Vol. 165, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1989 Pages 257-263

SUBSTITUTION OF AN EXTRACELLULAR CYSTEINE IN THE β_2-ADRENERGIC RECEPTOR ENHANCES AGONIST-PROMOTED PHOSPHORYLATION AND RECEPTOR DESENSITIZATION

S.B. Liggett[1], M. Bouvier, B.F. O'Dowd, M.G. Caron,
R.J. Lefkowitz and A. DeBlasi

Departments of Medicine, Cell Biology and
Biochemistry, Duke University Medical Center
and HHMI, Durham, North Carolina

Received September 25, 1989

SUMMARY: We constructed and expressed in a permanent cell line a β_2-adrenergic receptor with a valine substitution for cysteine 184 of the second putative extracellular loop. The mutant receptor was partially uncoupled from adenylyl cyclase with impaired ability to form the high affinity agonist-receptor-G protein complex, yet displayed more rapid and extensive agonist-induced desensitization. The enhanced desensitization was accompanied by increased agonist promoted, but not cAMP promoted, receptor phosphorylation in intact cells. Thus, not only is impaired desensitization associated with decreased phosphorylation, as we have shown with several mutant β_2-adrenergic receptors recently, but enhanced desensitization is accompanied by increased agonist promoted receptor phosphorylation. In the case of this cysteine mutant, this may be due to the greater accessibility of the uncoupled receptor for phosphorylation by the β-adrenergic receptor kinase.

The phenomenon of desensitization, which is a dampening of a biological response despite continuous activation, has been readily demonstrated in the β-adrenergic receptor (βAR)/adenylyl cyclase system (1,2,3). Of the several mechanisms that appear to play roles in the desensitization process, phosphorylation of the receptor by protein kinase A (PKA) and the β-adrenergic receptor kinase (βARK) appears to be important during short term agonist exposure. We have recently shown, for example, that cells bearing mutated β_2AR lacking the putative PKA or βARK phosphorylation sites have depressed agonist-promoted phosphorylation, which is correlated with the loss of agonist-promoted desensitization (1,2). Similar results have been shown by measuring cAMP accumulation in intact cells (3).

In the course of evaluating the importance of extracellular cysteines in the human β_2AR, we developed a mutant β_2AR lacking cysteine 184, normally present in the putative second extracellular loop. In stably transfected

[1] To whom correspondence should be addressed.

0006-291X/89 $1.50

cells, this mutant receptor desensitized more rapidly and to a greater extent than did the wild type receptor upon short term exposure to agonist. Studies of agonist-promoted phosphorylation showed that the enhanced desensitization of the mutant β_2AR was accompanied by an enhanced receptor phosphorylation. Thus, not only is a loss of phosphorylation accompanied by a loss of desensitization, but for the first time we have shown that increased phosphorylation accompanies enhanced agonist-induced desensitization.

METHODS

Mutagenesis, transfection and cell culture

Wild type β_2AR cDNA was subcloned into the plasmid PTZ at the Eco RI/Hind III restriction sites, and oligonucleotide directed mutagenesis of the β_2AR cDNA carried out by methods previously utilized (1,2). The βAR mutant described here has a valine substituted for cysteine 184 in the second extracellular loop and is designated ^{184}CYS(VAL). The mutation was verified by dideoxy sequencing. Mutant cDNA was then cloned into the plasmid pBC12MI and co-transfected with pSVNeo into Chinese hamster fibroblast (CHW) cells by co-precipitating the DNA with calcium phosphate. Clonal cells were selected in media containing geneticin. CHW cells transfected with the normal human β_2AR gene, as previously described, were used as control cells (4). Cells were grown in monolayers in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml Streptomycin in a 95% air, 5% CO_2 atmosphere at 37°C.

Agonist-promoted desensitization

^{184}CYS(VAL) and wild type expressing cells were exposed to 2 μM isoproterenol in DMEM supplemented with 0.5 mM ascorbic acid for 60, 30, 15, 6, 3 and 0 min. at 37°C, then washed three times by rinsing the plates with cold PBS. Cells were then scraped in hyptonic buffer (5 mM Tris, 2 mM EDTA) and homogenized for 5 sec with a polytron. A 400 xg centrifugation pelleted nuclei and clumped cells, and membranes of the supernatant were pelleted by centrifugation at 38,000 xg, washed once by a similar centrifugation, and resuspended in 75 mM Tris, 12.5 mM $MgCl_2$, 2 mM EDTA buffer. Adenylyl cyclase activities were determined by the method of Salomon as modified (4). Activities were determined in the presence of 10 μM isoproterenol, 100 μM Forskolin or buffer alone.

