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Desensitization of β_2-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 β_2-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 PKA$^-$ 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 β_2-adrenergic receptor (βAR)[1] 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 β_2-adrenergic receptor; HA, hemagglutinin; GRK, G protein-coupled receptor kinase; WT, wild type; PKA, cAMP-dependent protein kinase; ^{125}ICYP, [^{125}]iodocyanopindolol; PAGE, polyacrylamide gel electrophoresis; CGP, [^{3}H]CGP-12177; DMEM, Dulbecco's modified Eagle's medium; AT, ascorbic acid/thiourea; WGA, wheat germ agglutinin; PVDF, polyvinylidene difluoride; PCR, polymerase chain reaction; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate.

nalization were 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 βAR do not involve the sites identified by *in vitro* phosphorylation.

EXPERIMENTAL PROCEDURES

Construction of the Mutant βARs—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_6-tagged βAR was excised from the pBC12B1 plasmid as an *Nco*I/*Sal*I fragment, made blunt-ended, and ligated into the expression vector pKNH that had been *Hin*dIII-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_6 βAR in pBC12B1 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 *Acc*I and subcloned into the plasmid pGEM3Z (Promega). The mutagenized receptor was excised as a *Bam*HI/*Hin*dIII fragment, blunt-ended, and subcloned into *Hin*dIII-digested, blunt-ended pKNH. The S261A and S262A mutant HA-His_6-tagged βAR in pKNH served as a template for subsequent mutagenesis. All other mutageneses were performed with single PCR reactions using mutagenizing sense and antisense oligonucleotides and *Pfu* I polymerase (Stratagene). After PCR, digestion with *Dpn*I (which requires methylated DNA) was performed to remove the non-mutagenized template DNA, followed by transformation into XL1 Blue competent cells. The entire length of each mutant βAR was sequenced to verify the changes and to ensure that no other alterations were introduced by PCR.

The mutant βAR designated PKA^- had alanine substituted for the serines of both PKA consensus sites and can be described as Ser-261 → Ala, Ser-262 → Ala, Ser-345 → Ala, and Ser-346 → Ala. The mutant βAR designated $GRK2^-$ was constructed from the PKA^- 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 $GRK5^-$ was constructed from $GRK2^-$ and, in addition, has threonine 393 and serine 411 changed to alanine. The six 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% CO_2. Each mutant plasmid was linearized by *Pvu*I digestion and transfected into the HEK 293 cells using the $CaPO_4$ method. The day after transfection the cells were shocked with 25% glycerol in DMEM and placed in media containing 0.4 mg/ml G418 the day after shocking. Stable transfectants were identified using an intact cell [^{125}I]iodocyanopindolol (^{125}ICYP) binding assay described below.

Measurement of Receptor Levels—To measure intact cell receptor number by ^{125}ICYP binding, cells were grown in 12-well dishes. After rinsing with serum-free DMEM, the cells were removed by pipetting up and down with 200–500 μl of serum-free DMEM. Triplicate reactions were performed in DMEM containing ≈200 pM ^{125}ICYP, 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 $MgCl_2$. The ^{125}ICYP-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/$MgCl_2$ buffer and counted in a Beckman 4000 Gamma counter. Protein was measured in duplicate or triplicate with 100 μl of cells. To measure βAR levels in membranes, 5 μg of membrane protein was used per reaction containing 0.1 mM phentolamine, 40 mM Hepes, pH 7.2, 2 mM EDTA, 0.2 mM ascorbate, and 2 mM thiourea, and ≈200 pM ^{125}ICYP in the presence or absence of 1 μM alprenolol. The reactions were incubated at 30 °C for 50 min and terminated as described for the intact cell binding.

Measurement of Equilibrium Binding Constants for Epinephrine—The K_d values for epinephrine were determined by displacement of ^{125}ICYP using methods previously described (8). The ^{125}ICYP was prepared according to the method of Barovsky and Brooker (11) and Hoyer *et al.* (12). Alprenolol (1 μM) was included to measure nonspecific binding. The reactions were incubated for 50 min at 30 °C and stopped as described above. The reactions included 40–50 pM ^{125}ICYP, 10 μM GTPγS, and concentrations of epinephrine ranging from 0.1 to 100 μM. The data were fit to a one-component competition sigmoidal curve with a Hill coefficient of −1 using GraphPad software and K_d values determined using the Cheng-Prusoff formulation.

