0026-895X/89/040641-06$02.00/0

MOLECULAR PHARMACOLOGY, 36:641–646

Altered Patterns of Agonist-Stimulated cAMP Accumulation in Cells Expressing Mutant β_2-Adrenergic Receptors Lacking Phosphorylation Sites

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Received March 24,1989; Accepted July 11,1989

SUMMARY

As with many other receptor-effector systems, the responsiveness of the β-adrenergic receptor (βAR)/adenylyl cyclase system undergoes desensitization upon agonist exposure. Phosphorylations of the receptor by the cAMP-dependent protein kinase (protein kinase A) and the βAR kinase appear to play roles in such desensitization phenomena, but the functional significance of the receptor phosphorylation in intact cells has not been previously assessed. In this study, we constructed and expressed in a mammalian fibroblast line the normal (wild type) human β_2AR and mutant forms of the receptor that lack the putative phosphorylation sites for these two protein kinases. The two consensus sequences for phosphorylation by protein kinase A were altered by changing serines 261, 262 and 345, 346 to alanines. In another mutant, the 11 serines and threonines at the carboxy terminus of the protein that constitute the putative βAR kinase phosphorylation sites were changed to alanines or glycines. The mutated receptors did not differ from the wild type in their affinities for agonists or antagonists or in their ability to mediate agonist stimulation of adenylyl cyclase. Moreover, their levels of expression in the cultured cells were the same. When stimulated with the βAR agonist isoproterenol, cells bearing either the wild type or mutant receptors generated cAMP at essentially identical rates for the first 2 min. Cells bearing wild type receptors then showed a rapid desensitization characterized by a markedly diminished rate of cAMP production after the first few minutes of stimulation. However, cells bearing either of the mutated forms of the receptor showed much less desensitization and continued to generate cAMP at a rate 3–4 times greater than that observed in cells expressing the wild type receptor. In contrast, intact cell cAMP levels stimulated by prostaglandin E_1 and forskolin were not different between cells bearing wild type or mutant βAR. These results suggest an important physiological role for phosphorylation of the βAR in regulating rapid agonist-induced desensitization in intact cells.

A major property of drug, hormone, and neurotransmitter receptors that signal to the interior of the cell via guanine nucleotide regulatory proteins is that their actions become attenuated with time, despite the continued presence of a stimulatory agonist. Such desensitization phenomena may be specific for the effects of the stimulating agent (homologous) or may involve decreased responsiveness to several classes of activators (heterologous). Desensitization may significantly shorten the duration of effective therapeutic action of adrenergic agonists and many other drugs (1).

The underlying biochemical mechanisms that produce desensitization have been extensively studied for the adenylyl cyclase-coupled βAR system (1–4). During desensitization, covalent modifications of the β_2AR itself, the stimulatory guanine nucleotide regulatory protein, and the adenylyl cyclase moiety have been reported to occur (3). At the level of the receptor, rapid stoichiometric phosphorylation has been associated with the onset of both homologous and heterologous desensitization. Three different protein kinases, PKA, protein kinase C, and βARK, have been shown to phosphorylate the β_2AR *in vitro* and have been proposed to play distinct roles in receptor regulation and desensitization (5–8). Although two consensus sequences for phosphorylation by PKA exist in the cytoplasmic domains of the receptor (see Fig. 1), the sites of phosphorylation by βARK have not been unequivocally assigned. Nonetheless, several lines of evidence suggest that a cluster of serine and threonine residues located near the cytoplasmic carboxy terminus of the receptor may represent these sites of phosphorylation. In the analogous guanine nucleotide regulatory protein-coupled rhodopsin photoreceptor system, rhodopsin kinase (analogous to βARK) phosphorylates this receptor at multiple serines and threonines in the carboxy tail (9). Moreover, Dohlman *et al.* (10) have shown that limited proteolysis of purified β_2AR by carboxypeptidase Y (which removes the cytoplasmic

ABBREVIATIONS: βAR, β-adrenergic receptor; βARK, β-adrenergic receptor kinase; PKA, protein kinase A; PBS, phosphate-buffered saline; CHW, Chinese hamster fibroblasts; ICYP, ^{125}I-cyanopindolol.

tail) eliminates *in vitro* βARK phosphorylation sites (10). Finally, a mutant β_2AR, which lacks seven serines and threonines due to truncation of the carboxy tail at residue 365, phosphorylates minimally *in vivo* in response to agonist exposure (8).