Agonist-promoted phosphorylation

Cells were detached by incubation with collagenase (1.0 mg/ml) and soybean trypsin inhibitor (.05 mg/ml), washed three times and resuspended in phosphate free DMEM at a density of 3 X 10^6 cells/ml. Cells were then equilibrated for two hours at 37°C with carrier free $^{32}P_i$ (7 mCi/sample), then samples exposed to either 2 μM isoproterenol or 1 mM dibutyryl cAMP or buffer for 8 min. After a 5 min centrifugation at 400 xg, the reaction was stopped by addition of cold PBS, the cells washed an additional two times and then disrupted by sonication in 20 mM Tris, 5 mM EDTA, 10 mM Na_2PO_4 buffer. This and all subsequent buffers included the protease inhibitors benzamidine 10 μg/ml, leupeptin 5 μg/ml and soybean trypsin inhibitor 5 μg/ml. The particulate suspension was then washed and solubilized in 2% digitonin. Solubilized receptor was purified by Sepharose/alprenolol chromatography as previously described (1,2), and examined by 10% SDS-polyacrylamide gel electrophoresis and autoradiography.

Radioligand binding

For membrane studies, cells were detached and homogenized as above for adenylyl cyclase activities and radioligand binding studies carried out with [^{125}I]-pindolol, as described (2,4). Intact cell binding was carried out using cells detached by gentle scraping as previously described (2,3). Briefly, cells were incubated with [^{125}I]-pindolol (300 pM) in the absence or presence of 0.5 μM CGP12177 (for quantitation of cell surface receptors) or 1

μM propranolol (for quantitation of all receptors, both cell surface and sequestered) for three hours at 13°C. Reactions were terminated as above. Solubilized receptor binding was performed using [^{125}I]-cyanopindolol in the absence or presence of 10 μM alprenolol, with bound ligand separated by Sephadex G50 column chromatography (1,2).

RESULTS

Receptor densities of membranes dervied from cells bearing wild type (2.2 $\pm$ 0.2 pmol/mg) or ^{184}CYS(VAL) (1.9 $\pm$ 0.2 pmol/mg) β_2AR were similar, as were the dissociation constants for [^{125}I]pindolol (47 $\pm$ 10 pM vs 69 $\pm$ 5 pM, respectively). However, as shown in Table 1, basal and maximal isoproterenol stimulated adenylyl cyclase activities were lower in ^{184}CYS(VAL) as compared to cells bearing wild type receptors. The EC_{50}'s for isoproterenol stimulation were not different (24 $\pm$ 5 nM for wild type and 17 $\pm$ 3 nM for ^{184}CYS-(VAL)). This apparent functional uncoupling was proximal to the catalytic unit, since forskolin stimulated activities were the same. Consistent with the lower basal adenylyl cyclase activity of the mutant, basal intracellular cyclic AMP contents of ^{184}CYS(VAL) cells were significantly lower (17.2 $\pm$ 3.4 pmol/10^7 cells) than those of the wild type (50.1 $\pm$ 10.1 pmol/10^7 cells).

To explore this functional uncoupling further, isoproterenol competition studies were performed in membranes in the absence or presence of 100 μM GTP (Figure 1). The data derived in the presence of GTP were best fit by a one site model and showed that mutant and wild type β_2AR had similar low affinity binding. In the absence of GTP, the data were fit to two sites providing high affinity (K_H) and low affinity (K_L) dissociation binding constants. Under these conditions, the K_L for ^{184}CYS(VAL) was ~2 fold higher than wild type. In addition, K_H for ^{184}CYS(VAL) was ~9 fold higher than that of the wild type β_2AR, revealing a substantially lower affinity for agonist of the mutant receptor. The proportions of receptor in the high affinity form were similar for wild type (30 $\pm$ 2%) and ^{184}CYS(VAL) (42 $\pm$ 7%).

TABLE I

	Wild Type	^{184}CYS(VAL)
Basal	26.2 $\pm$ 4.4	9.4 $\pm$ 3.1
Isoproterenol (10 μM)	114.4 $\pm$ 12.1	40.7 $\pm$ 2.6
Forskolin (100 μM)	266.1 $\pm$ 35.0	262.5 $\pm$ 85.1

Adenylyl cyclase activities of membranes derived from CHW cells expressing wild type or ^{184}CYS(VAL) mutant β_2AR. Activities are expressed as pmol/min/mg and are means of 5 independent experiments. Both basal ($p < 0.02$) and isoproterenol stimulated ($p < 0.001$) activities were significantly less in the mutant as compared to the wild type.

