mediate stimulation of adenyl cyclase (see "Experimental Procedures"). These results, and the demonstration that all four receptors show identical kinetics and extent of agonist-induced sequestration (Fig. 1B), strongly suggest that the alteration of the putative phosphorylation sites does not alter the ability of the receptor to undergo agonist-induced conformational changes.

**Receptor Phosphorylation Induced by a Low Concentration of Agonist**—We first utilized photoaffinity labeling techniques to determine the mobility on SDS-polyacrylamide gels of \( \beta_2 \)AR expressed in these cells. Particulate fractions were prepared from wild type, mutant A, mutant B, and mutant AB cells and covalently labeled with the photoaffinity ligand \[^3^2\]P-cyanopindolol diazirine, as described under "Experimental Procedures," in the presence (+) or absence (−) of 10 \( \mu \)M alprenolol (Alp) to define specific labeling. First and second, third and fourth, fifth and sixth, seventh and eighth lanes show labeled membranes prepared from wild type, mutant B, mutant A, and mutant AB cells, respectively. Band, whole cell phosphorylation. Wild type and mutant cells were incubated with \[^3^2\]P and exposed to 0.1 mM ascorbic acid with (+) or without (−) 10 nM isoproterenol (ISO) for 10 min. Receptors were then purified by affinity chromatography as described under "Experimental Procedures" and evaluated by gel electrophoresis and autoradiography. Approximately 0.1 pmol of purified \( \beta_2 \)AR (as determined by \[^3^2\]P-cyanopindolol binding) are loaded in each lane.

As noted earlier (27), which is within the range of that seen with photoaffinity labeling. Therefore, in all phosphorylation experiments the regions of each autoradiogram corresponding to that enclosed by the bracket labeled \( \beta_2 \)AR in this figure were scanned by laser densitometry for quantitative purposes. In some experiments, a contaminating band that migrates at approximately 40 kDa was also apparent; this band, possibly actin (data not shown), was not a consistent finding with any of the mutants, nor was it preferentially associated with receptors purified from cells that had been pretreated with agonist.

The results of several such experiments involving exposure of cells to the low concentration of agonist are summarized in Fig. 4. In general, receptors purified from all four cell lines show similar levels of basal phosphorylation (75–100% of the level seen with wild type cells). This suggests that most receptor phosphorylation in the absence of agonist occurs on sites not located in either of the two domains examined here. Receptors purified from wild type cells exposed to a low concentration of agonist for 10 min exhibit an approximately 3-fold increase in their level of phosphorylation. Under similar conditions, both mutant A and mutant B.

**Fig. 2.** Wild type and mutant human \( \beta_2 \)AR. Membrane topography of human \( \beta_2 \)AR. Filled circles represent serine or threonine residues substituted in various mutants by alanine, except for those marked with an asterisk, which were substituted for by glycine. The residues substituted in mutant AB included all those substituted in both mutant A and mutant B.

**Fig. 3.** Photoaffinity labeling and phosphorylation of wild type and mutant \( \beta_2 \)AR. A, photoaffinity labeling of wild type and mutant \( \beta_2 \)-adrenergic receptors in membranes. Labeling was conducted with \[^3^2\]P-cyanopindolol diazirine, as described under "Experimental Procedures," in the presence (+) or absence (−) of 10 \( \mu \)M alprenolol (Alp) to define specific labeling. First and second, third and fourth, fifth and sixth, seventh and eighth lanes show labeled membranes prepared from wild type, mutant B, mutant A, and mutant AB cells, respectively. Band, whole cell phosphorylation. Wild type and mutant cells were incubated with \[^3^2\]P and exposed to 0.1 mM ascorbic acid with (+) or without (−) 10 nM isoproterenol (ISO) for 10 min. Receptors were then purified by affinity chromatography as described under "Experimental Procedures" and evaluated by gel electrophoresis and autoradiography. Approximately 0.1 pmol of purified \( \beta_2 \)AR (as determined by \[^3^2\]P-cyanopindolol binding) are loaded in each lane.
conditions receptors from A or AB cells, both of which lack the sites for protein kinase A phosphorylation, were also increased in their level of phosphorylation. However, for both cell types the increase over basal was only 30-40% of that seen with wild type cells. In contrast, receptors from B cells, which lack the putative βAR kinase sites but retain the protein kinase A sites, show an increase in phosphorylation over basal that was 80% of that seen with wild type cells.

