Desensitization of β2-Adrenergic Receptors with Mutations of the Proposed G Protein-coupled Receptor Kinase Phosphorylation Sites

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Tentative identification of the G protein-coupled receptor kinase 2 and 5 (GRK2 and GRK5) sites of phosphorylation of the β2-adrenergic receptor (βAR) was recently reported based on in vitro phosphorylation of recombinant receptor (Fredericks, Z. L., Pitcher, J. A., and Lefkowitz, R. J. (1996) J. Biol. Chem. 271, 13796–13803). Phosphorylated residues identified for GRK2 were threonine 384 and serines 396, 401, and 407. GRK5 phosphorylated these four residues as well as threonine 393 and serine 411. To determine if mutation of these sites altered desensitization, we have constructed βARs in which the threonines and serines of the putative GRK2 and GRK5 sites were substituted with alanines. These constructs were further modified to eliminate the cAMP-dependent protein kinase (PKA) consensus sites. Mutants βARs were transfected into HEK 293 cells, and standard kinetic parameters were measured following 10 μM epinephrine treatment of cells. The mutant and wild type (WT) receptors were all desensitized 89–94% after 5 min of 10 μM epinephrine stimulation and 96–98% after a 30-min pretreatment. There were no significant changes observed for any of the mutant βARs relative to the WT in the extent of 10 μM epinephrine-induced internalization (77–82% after 30 min). Epinephrine treatment for 1 min induced a rapid increase in the phosphorylation of the GRK5 and PKAβAR mutant βARs as well as the WT. We conclude that sites other than the GRK2 and GRK5 sites identified by in vitro phosphorylation are involved in mediating the major effects of the in vivo GRK-dependent desensitization of the βAR.

Epinephrine stimulation of the β2-adrenergic receptor (βAR) in intact cells activates the receptor and rapidly induces its desensitization. The decreased responsiveness of the receptor after stimulation by near-saturating concentrations of epinephrine appears to be caused by rapid cAMP-dependent protein kinase (PKA) and G protein-coupled receptor kinase (GRK) phosphorylation. GRK phosphorylation in turn promotes β-arrestin binding and receptor internalization (1, 2). Identification of the specific amino acids phosphorylated by these protein kinases has been the focus of numerous studies. Through the use of several deletion and substitution mutants, the sites for PKA-mediated desensitization of the βAR in intact cells were shown to be serines 261 and 262 in the third intracellular loop PKA consensus site (3–5). For the GRKs, mutagenesis studies indicate the involvement of 11 serines and threonines in the carboxyl terminus (5, 6). By utilizing in vitro GRK phosphorylation of recombinant βAR reconstituted into liposomes followed by sequencing of proteolytic fragments of the carboxyl tail, it was found that four sites were phosphorylated by GRK2 (βAR kinase 1), serines 396, 401, and 407, and threonine 384, and six by GRK5 that included the same four phosphorylated by GRK2 and additionally threonine 393 and serine 411 (7). On the basis of this study it was proposed that these amino acids were the sites of GRK-mediated phosphorylation in intact cells; however, the effects of mutating these sites on the desensitization of the βAR in vivo was not addressed.

In the studies presented here, we have determined the effects of substitutions of the putative GRK phosphorylation sites identified by the in vitro approach of Fredericks et al. (7) on the desensitization, internalization, and phosphorylation of the respective mutant βARs. The serine or threonine residues tentatively identified as the GRK2 and GRK5 phosphorylation sites were replaced with alanine. To aid our analysis of the effects of these mutations, we also replaced the serine residues of the two consensus PKA phosphorylation sites with alanine to eliminate PKA-mediated desensitization and phosphorylation. The GRK/PKA mutants (designated as GRK2(−) or GRK5(−)), as well as a mutant βAR containing only the PKA substitutions (PKA−), were constructed in the WTβAR that had been modified by placement of the hemagglutinin (HA) antigen at the amino terminus and six histidine residues at the carboxyl terminus. We recently established that the desensitization, internalization, and phosphorylation of this double epitope-modified βAR, stably transfected into HEK 293 cells, was indistinguishable from the wild type receptor (8). Furthermore, the HEK 293 cell line offers a system in which the effects of overexpressed GRK2 on βAR phosphorylation and internalization have been studied (9) and in which endogenous GRK2 expression has been shown (10). Our results demonstrate that the GRK2 substitutions did not significantly alter epinephrine-induced desensitization of the βAR, although a slight reduction of the rate and extent of desensitization was observed with the GRK5 substitutions. Consistent with these observations, we found that the mutant βARs were rapidly phosphorylated and that the rates of inter-