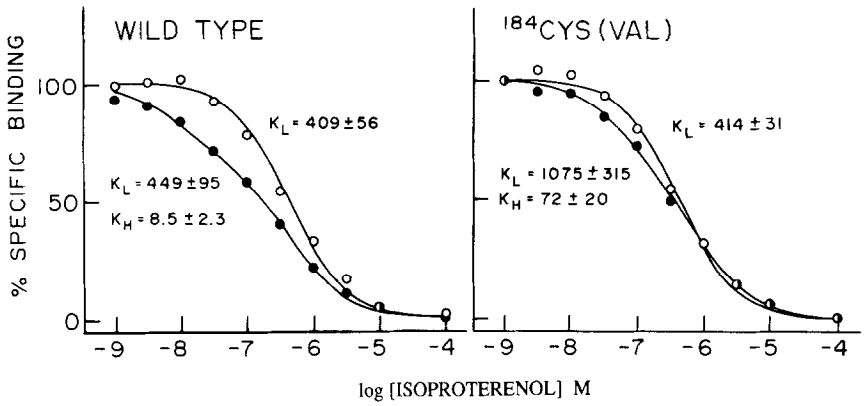

Figure 1. Agonist-induced desensitization of β_2AR. Wild type and mutant β_2AR bearing cells were incubated with 2 μM isoproterenol for the times indicated, washed extensively and membranes prepared. Maximal isoproterenol stimulated adenylyl cyclase activities were then determined as described in Methods. Shown are the means of five experiments.

When cells expressing this mutant β_2AR were exposed to 2 μM isoproterenol, a more rapid and extensive desensitization developed as compared to that which occurred with cells bearing the wild type receptor (Figure 2). As can be seen, in wild type receptor bearing cells, isoproterenol stimulated adenylyl cyclase activity gradually decreased over the course of agonist exposure to 73 $\pm$ 8% of the control level after 1 hour exposure. In contrast, isoproterenol stimulated adenylyl cyclase from membranes of ^{184}CYS(VAL) cells

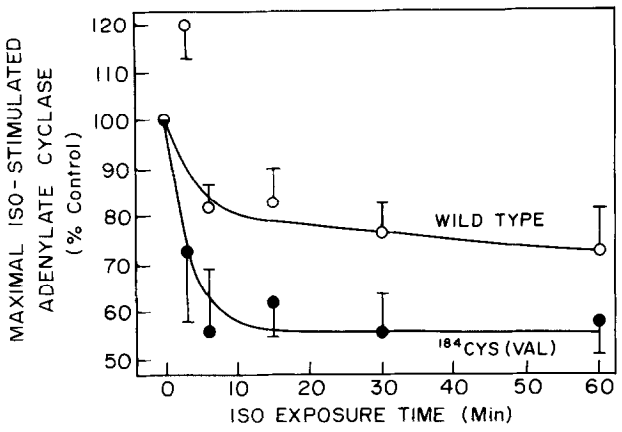

Figure 2. Isoproterenol competition for [^{125}I]pindolol binding to β_2AR in membranes from wild type and ^{184}CYS(VAL) bearing cells. Membranes were incubated in the absence (●) or presence (○) of 100 μM GTP, and the results analyzed by iterative least squares techniques. K_L, K_H = low and high affinity dissociation constants in nM. Results are means of three experiments.

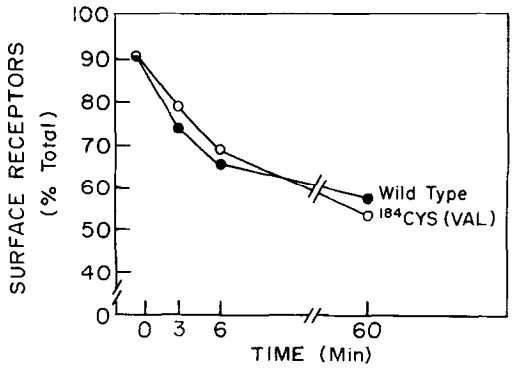

Figure 3. Agonist-induced loss of cell surface β_2AR. Cells bearing wild type or ^{184}CYS(VAL) β_2AR were incubated with 2 μM isoproterenol for the indicated times, washed extensively, and binding with [^{125}I]pindolol carried out as described in Methods. Shown are the means of three experiments.

reached a nadir after 6 minutes of prior agonist exposure, and remained at ~55% of control levels after exposure for up to 1 hour. This altered pattern of desensitization was not due to a difference in the agonist promoted loss of cell surface receptors, since this parameter was identical at the time points shown for both wild type and mutant receptor bearing cells (Figure 3).