These results suggest that exposure of wild type cells to a low concentration of agonist results in preferential phosphorylation of one or both protein kinase A sites, with little phosphorylation occurring on the carboxyl-terminal βAR kinase sites. In addition, some of the increase in phosphorylation occurs at a site or sites distinct from either of the two domains.

Decreases in Sensitivity Induced by a Low Concentration of Agonist—Cells expressing the mutant receptors were therefore tested for their abilities to mediate desensitization of the adenylyl cyclase response following a 15-min exposure to the low concentration of agonist. In wild type cells (Fig. 5, top left) exposure to 10 nM isoproterenol causes a significant \((p < 0.05)\) change in the sensitivity of the enzyme response. For purposes of comparison the changes in sensitivity were quantitated as the relative loss in ability of a low concentration of agonist (100 nM) to restimulate the enzymatic response in membranes from agonist-treated versus untreated cells (Fig. 6, left panel).

Interestingly, exposure of cells expressing the receptors lacking the putative protein kinase A sites (A and AB cells) to the low concentration of agonist causes only a small rightward shift of the concentration-response curve (Fig. 5, right panels). This result is reflected in the finding that the ability of 100 nM agonist to restimulate the enzyme response is reduced by only 10-25%, as compared with the 45% decrease seen with wild type cells \((p < 0.05; n = 7)\) (Fig. 6, left). However, the B cells, which express receptors lacking the carboxy-terminal βAR kinase sites, show the same loss (50%) in sensitivity as the wild type cells following treatment with a low concentration of agonist (Fig. 5, bottom left, and Fig. 6, left).

These results imply that the changes in sensitivity resulting from exposure of wild type cells to the low concentration of agonist originate, at least in part, from a mechanism which involves phosphorylation of one or both putative protein kinase A sites. While some other mechanism(s) must also participate (to explain the residual loss of sensitivity in mutant A cells), the putative βAR kinase sites in the carboxyl-terminal region are clearly not required for these changes in sensitivity to occur.

Receptor Phosphorylation Induced by a High Concentration of Agonist—We next sought to determine the origins of the decrease in the maximal responsiveness of adenylyl cyclase stimulation seen following exposure of cells to high concentrations of agonist. We therefore assessed the levels of whole cell phosphorylation of receptors purified from each of the four cell types following exposure to a high concentration of agonist, and a summary of the results is shown in Fig. 7. Under these conditions, receptors purified from wild type cells show a 3-4-fold increase in the level of receptor phosphorylation. Receptors from A cells exposed to similar conditions show an increase in phosphorylation over basal that is 55% of that seen with wild type receptors. Similarly, receptors from B cells show 45-50% of the increase in phosphorylation over basal seen with wild type receptors. In contrast, receptors from AB cells exhibit only about 10% of the increase in phosphorylation state observed with wild type cells.
Phosphorylation for each cell type was subtracted from that seen in basal phosphorylation on both the protein kinase A sites and on the P2AR kinase sites in the carboxyl-terminal region. How-

ever, phosphorylation on either set of sites is clearly not required for receptor sequestration to occur (Fig. 1B).

Losses in Sensitivity and Maximal Responsiveness with a High Concentration of Agonist—The pattern of desensitization of the mutant cells following exposure to the high concentration of agonist was examined next. Membranes prepared from wild type cells treated in this way show both a 60–70% loss in the ability of 100 nM of the agonist to restimulate the enzyme response (sensitivity) and a 25–30% decrease in the maximum responsiveness to the agonist (Fig. 6, right, and Fig. 8, top left). In contrast, there is no loss in the maximum responsiveness in membranes prepared from any of the mutant cells following exposure to the high concentration of agonist. Even preexposure of either the A or the B cells to the high concentration of agonist for 60 min failed to significantly decrease the maximum adenyl cyclase response (data not shown).

With regard to alterations in sensitivity, under these desensitizing conditions membranes from A and AB cells (Fig. 8, top right and bottom right, respectively) both exhibit significant rightward shifts in the agonist concentration-response curves, which correspond to 45% (A cells) and 25% (AB cells) decreases in the adenyl cyclase response seen upon restimulation with 100 nM isoproterenol (Fig. 6). Nevertheless, these losses in sensitivity are still smaller than that observed (65%) with the wild type cells (p < 0.05). Mutant B cells, in contrast, exhibit a loss of sensitivity to agonist (60%) similar to that seen with the wild type cells (Fig. 8, bottom left).