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1 The abbreviations used are: βAR, human β2-adrenergic receptor; HA, hemagglutinin; GRK, G protein-coupled receptor kinase; WT, wild type; PKA, cAMP-dependent protein kinase; 125I-ICYP, [125]iodocyanopindolol; PAGE, polyacrylamide gel electrophoresis; CGP, 1H(CGP-12177; DMEM, Dulbecco's modified Eagle's medium; AT, ascorbic acid/thiouric; WGA, wheat germ agglutinin; PVDF, polyvinylidene difluoride; PCR, polymerase chain reaction; GTPγS, guanosine 5′-3′-(thio)triphosphate.
eralization was unimpaired. The lack of any major effects on these parameters suggests that the GRK site(s) that mediate the desensitization and subsequent internalization of the β2AR do not involve the sites identified by in vitro phosphorylation.

**EXPERIMENTAL PROCEDURES**

**Construction of the Mutant β2ARs**—The construction of the plasmid containing the HA and six histidine-tagged βAR has been described previously (8). This plasmid is designated here as WTβAR. For construction of the mutant βARs, the HA-His+ tagged βAR was excised from the pBIC12B1 plasmid as an EcoRI/SalI fragment, made blunt-ended, and ligated into the expression vector pKHN that had been HindIII-digested and blunt-ended. All mutants were constructed using polymerase chain reaction (PCR) methods. To change the serines at 261 and 262 to alanines (third intracellular loop PKA consensus site), a two-step PCR mutagenesis method was used with the HA-His+ βAR in pBIC12B1 as template. In the first step, two independent reactions were carried out, one using a sense mutagenizing oligonucleotide paired with a downstream oligonucleotide, and the other using an antisense mutagenizing oligonucleotide paired with an upstream oligonucleotide. In the second step, the products of the first PCR reactions were amplified using a pair of oligonucleotides nested within the upstream and downstream oligonucleotides. The resulting product was digested with AccI and HindIII, and the linearized plasmid pC6Ha was treated with T4 DNA ligase. The mutant receptor was excised as a BamHI/HindIII fragment, blunt-ended, and subcloned into HindIII-digested, blunt-ended pKHN. The S261A and S262A mutant HA-His+ tagged βAR in pKHN served as a template for subsequent mutagenesis. All other mutageneses were performed with single PCR reactions using mutagenizing sense and antisense oligonucleotides and/or primers and, in addition, has threonine 393 and serine 408 changed to alanine. The four residues Thr-384, Ser-396, Ser-401, and Ser-407 are alanine. The mutant & βAR designated PKA-α1—has alanine substituted for the serines of both PKA consensus sites and can be designated as Ser-261A, Ser-262A, Ala, Ser-345→Ala, Ser-346→Ala, and Ser-346→Ala. The mutant βAR designated GRK2-2 was constructed from the PKA-α1 mutant and, in addition, has threonine 384 and serines 396, 401, and 407 changed to alanine. The four residues Thr-384, Ser-396, Ser-401, and Ser-407 are those identified by Fredericks et al. (7) as the sites of in vitro phosphorylation of the βAR by GRK2. The mutant designated GRK2-2 was constructed from GRK2-2 and, in addition, has alanine substituted for the serine at position 382. The four residues Thr-384, Ser-396, Ser-401, and Ser-411 were changed to alanine. The five residues Thr-384, Thr-393, Ser-396, Ser-401, Ser-407, and Ser-411 are those identified by Fredericks et al. (7) as the sites of in vitro phosphorylation of the βAR by GRK5.