To assess whether alterations in receptor phosphorylation accompanied this enhanced desensitization profile, cells which had been loaded with $^{32}P_i$ were exposed to isoproterenol for 8 minutes, then the β_2AR purified and run on SDS-PAGE. As shown in Figure 4, the phosphorylated receptor migrates as a broad band with Mr = 70,000. As previously reported (1,2,4), upon exposure to agonist wild type receptor phosphorylation is increased. In the present study, basal phosphorylation of the ^{184}CYS(VAL) receptor was markedly lower than that of the wild type. The agonist-induced phosphorylation of ^{184}CYS-

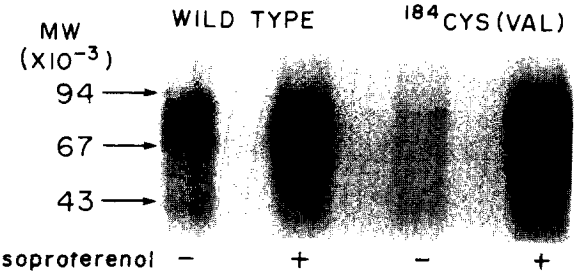

Figure 4. Autoradiographs of phosphorylated β_2AR. Cells loaded with [$^{32}P_i$] bearing wild type or ^{184}CYS(VAL) β_2AR were incubated with or without 2 μM isoproterenol, purified and subjected to 10% SDS-PAGE.

(VAL), expressed as net phosphorylation (isoproterenol stimulated minus basal) was greater than that of the agonist-induced phosphorylation of the wild type receptor. In three such experiments, the agonist-induced phosphorylation was 2.1 $\pm$ 0.07 fold higher in the ^{184}CYS(VAL) mutant as compared to wild type. To differentiate whether this was due to enhanced βARK mediated, or PKA mediated phosphorylation, ^{184}CYS(VAL) and wild type βAR phosphorylation in response to dibutyryl cyclic AMP was examined. In contrast to the marked enhancement of phosphorylation of the mutant β_2AR by isoproterenol, the increment induced by dibutyryl cyclic AMP was no different than that found with the wild type (data not shown).

DISCUSSION

Phosphorylation/dephosphorylation processes play key regulatory roles in a number of receptor systems (5). In the βAR/adenylyl cyclase system several key findings implicate phosphorylation of the receptor as a regulatory mechanism occurring during agonist promoted desensitization. In the turkey erythrocyte, the time courses of agonist promoted desensitization of adenylyl cyclase and phosphorylation are identical, as are the rates of resensitization and return to control phosphate/receptor stoichiometry (6). Purified βAR can be phosphorylated by PKA and βARK and when such receptors are reconstituted with G_s, phosphorylated receptors show an impaired ability to couple to G_s (7,8).

The mutant receptor described here provides the first link between enhanced desensitization and increased phosphorylation in a whole cell setting. The enhanced desensitization occurred despite the marked uncoupling of the receptor. This is in contrast to the studies of Cheung et al. (9), which have shown that agonist-induced desensitization is delayed as a consequence of mutations that depress receptor-G_s coupling, suggesting that these two phenomena share common domains of the receptor. Current studies with the ^{184}CYS(VAL) mutant do not fully elucidate the mechanisms whereby substitution of a single extracellular cysteine causes enhanced desensitization and phosphorylation. This cysteine, as well as others located in the extracellular domain, has been implicated in formation of disulfide bonds, important for maintaining tertiary structure and ligand binding properties (10,11). Moreover, the cysteine in position 184 is universally conserved in all known adrenergic receptors cloned thus far, further suggesting its critical role. In the present study, ^{184}CYS(VAL) β_2AR permanently expressed in cells is partially uncoupled from adenylyl cyclase. Competition experiments suggest that the ability to form the high affinity agonist-receptor-G_s complex (ternary complex) is impaired in this mutant. While the percentage of receptor converted to the high affinity form is similar to that of the wild

type, the affinity of this form is ~9 fold less than the wild type receptor. This unique form of uncoupling may be responsible for the enhanced isoproterenol induced desensitization. We hypothesize that because of its inability to stabilize the ternary complex, it is rapidly released from binding to G_s and thus more available for phosphorylation. This increase in phosphorylation appears to be βARK mediated, since dibutyryl cAMP mediated phosphorylation is not enhanced in a like manner in this mutant. This is similar to that reported for the analogous G-protein coupled rhodopsin system, where uncoupled rhodopsin is a better substrate for rhodopsin kinase than the rhodopsin-transducin complex (12). These current studies with the ^{184}CYS(VAL) mutant should not imply that receptor uncoupling due to other structural alterations in the β_2AR would necessarily lead to enhanced desensitization and phosphorylation. Rather, as in this case, specific alterations of the receptor may enhance desensitization despite receptor uncoupling, suggesting that the regions of the molecule responsible for these properties are distinct.

Acknowledgments: The authors thank Grace Irons for tissue culture technical assistance and Mary Holben for manuscript preparation. Stephen B. Liggett is supported by an RJR-Nabisco Research Scholar Award and Michel Bouvier is supported by a Centennial Fellowship from the Medical Research Council of Canada.

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