These results demonstrate that both protein kinase A and βAR kinase phosphorylation sites in βAR are required for the decrease in the maximum responsiveness of adenyl cyclase elicited by exposure of wild type cells to high concentrations of agonists. Intact protein kinase A phosphorylation sites are also necessary for the full rightward shift of the concentration-response curves seen under these desensitizing conditions. In addition, other mechanisms such as sequestration might also be causally involved in this loss of sensitivity.
Mechanisms of β-Adrenergic Receptor Desensitization

Changes in the Sensitivity of the Adenylyl Cyclase Response to Agonist Stimulation Are Mediated by Phosphorylation of Protein Kinase A Sites—The low concentrations of agonist used in this study (10–20 μM isoproterenol) maximally stimulate cellular cAMP levels (data not shown). According to the model, protein kinase A is thereby activated and phosphorylates β2AR on one or both of the putative protein kinase A sites. This phosphorylation is largely responsible for the decreased sensitivity of the adenylyl cyclase response to agonist. Similarly, exposure of cells to high concentrations of agonist also results in phosphorylation of the putative protein kinase A sites. While at the high concentrations a second mechanism (such as sequestration) may also contribute to the loss of sensitivity, phosphorylation of the sites still plays a role in this desensitization. In contrast, the putative β2AR kinase sites in the carboxyl-terminal region of the receptor do not seem to be required for the full decrease in sensitivity of the adenylyl cyclase response following exposure of the cells to either low or high agonist concentrations.

Both consensus sites for protein kinase A adjoin the regions of β2AR proposed to interact with G, (34, 35). For this reason it is conceivable that covalent modification of the serine residues in one or both locations could directly impair coupling of the receptor with the regulatory protein. Additional studies with purified receptor and G, in a reconstituted system (20) will be needed to directly determine whether phosphorylation at one or both protein kinase A sites may have a more pronounced effect on the apparent Kᵢ than the Vₘₐₓ of the agonist receptor complex for the G protein.

The results obtained here with mutated β2AR are in agreement with those recently described by Clark and colleagues (36) in their studies of the desensitizing effects of low concentrations of catecholamines on the cyc- and kin- variants of S49 lymphoma cells, which lack G, and protein kinase A, respectively. These authors concluded that protein kinase A played a fundamental role in mediating the effects of low concentrations of agonist on desensitization of both β-adrenergic receptors and, concomitantly, of other hormone receptors.

Decreases in the Maximal Stimulation of Adenylyl Cyclase by Agonist Require Intact Protein Kinase A and β2AR Kinase Phosphorylation Sites—With regard to the loss of the maximal responsiveness of the enzyme resulting from exposure of cells to high concentrations of agonist, these data and our previous results with mutated receptors expressed in mammalian cells (27) suggest a prominent role for phosphorylation catalyzed by β2AR kinase. In addition, these results are consistent with a requirement for the receptor to be occupied by agonist in order for phosphorylation to occur, as demonstrated in vitro (19). For example, with an apparent binding dissociation constant (Kᵢ) for isoproterenol to intact cells of approximately 100 nM, exposure of the cells to the low concentration (10 nM) of agonist used here would result in the occupancy of only 10% of the receptors. This could explain our finding that when intact cells are exposed to such low concentrations little phosphorylation occurs on β2AR kinase sites. Conversely, exposure of cells to the high concentration of agonist used here (2 μM isoproterenol) should result in occupancy of greater than 90% of the receptors, and, under those conditions, substantial phosphorylation of the receptor on the β2AR kinase sites occurs. Recent experiments with inhibitors of β2AR kinase in permeabilized cells also support the idea that the novel kinase plays a central role in the desensitization process (37).

Strader and colleagues have recently characterized the agonist-induced loss of maximal responsiveness of adenylyl cyclase in three mutated forms of the hamster β2AR (12). In one mutant receptor, amino acid residues containing the putative β2AR kinase sites in the carboxyl-terminal region of the wild type receptor were deleted. A second mutant receptor involved a deletion of 30 amino acids in the third cytoplasmic loop, including a putative protein kinase A site. Both receptor mutants showed delayed onsets of desensitization. Finally,
when both mutations were combined in a single receptor a more dramatic impairment of desensitization was seen than with the truncation mutant alone (12). In principle, these results appear to agree with the major conclusions obtained with the substitution mutants of the human β2AR analyzed in our study: alteration of either β2AR kinase or protein kinase A sites yields dramatic effects on desensitization.