Multiple mechanisms appear to mediate the overall process of cellular desensitization to agonist action. Thus, studies with isolated membranes may not necessarily provide a complete picture of how these mechanisms may contribute to the evolving cellular adaptation. Accordingly, the present studies were performed in order to assess the importance of βARK- and PKA-mediated receptor phosphorylation to the overall phenomenon of desensitization as it operates in whole cells. This has been accomplished by studying the process of agonist-stimulated cAMP production by intact cells bearing normal receptors or mutated receptors in which the putative PKA and βARK phosphorylation sites have been altered. The results provide strong evidence for the physiological importance of these regulatory enzymes in mediating aspects of agonist-promoted desensitization.

Materials and Methods

Cells. Wild type or mutant human β_2AR were expressed in CHW cells, as previously described (11). Cells were grown as monolayers in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin until 90% confluent in a 95% air/5% CO_2 atmosphere at 37°.

β_2AR mutagenesis and expression. Wild type β_2AR cDNA (12) was subcloned into the *Eco*RI/*Hind*III sites of the plasmid pTZ and mutant β_2AR cDNA was made by modifications of the Amersham oligonucleotide-directed mutagenesis system, as previously utilized (8). Mutations were verified by dideoxy sequencing. The mutant cDNA were then cloned into the eukaryotic expression vectors pBC12MI (13) or pKSV10 (Pharmacia) and the resulting plasmids were cotransfected with pSV Neo into CHW cells by coprecipitating the DNA with calcium phosphate. Clonal cells were selected in media containing geneticin (150 μg/ml). The mutants constructed are illustrated in Fig. 1. A mutated receptor that lacks the putative PKA sites of phosphorylation (designated Mutant A) was made by substitution of serines 261 and 262 of the third cytoplasmic loop and serines 345 and 346 of the proximal cytoplasmic tail with alanines. Potential βARK sites in the distal cytoplasmic tail were altered in Mutant B by substitution of the 11 serines and threonines with alanines or glycines, beginning with residue 355 (8). For the current study, clones were selected with β_2AR density similar to that of the wild type.

cAMP accumulation experiments. Isoproterenol-induced cAMP accumulation was measured in attached cells grown as monolayers in 16-mm diameter culture wells. Cells were rinsed three times with PBS, and then 0.4 ml of PBS supplemented with 0.8 mM ascorbic acid (for isoproterenol oxidation protection) was added and the plates were incubated at 37°. At the indicated times, isoproterenol (2 μM final concentration) was added and all reactions were terminated simultaneously by the addition of perchloric acid (0.6 M final concentration). The samples were neutralized by potassium bicarbonate and the precipitate was pelleted by centrifugation. An aliquot was then assayed for cAMP with a radioimmunoassay, using ^{125}I-succinyl cAMP tyrosine methyl ester (14). In other experiments, cells were incubated for 20 min with 0.1 mM isobutylmethylxanthine before addition of isoproterenol. Studies were also carried out in a similar manner using suspended cells derived from scraping or collagenase digestion.

Radioligand binding studies. Cells were detached by scraping and for intact cell studies βAR density was determined by incubation of cells with 400 pM ICYP, in the presence or absence of 1 μM alprenolol, to determine nonspecific binding. The receptor density is reported here as fmol/mg of whole cell protein. Incubations were carried out for 2 hr at 25° and were terminated by dilution. Unbound ligand was separated by vacuum filtration. To assess the degree of agonist-induced sequestration of cell surface receptors, cells in monolayers were incubated with 2 μM isoproterenol, washed extensively with cold PBS, and detached by scraping. ICYP binding was then carried out for 5 hr at 13°C, a temperature that maintains sequestered receptors in the intracellular pool. Cells were incubated with 400 pM ICYP, with total receptor binding defined as that displaced by the hydrophobic antagonist propranolol (1.0 μM) and cell surface receptor binding defined as that displaced by the hydrophilic antagonist CGP-12177 (0.3 μM) (15).