**Transfection into HEK 293 Cells**—The HEK 293 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum at 37 °C in 5% CO2. Each mutant plasmid was linearized by Alu digestion and transfected into the HEK 293 cells using the CaPO4 precipitation method. The day after transfection the cells were shocked with 25% ethanol for 2 min. Following ethanol shock, the cells were washed with serum-free DMEM, the cells were removed by pipetting up and down with 200–500 μl of serum-free DMEM. Trypsinization reactions were performed in DMEM containing ~200 μM 125IICYP, in a total assay volume of 200 μl. Nonspecific binding was measured with the addition of 1 μM alprenolol. The reactions were incubated on ice for 50 min and terminated by dilution with 2.5 ml of ice-cold 50 mM Tris-HCl, pH 7.5, 10 mM MgCl2. The 125IICYP-bound receptor protein was isolated by filtration through Whatman GF/C filters. The filters were rinsed three times with 2.5 ml of the Tris/MgCl2 buffer and counted in a Beckman 4000 Gamma counter. Protein was measured in duplicate or triplicate with a coating enzyme immunoassay (ELISA). The fraction at the 224 nm interface was removed, flash-frozen in liquid nitrogen, and stored at −80 °C.

**Quantitation of Desensitization**—As we have previously shown, the expression for coupling efficiency can be combined with that for Vmax to give Equation 1 (8).

\[
V_r = \frac{V_{\text{max}} (K_r) (C)}{(V_{\text{max}} (K_r) (C)). (E_{50})} 
\]  

This equation describes the coupling capacity, \( V_r \), where \( V_{\text{max}} \) is the maximum adenyl cyclase activity measured for saturation against 10 μM ATP, and \( V_{\text{max}} \) is the theoretical value for adenyl cyclase. The increase in \( E_{50} \) and the decrease in \( V_{\text{max}} \) that occurs during desensitization can be quantitated using the expression for coupling capacity. The extent of desensitization can be expressed as the ratio of receptor coupling capacity in the desensitized relative to naive state. Since the \( K_r \) and \( V_{\text{max}} \) values do not change upon desensitization, the ratio can be expressed as shown in Equation 2.

\[
\frac{(k_r)_{E_{50}}}{(k_r)_{E_{50}}} = \frac{(V_{\text{max}} (K_r) (C))}{(V_{\text{max}} (K_r) (C))}. 
\]  

The expression \( (k_r)_{E_{50}} \) is defined as the fraction activity remaining.
Membranes were prepared from naive HEK 293 cells expressing the wild type or mutated βARs. Basal adenyl cyclase activity and the $V_{\text{max}}$ and EC$_{50}$ values for epinephrine stimulation were measured as described under “Experimental Procedures.” The results are the mean ± S.E., with the number of determinations (n) in parentheses.

| Cell line | Basal adenyl cyclase | $V_{\text{max}}$ for epinephrine | EC$_{50}$ for epinephrine | Receptor level | $K_f$ for epinephrine |
|-----------|----------------------|---------------------------------|--------------------------|----------------|-----------------------|
| WT βAR    | 8.3 ± 1.2 (5)        | 7.5 ± 0.3 (5)                   | 3.5 ± 0.3 (5)            | 3363 ± 118 (5) | 339 ± 49 (4)          |
| PKA       | 8.6 ± 0.8 (6)        | 11.9 ± 11.6 (6)                 | 15.7 ± 1.2 (6)          | 2311 ± 220 (6) | 503 ± 46 (3)          |
| GRK2      | 7.2 ± 0.9 (7)        | 91.1 ± 11.4 (7)                 | 17.1 ± 2.0 (7)          | 1532 ± 167 (6) | 374 ± 24 (3)          |
| GRK5      | 4.9 ± 0.78 (7)       | 84.8 ± 10.1 (7)                 | 22.9 ± 5.0 (7)          | 2497 ± 399 (6) | 489 ± 37 (3)          |

values. This calculation can be converted to percent desensitization by multiplying the fraction of βAR activity remaining by 100 and subtracting that value from 100. Desensitization data in Fig. 3 and Table II are presented as the mean of the fraction activity remaining ± S.E.

The apparent rates of desensitization and internalization were determined using GraphPad software for monoeponential decay. With several mechanisms contributing to desensitization and internalization, the data cannot be explained by a simple monoeponential decay. However, it is useful to give a $t_{1/2}$ for the sum of the total process.