However, Cheung et al. (12) came to the opposite conclusion in their study. They suggested that altered levels of sequestration, not receptor phosphorylation, were responsible for the impaired kinetics of desensitization of their mutants. Unfortunately, though, that group did not assess the phosphorylation states of their mutants. In fact, since most or all parameters of receptor activation they did measure were substantially impaired with these mutated receptors, it is likely that phosphorylation was affected as well (38). Given the global effects of these mutations, then, it is unclear how that group was able to pinpoint the mechanisms underlying the altered desensitization patterns they observed.

In contrast, the models of desensitization proposed here are based on studies of receptors with mutations involving substitution of as few as 4 serine and/or threonine residues by alanine and glycine. While these mutant receptors yield strikingly altered patterns of phosphorylation and desensitization, other agonist-triggered parameters of β2AR activation (such as stimulation of adenylyl cyclase, ability of the agonists to form high affinity complexes with Gs, and sequestration) appear to be completely unaffected. The functional specificity of these mutations clearly demonstrates that distinct molecular determinants are involved in desensitization of the adenylyl cyclase response versus receptor sequestration, contrary to the hypothesis proposed by others (12, 33).

Receptor Sequestration—What, then, is the role of receptor sequestration? Sequestration in wild type cells has been proposed to represent a rapid translocation of predominantly phosphorylated receptors to a subcellular compartment rich in phosphatases where the receptors are dephosphorylated and from which they are then recycled back to the plasma membrane (39). Consistent with this proposal, we suggest that prevention of sequestration in wild type cells demonstrably has had little effect on desensitization (see the Introduction) because most or all sequestered receptors are already uncoupled from Gs, via phosphorylation mechanisms. Yet in cells expressing mutated forms of the β-adrenergic receptor that lack phosphorylation sites, agonist-induced sequestration would constitute internalization of unphosphorylated receptors that are presumably still coupled. Sequestration of receptors in these cells could thus have direct and profound consequences on the agonist-stimulated adenylyl cyclase response, especially in cells where the receptor-Gs ratio is already relatively low. Such effects of sequestration may have contributed to the desensitization response mediated by the mutated receptors described by Cheung et al. (12) as well as to the rapid, agonist-induced uncoupling seen with the kin mutant of S49 cells (40–41). In both cell types, receptors are expressed at only about one-tenth of the level seen in the high expressing CHW cell lines studied here (~1.7 pmol of receptor/mg protein).

In contrast to these studies of desensitization following short term exposure of cells to agonist, it is difficult to assess the importance of phosphorylation on β2AR kinase and protein kinase A sites in the desensitization induced by prolonged exposure to agonist (>1 h). For example, some of the differences in the patterns of desensitization among the mutated and wild type receptors described here and elsewhere are no longer apparent following exposure of the cells to agonist for 60 min (12) or 3 h (27, 33). To some extent, this may be due to desensitizing effects of the disproportionately high levels of cAMP which accumulate only in cells expressing mutant receptors that do not desensitize at early times.4 It is also likely that additional molecular mechanisms, such as modification of G proteins or the cyclase moiety, as well as receptor down-regulation, are involved in longer term desensitization. Alternatively, it is possible that phosphorylation of β2AR simply enhances the rate of desensitization induced by agonist occupancy by some other mechanism, as has been observed with the nicotinic cholinergic receptor (42). Clearly, there is no obvious correlation between the loss of maximal β2AR responsivity following long term exposure to agonist and any single molecular mechanism.

Finally, there may be physiological implications in the existence of separate biochemical mechanisms for desensitization, one attuned to low concentrations of catecholamines and another to high concentrations. It is tempting to speculate that the former mechanism, which we propose involves protein kinase A, is more active in peripheral tissues, which are predominantly exposed to circulating concentrations of catecholamines that are relatively low. Conversely, a desensitization mechanism involving β2AR kinase may be generally more employed where the concentrations of catecholamines are high, such as in neural synapses. In this regard, it is interesting that brain is an excellent tissue source for β2AR kinase (19). Such tissue compartmentalization of desensitization mechanisms might also have important therapeutic implications with regard to the potential pharmacological selectivity of β2AR kinase inhibitors (37).

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