Adenylyl cyclase. Adenylyl cyclase studies were performed with membranes prepared essentially as described (11). Attached cells were washed twice with PBS, scraped, and lysed by Polytron homogenization in hypotonic buffer. The suspensin was centrifuged at 400 × *g* to remove unbroken cells and nuclei, and the supernatant was centrifuged at 40,000 × *g*. Pelleted membranes were washed twice by similar centrifugations, with the final resuspension being in 75 mM Tris, 12.5 mM $MgCl_2$, 2 mM EDTA, pH 7.4, buffer. Adenylyl cyclase activities were determined exactly as previously described (11), in the presence of 10 μM isoproterenol, 10 mM NaF, 100 μM forskolin, or buffer alone. Protein determination was by the method of Bradford (16).

Data analysis. Inspection of the isoproterenol-induced cAMP accumulation profiles indicated that they were linear between 0 and 2 min and between 10 and 40 min, albeit at a much reduced rate during the latter period. In order to facilitate comparison of the data from various clones, accumulation rates were thus calculated by linear regression analysis ($r > 0.9$ in all cases) of data obtained at 0–2 min and 10–40 min and reported as pmol of cAMP/mg of protein/min. Isoproterenol/cAMP dose-response curves were analyzed by a computer-assisted four-parameter logistic method (17). Data were compared by *t* tests, with significance imparted at the $p < 0.05$ level. Data are presented as the means ± standard errors.

Results

As shown in Table 1, membranes derived from cells bearing wild type, Mutant A, and Mutant B β_2AR had similar adenylyl cyclase activities in the basal state and after stimulation by isoproterenol, NaF, or forskolin. In order to provide additional confirmation of the functional equivalence of the mutant receptors, as compard with the wild type, isoproterenol-cAMP dose-response studies were carried out in intact cells with an incubation time of 2 min. As shown in Fig. 2, the EC_{50} for Mutant A and Mutant B receptor-bearing cells was the same as for the wild type. The intact cell receptor densities were also similar and are equivalent to ~75,000 sites/cell (Table 1).

When cells bearing wild type or mutant β_2AR were exposed to agonist, cAMP accumulated rapidly, increasing ~8-fold within 1 min (Fig. 3, *inset*). As shown in Figs. 3 and 4, the rates of cAMP accumulation were identical for cells bearing wild type or mutant receptors during this period (119 ± 23 for wild type, 125 ± 23 for Mutant A, and 124 ± 12 pmol/mg/min for Mutant B). This rate of accumulation decreased at later time points in all three cell lines but dampened most dramatically in cells bearing wild type receptor (Figs. 3 and 4). Consequently, whereas the wild type receptor cells reached a maximal level of cAMP of 846 ± 126 pmol/mg at 40 min, Mutant A cells achieved levels of 1740 ± 590 pmol/mg and Mutant B cells had levels of 2489 ± 400 pmol/mg ($p < 0.025$ and $p < 0.001$, respectively, versus wild type receptor cells). The steady state cAMP accumulation rates (10–40 min) for Mutant A (28.9 ± 7.8 pmol/mg/min; $p < 0.01$) and Mutant B (45.0 ± 10.4 pmol/mg/min; $p < 0.001$) cells were significantly greater, as compared with those of the wild type cells (11.1 ± 2.2 pmol/mg/min), as shown in Fig. 4. These results are in contrast to those obtained when intact cells were exposed to prostaglandin E_1 or forskolin for