**Determination of βAR Phosphorylation**—To measure phosphorylation of the βAR, confluent cells were washed three times in phosphate-free DMEM, incubated for 3 h with $[^{32}P]\text{H}_{3}\text{PO}_{4}$ (0.5–1.0 mCi/100-mm dish), and pretreated for the indicated times with either 10 μM epinephrine or AT carrier. The cells were solubilized, and the extracts were subjected to a two-step purification using nickel nitrilotriacetic acid agarose and wheat germ agglutinin-agarose (WGA) as described previously (8) with the following modifications. The nickel nitrilotriacetic acid eluent fractions containing the βAR were mixed with 100 μl of WGA (packed volume) and incubated for 90 min at 4 °C with rocking. The WGA/WGA conjugates were collected and washed five times with 5 ml of nickel column buffer (0.05% n-dodecyl-β-d-maltoside, 20 mM Hepes, pH 7.4, and 150 mM NaCl) at 4 °C. The WGA was further washed twice with 400 μl of 0.5% sodium dodecyl sulfate (SDS) at 37 °C for a total incubation time of 10 min. The WGA pellet was collected and the βAR eluted with SDS sample buffer (50 mM Tris, pH 6.8, 2% SDS, 0.025% bromphenol blue, 6 μl urea, 14.3 μM β-mercaptoethanol). The receptor was resolved on 7.5% SDS-polyacrylamide gels with the addition of pre-stained low molecular weight standards (Bio-Rad). The gels were dried and exposed to a phosphor screen for 2–24 h, and the data were analyzed using a Molecular Dynamics Storm PhosphorImager model 860 and Imagequant software. Autoradiograms of the dried gels were also obtained (24–48 h). In some experiments the gels following SDS-PAGE were transferred to 0.22-micron PVDF membranes, and the identity of the radiolabeled band as the βAR was confirmed by Western analysis using a polyclonal anti-HA polyclonal antibody (Babco) and a horseradish peroxidase-conjugated goat anti-rabbit (Bio-Rad) as the secondary antibody as described previously (8).

**RESULTS**

**Determination of the Coupling Efficiency for Epinephrine Activation of Adenyl Cyclase for the Mutant and WT βARs**—As we have previously shown, determination of the coupling efficiency for agonist activation of adenyl cyclase requires the measurement of receptor levels, the low affinity $K_f$ for agonist binding, and the EC$_{50}$ for activation of adenyl cyclase (8). A summary of these determinations using membranes prepared from each cell line is shown in Table I. At least two clones expressing each receptor were examined, and those shown in Table I were selected for all subsequent experiments since they expressed reasonably similar levels. A representative experiment for the determination of the low affinity $K_f$ for epinephrine binding is shown in Fig. 1. We found no significant differences in the $K_f$ values for the mutant receptors relative to the WT. The WT and mutant WT cell lines displayed similar values for basal activity and the $V_{\text{max}}$ for epinephrine stimulation. The EC$_{50}$ values for epinephrine stimulation of the mutant receptors were found to be consistently 4–6.5 times higher than that of the WTβAR (see Fig. 2 for data summary). Using the formulations we described previously (8, 19), we calculated from the data in Table I that the coupling efficiencies of the mutants were reduced by a factor of 2–4-fold relative to WTβAR.

**Desensitization of the Mutant and WT βARs**—To assess desensitization, HEK 293 cells stably expressing the WT or mutant βARs were pretreated with 10 μM epinephrine for various times from 0.5–30 min. Following pretreatment, membranes were prepared and assayed for epinephrine-stimulated adenyl cyclase activity using a range of epinephrine concentrations. The data summary for desensitization in response to 2 and 30 min pretreatment with 10 μM epinephrine is shown in Fig. 2. These data as well as data from the other time points of 10 μM epinephrine pretreatment are summarized in Fig. 3. The extent of desensitization was quantitated as fraction activity remaining by measuring the right-shift in EC$_{50}$ and decrease in $V_{\text{max}}$ as described under “Experimental Procedures” (8). The fraction activity remaining for the 2- and 30-min time points for the various receptors are also shown in Table II, along with the results of calculations of the $t_{1/2}$ values for the apparent rates of desensitization. The $t_{1/2}$ values were determined by fitting the data to an equation for monoeponential decay. Although the 30-min data did not fit well to the theoretical curve, this method allowed calculation of approximate apparent rates of desensitization for comparison of the WT and mutant βARs. The data demonstrate that there was no significant difference in the apparent rate or extent of desensitization for the PKA$\odot$ relative to the WTβAR. Although there was a slightly reduced apparent rate and extent of desensitization for the GRK2$\odot$ and the GRK5$\odot$, the decrease was only significant for the GRK5$\odot$ βAR.