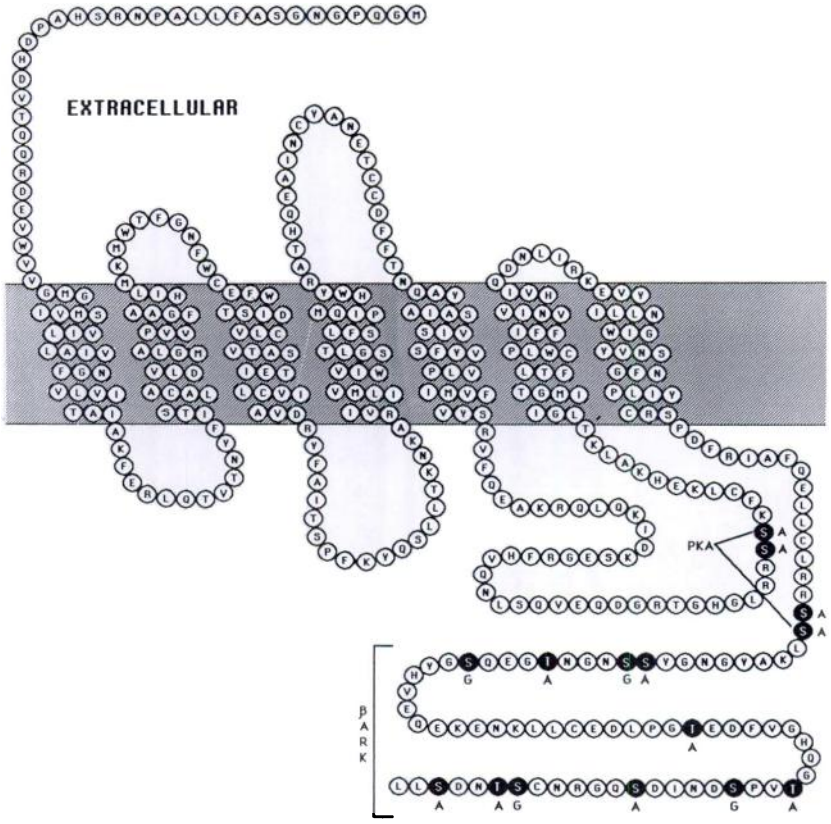

Fig. 1. Amino acid sequence and proposed membrane topography of the human β_2AR. The putative sites of phosphorylation by βARK are the serines and threonines in the cytoplasmic tail, as indicated by the *bracket*, and for PKA are the serines denoted in the consensus sequences R-R-X-S, as shown. *Darkened circles* indicate the sites of specific amino acid substitutions with glycines or alanines, as shown.

TABLE 1

Characteristics of wild type and mutant human β_2-AR expressed in CHW cells

In five or six experiments, membranes from cells expressing wild type receptor or receptors lacking the putative PKA (Mutant A) or βARK (Mutant B) phosphorylation sites were prepared and basal, isoproterenol-, NaF-, and forskolin-stimulated adenylyl cyclase activities were determined. Receptor density, quantitated in whole cells using ICYP, is expressed as fmol/mg of cellular protein and corresponds to ~1500 fmol/mg when assayed using membranes (11). Cyclic AMP studies were performed by incubating cells in the absence of isobutylmethylxanthine with buffer, prostaglandin E_1, or forskolin for 40 min at 37° and cAMP was assayed by radioimmunoassay, as described in Materials and Methods. NaF-stimulated adenylyl cyclase activities were slightly lower in the mutants, but otherwise there were no statistically significant differences between corresponding parameters of the mutant receptors and those of the wild type.

Parameter	Wild type	Mutant A	Mutant B
Whole cells			
β_2AR density (fmol/mg of protein)	516 ± 81	641 ± 92	579 ± 80
cAMP (pmol/mg of protein)			
Basal	23.2 ± 4.2	27.1 ± 5.6	22.2 ± 4.0
10 μM Prostaglandin E_1	41.7 ± 2.5	47.7 ± 3.5	41.6 ± 4.8
100 μM Forskolin	2771 ± 210	3021 ± 197	2901 ± 259
Membranes			
Aldenylyl cyclase activity (pmol/min/mg of protein)			
Basal	7.8 ± 1.5	8.0 ± 0.7	6.9 ± 2.2
10 μM Isoproterenol	23.1 ± 3.5	28.7 ± 3.4	26.3 ± 3.4
10 μM NaF	24.5 ± 3.8	18.5 ± 1.3	17.7 ± 2.1
100 μM Forskolin	141 ± 32	120 ± 3	119 ± 4

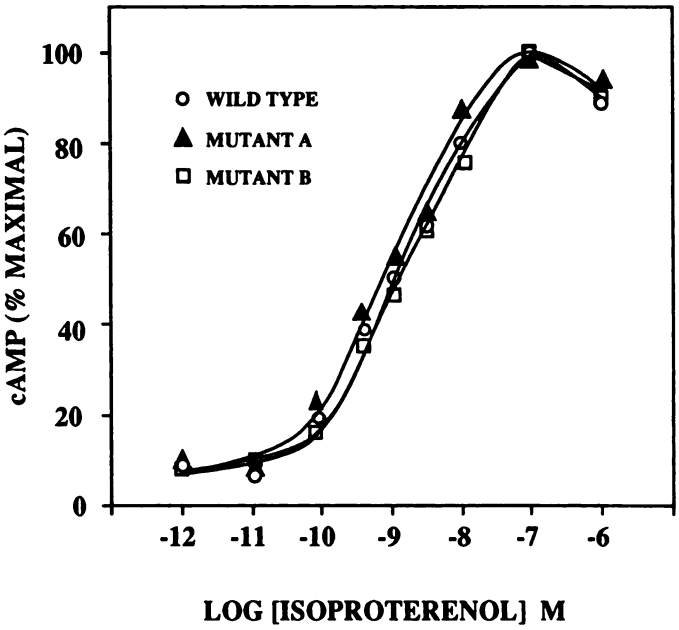

Fig. 2. Isoproterenol-cAMP dose-response in cells bearing wild type or mutant β_2AR. Cells were exposed to the indicated concentrations of isoproterenol for 2 min at 37°. Shown are single experiments. For four such experiments, the EC_{50} values were wild type, 1.34 ± 0.7, nM; Mutant A, 1.0 ± 0.4 nM; and Mutant B, 1.77 ± 0.8 nM.

40 min. Under these circumstances, the amounts of cAMP accumulated were the same for cells bearing wild type or either mutant receptor (Table I). This indicates that alterations in the desensitization patterns are observed only for the βAR-coupled pathway.

As shown in Fig. 5, these altered patterns in response to

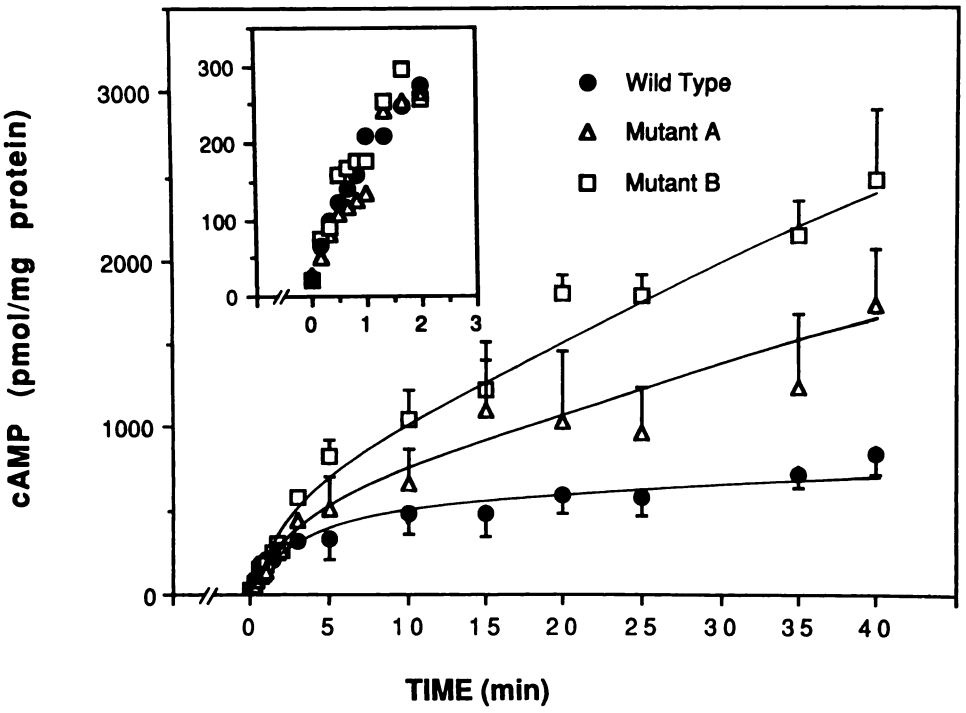

Fig. 3. Time course of isoproterenol-stimulated cAMP accumulation in CHW cells expressing wild type and mutated human β_2AR. Intact cells bearing normal (wild type) receptors and receptors with altered PKA- (Mutant A) or βARK- (Mutant B) mediated phosphorylation sites were incubated with 2 μM isoproterenol for the indicated times at 37° and cAMP was determined as described in Materials and Methods. Results are means ± standard errors for four or five experiments. For clarity, standard errors (typically ± 15%) are omitted from early time points, which were obtained every 10 or 20 sec for the first 2 min. *Inset*, cAMP levels at early time points on an expanded scale.