**Internalization of the WT and Mutant βARs**—Internalization of the mutant and WT βARs in response to 10 μM epinephrine was measured by CGP binding and is plotted as the loss of surface receptors (Fig. 4). The apparent rate of internalization was de-
determined by fitting the data shown in Fig. 4 to an equation for monoexponential decay. The fit to monoexponential decay did not take into account the initial lag observed for internalization of all the receptor types. This method, however, allowed calculation of approximate apparent rates of internalization for comparison of the WT and mutant bARs. The apparent rate of internalization of the WT bAR (2.96 min ± 0.17, n = 3) was found to be similar to those measured for the GRK2 (2.96 min ± 0.30, n = 3), the GRK5 (3.76 min ± 0.25, n = 3), and the PKA (3.69 min ± 0.28, n = 3) bAR mutants. The extent of internalization was also similar, with 80% ± 1.9 (n = 9) of the WT bAR internalized after 30 min of 10 μM epinephrine pretreatment compared with 84% ± 0.5 (n = 6), 82% ± 1.1 (n = 5), and 77% ± 0.5 (n = 4) for the GRK2, GRK5, and PKA mutants, respectively. The internalization was not the result of receptor loss or down-regulation, as determined by CGP binding in the presence and absence of digitonin as described under “Experimental Procedures” (data not shown).

Phosphorylation of the Mutant and Wild Type bARs in Response to 10 μM Epinephrine—Cells expressing the WT, GRK5, and PKA bARs were labeled with 32P for 3 h and subsequently treated with either carrier or 10 μM epinephrine for 1 min. Phosphorylation of the bAR was assessed by solubilization and purification of the receptors using the two-step affinity chromatography procedure described under “Experimental Procedures.” The purified receptor was subjected to SDS-PAGE, and the proteins were transferred to PVDF membranes as described under “Experimental Procedures.” A representative experiment performed in duplicate is shown in Fig. 5. The PhosphorImage scan of the gel after transfer to the PVDF membrane is shown in Fig. 5A, and the Western blot of the same membrane is seen in Fig. 5B. The time course of phosphorylation for the mutant bARs was similar to what we have previously reported for the WT bAR (8), with the peak at about 1 min, declining after 5 min (data not shown).

DISCUSSION

Our experiments demonstrate that mutant bARs containing alanine substitutions for the serine/threonine residues, tentatively identified by in vitro phosphorylation as the sites of GRK2 or GRK5 phosphorylation (7), undergo extensive and rapid agonist-induced desensitization and internalization. We had expected that these substitutions of the putative GRK and PKA sites would eliminate the desensitization of the bAR. Consistent with the desensitization data, we found that the GRK5 mutant was rapidly phosphorylated. We propose that sites other than or in addition to those identified in vitro by Fredericks et al. (7) are required for in vivo GRK2 or GRK5 phosphorylation and desensitization of the bAR.

It is possible that one or more of the crucial GRK sites involved in the functional desensitization of the receptor were missed in the in vitro study of GRK2 and GRK5 phosphorylation (7). The sequencing of peptides in this study was focused on a fragment located in the distal portion of the receptor carboxyl terminus (residues 374–413). Thus, it remains possible that serines 355, 356, and 364 and threonine 360 residues...
Table II
Characterization of 10 μM epinephrine-induced desensitization of βARs expressed in HEK 293 cells

| Cell line | Time of pretreatment | fraction activity remaining | τ_{1/2} |
|-----------|----------------------|-----------------------------|---------|
|           | 2 min                | 30 min                      | |
| WT βAR    | 0.13 ± 0.02 (3)      | 0.024 ± 0.003 (3)           | 0.38 ± 0.04 (3) |
| PKA−      | 0.16 ± 0.05 (2)      | 0.027 ± 0.003 (3)           | 0.45 ± 0.06 (3) |
| GRK2−     | 0.21 ± 0.05 (3)      | 0.04 ± 0.002 (3)            | 0.56 ± 0.07 (3) |
| GRK5−     | 0.21 ± 0.02 (5)\*    | 0.042 ± 0.004 (5)\*         | 0.65 ± 0.06 (4) \* |

\*The fraction activity remaining (extent of desensitization) measured for the WT βAR and the GRK5− mutant after 2 and 30 min 10 μM epinephrine pretreatment were compared using an unpaired t test and were found to be significantly different, p < 0.05.

\*The apparent rate of desensitization calculated from the data in Fig. 5 using the equation for monoexponential decay (not shown).

Fig. 3. Time course of epinephrine-induced desensitization. Cells expressing the WTβAR (●), the PKA− (○), the GRK2− (□), or the GRK5− (△) were pretreated with 10 μM epinephrine from 0.5 to 30 min, and membranes were prepared and assayed as described in Fig. 2. The extent of desensitization induced after pretreatment with 10 μM epinephrine was quantitated as fraction activity remaining using Equation 2 under “Experimental Procedures.” For each receptor type, 3–5 independent time courses were assayed in triplicate with a full epinephrine dose response at each time point. The data shown are the mean ± S.E. for 3–5 experiments, except for the 2 min pretreatment of PKA− (mean ± range, n = 2). The lines are drawn point-to-point and are not fit to a theoretical curve. The apparent rate of desensitization (Table II) was determined by fitting the data to an equation for monoexponential decay (not shown).

Fig. 4. Receptor internalization in response to epinephrine. Cells expressing the WTβAR (●), the PKA− (○), the GRK2− (□), or the GRK5− mutant (△) were pretreated with either carrier or 10 μM epinephrine for 1–30 min, and surface receptor number was measured in triplicate for each time point using CGP as described under “Experimental Procedures.” The data shown are the mean ± S.E. of 3–9 experiments, or the mean ± range (where n = 2). The lines are drawn point-to-point and are not fit to a theoretical curve. The apparent rate of internalization given in the text was determined by fitting the data to an equation for monoexponential decay (not shown).

Fig. 5. Phosphorylation of the WTβAR, GRK5−βAR, and the PKA−βAR. Cells expressing the various receptors were labeled for 3 h with 32P and pretreated for 1 min with either AT carrier or 10 μM epinephrine (epi) in duplicate. The βAR was solubilized, purified, resolved by SDS-PAGE, and transferred to PVDF as described under “Experimental Procedures.” The PhosphorImager scan of the PVDF membrane is shown in A, and the Western blot performed on the same membrane is shown in B. The data presented are representative of 3 independent experiments.
with the possibility that serines 355, 356, and 364 and threonine 360 may include the in vivo sites of GRK phosphorylation.

Another possible explanation for the discrepancy we have found between the in vitro phosphorylation of the βAR and our studies of the functional effects of these mutations when expressed and analyzed following intact cell treatment is that additional sites may be nonspecifically phosphorylated by GRK2 or GRK5 in vitro. precedent for this possibility is found in recent studies of the rhodopsin receptor that demonstrated important differences between in vivo and in vitro identification of GRK phosphorylation sites. Chemical identification of the sites phosphorylated in vivo by a member of the GRK family has been described for the rhodopsin receptor by Ohguro et al. (21). They found that two sites in the receptor carboxyl terminus were phosphorylated by rhodopsin kinase (GRK1). The two serines they identified were differentially regulated; serine 338 was phosphorylated in response to flashes of light, whereas serine 334 was phosphorylated more slowly, and only after continuous light exposure. In rather striking contrast to whereas serine 334 was phosphorylated more slowly, and only after continuous light exposure. In rather striking contrast to these studies, in vivo phosphorylation consistently identified 7–8 mol of phosphate/mol of rhodopsin receptor (22, 23). Based on these studies of rhodopsin at least, it is reasonable to expect that there may be substantial differences between in vivo and in vivo phosphorylations of the βAR.

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