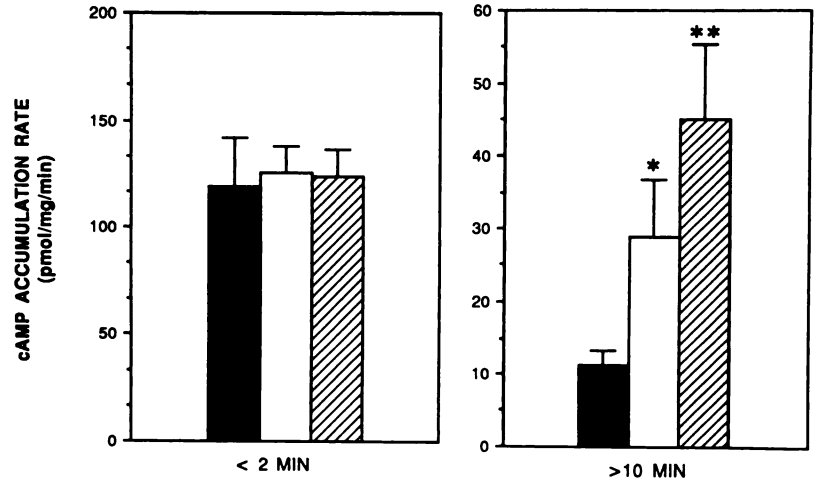

Fig. 4. Comparison of cAMP accumulation rates at early (2 min) and late (>10 min) time points for wild type (■), Mutant A- (□), and Mutant B- (▨) bearing cells during exposure to 2 μM isoproterenol. Rates were calculated as described in Materials and Methods. Note the different scales for the *oridinates*. $^*p < 0.01$; $^{**}p < 0.001$.

isoproterenol were not due to differences in agonist-promoted loss of cell surface β_2AR. Thus, sequestration of cell surface receptors was similar in all three cell lines. This is in confirmation of our recent findings, which have shown that these receptor mutations do not alter agonist-promoted sequestration (15). Moreover, no detectable down-regulation of total receptor density occurs over the same time period in cells bearing wild type or either mutant receptor (Fig. 5). Preincubation of cells with the phosphodiesterase inhibitor isobutylmethylxanthine increased cAMP levels ~50%, but the accumulation profiles were unaffected. Cells studied in suspension showed essentially the same phenomena. Another CHW cell line expressing the wild type β_2AR also displayed desensitization profiles virtually identical to those of the wild type receptor shown here.

Discussion

The processes involved in agonist-induced desensitization at the receptor level have been proposed to include receptor sequestration away from the cell surface into a compartment distinct from the plasma membrane, down-regulation of the total receptor complement, and functional uncoupling of the receptors from interaction with guanine nucleotide regulatory proteins without change in subcellular distribution of the receptors. Evidence that this latter phenomenon may be related to phosphorylation of the receptors has been recently reviewed (2, 3). Studies have suggested primary roles for the cAMP-dependent protein kinase (PKA) and a unique cAMP-independent protein kinase termed βARK in different aspects of desensitization (8, 18). Both kinases have been shown to be capable of phosphorylating purified receptor *in vitro* (5, 6). In each case, the covalent modification is associated with a decreased ability of the receptor to interact with G_s in a reconstituted phospholipid vesicle system (5, 19).

Studies of the influence of receptor phosphorylation on GTPase activity in such reconstituted system or on adenylyl cyclase activities in plasma membrane fractions have provided strong evidence for the potential importance of receptor phosphorylation in the desensitization process. Indeed, we have

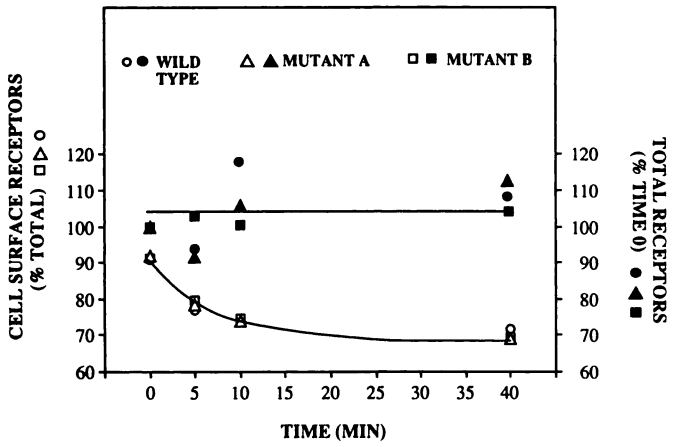

Fig. 5. Agonist-induced sequestration and down-regulation of wild type and mutant β_2AR. Cells were exposed to 2 μM isoproterenol for the indicated times, washed, and assayed as described in Materials and Methods for cell surface and total receptor densities. The degrees of cell surface receptor loss (sequestration) were identical between wild type and mutant β_2AR. The apparent fluctuations in total receptor densities were not statistically different than at time 0, indicating no significant down-regulation over this time course. Data presented are means of three independent experiments.

recently confirmed that βAR with altered PKA or βARK phosphorylation sites undergo decreased agonist-induced phosphorylation, as compared with wild type receptors (15). Moreover, the degree of phosphorylation correlates with the extent of desensitization, as assessed by the ability of isoproterenol to stimulate adenylyl cyclase activity in membranes derived from agonist-treated cells. Yet, these studies offer little information as to the physiological importance of events such as receptor phosphorylation to the overall pattern of cellular desensitization *in vivo*. Accordingly, the present studies were performed to examine the course of cAMP production by intact cells stimulated by β-agonists when the sites of receptor phosphorylation by PKA or βARK were absent.

As demonstrated in the current investigations, such mutated receptors show striking alterations in their patterns of desensitization when assessed in this whole-cell setting. Either mutation leads to a significantly higher rate of cAMP accumulation after the first few minutes of agonist exposure than that observed with wild type receptors. It should also be pointed out that, whereas the sites of phosphorylation on the receptor for the cAMP-dependent protein kinase can be predicted with some confidence (there are only two consensus sequences for this kinase in the receptor), the sites phosphorylated by βARK are not yet known with assurance. However, based on analogy with rhodopsin and several biochemical lines of evidence (8–10), it is most likely that the bulk of such phosphorylation normally occurs on the carboxy terminal serines and threonines, which were mutated in receptor Mutant B.

Recently, Cheung *et al.* (20) have proposed that βAR phosphorylation does not play a primary role in receptor desensitization. In their studies, large truncation or deletion mutants of the hamster β_2AR, which were lacking phosphorylation sites, appeared to undergo desensitization, as assessed by adenylyl cyclase activities, similar to that found in cells bearing wild type receptors after prolonged (1–16 hr) exposure to agonist. At earlier time points (0–1 hr), however, their mutant receptors indeed displayed delayed patterns of desensitization. Also, these mutant receptors were either partially functionally uncoupled in the basal state, were differentially expressed in cells, and/or displayed impaired agonist-induced sequestration. It is thus difficult to assess the role of phosphorylation in the desensitization of such radically altered receptors. Moreover, the L cells utilized by this group have lower (albeit more physiological) numbers of receptors, as compared with the CHW cells used in our studies (50–150 fmol/mg of protein as compared with ~1500 fmol/mg of membrane protein). Consequently, the process of receptor sequestration, which removes ~70% of cell surface receptors in L cells, plays a more dominant role than in our CHW cells, where only ~30% of receptors are sequestered under the influence of agonist.

Our results verify, for the first time in a whole-cell setting, the physiological importance of receptor phosphorylation events in controlling responsiveness to the actions of catecholamines. Moreover, they document that processes that lead to >90% reduction in the rate of β-agonist-stimulated cAMP accumulation are complete within 5–10 min of exposure to catecholamines. This time course is consistent with other studies of agonist-induced desensitization of βAR utilizing both intact cell (21, 22) and membrane (8, 23) preparations. Thus, the findings of Cheung *et al.* (20) that mutant receptors undergo desensitization equivalent to wild type at 1 to 16 hr are not really relevant to the issue of the involvement of receptor phosphorylation in rapid desensitization. As we have shown here, these rapid processes clearly seem to involve receptor phosphorylation, because they are markedly perturbed in the mutated receptors that are otherwise functionally normal in all regards. Some desensitization occurs, however, even with the mutant receptors. This may be due to the remaining phosphorylation sites present on either mutant receptor (i.e., PKA sites in Mutant B and βARK sites in Mutant A). In addition, either other processes besides receptor phosphorylation (such as sequestration) are also contributing to rapid desensitization or phosphorylation regulates only the rate of process. In any case, given the functional importance of such covalent modification of the receptors, it is possible to speculate that inhibitors of such kinases might markedly alter the patterns of cellular responsiveness and potentially be of therapeutic value.

Acknowledgments

The authors thank Mark Hnatowich for assistance in data analysis, Grace Irons for technical assistance, and Mary Holben for secretarial support.

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