Measurement of Receptor Internalization by [^{3}H]CGP-12177 Binding in Intact Cells—Cells were plated onto 60-mm dishes coated with poly-L-lysine (Sigma) to improve cell adhesion. The cells were pretreated with 10 μM epinephrine or carrier by additions made directly to growth medium from 100× stock solutions. The 100× epinephrine stock (1 mM) was prepared in 100× AT carrier, such that the final concentration of AT was 0.1 mM ascorbate and 1 mM thiourea, pH 7. Controls were treated with the AT carrier at the same final concentration. Pretreatment was performed at 37 °C for various times and was stopped by removal of media and 6 washes with 2 ml of ice-cold serum-free DMEM, pH 7. Surface receptor number was then measured with the addition of 2 ml of serum-free DMEM containing 5 nM [^{3}H]CGP-12177, designated CGP hereafter. Incubations were on ice for 1 h. To measure nonspecific binding, cells were incubated with 1 μM alprenolol added to the CGP mix. To measure total receptor number, including internalized βAR, digitonin was added to the binding mix (including alprenolol controls) to a final concentration of 0.2% as described previously (8, 13). The reactions were stopped by removal of the binding mix followed by 3 washes with ice-cold DMEM, 2 ml each. The cells were scraped into 0.75 ml of trypsin and counted in 5 ml of scintillation fluid. Measurements were performed in triplicate for each time point. Additional plates that were washed identically to the experimental plates were used to measure protein. The surface receptor number is expressed relative to the AT-treated control in each experiment. GraphPad software was used to fit the data to an equation for monoexponential decay and determine the apparent rate of internalization.

Membrane Preparation—Cells were plated into 150-mm dishes coated with poly-L-lysine and were pretreated at 37 °C with 10 μM epinephrine or AT carrier for the indicated times. The pretreatment was stopped with 6 washes of 10 ml of ice-cold HME buffer (20 mM Hepes, pH 8.0, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM benzamidine, 10 μg/ml trypsin inhibitor, 0.1 mg/ml bovine serum albumin). The washed cells were scraped into HME plus 10 μg/ml leupeptin, 20 mM tetrasodium pyrophosphate, and 0.1 μM okadaic acid and homogenized with 7 strokes in a type B Dounce homogenizer. The homogenates were layered onto step gradients of 23 and 43% sucrose prepared in HE buffer (20 mM Hepes, pH 8.0, 1 mM EDTA) and centrifuged at 25,000 rpm in a Beckman SW28.1 rotor for 35 min. The fraction at the 23/43% interface was removed, flash-frozen in liquid nitrogen, and stored at −80 °C.

Adenylyl Cyclase Assay—Adenylyl cyclase activity was assayed by a modification of the method described by Salomon *et al.* (14). Membranes were diluted to a final protein concentration of 0.2–0.4 mg/ml and were incubated for 10 min at 30 °C with 40 mM Hepes, pH 7.7, 1 mM EDTA, 1.34 mM $MgCl_2$, 8 mM creatine phosphate, 16 units/ml creatine kinase, 100 μM ATP, 1 μM GTP, 0.1 mM 1-methyl-3-isobutylxanthine, 2 μCi of [α-^{32}P]ATP (NEN Life Science Products, 30 Ci/mmol) in a total volume of 100 μl. The final free Mg^{2+} concentration was calculated to be 0.3 mM to optimize desensitization measurements (3, 15, 16). Each point was assayed in triplicate, with 6–8 concentrations of epinephrine bracketing the EC_{50}. The [^{32}P]cAMP produced in the reaction was purified over Dowex and alumina columns (17). The V_{max} and EC_{50} values were determined with GraphPad software.

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

$$(k_1)r = (V_{max})(K_d)/(V_{100})(EC_{50}) \quad \text{(Eq. 1)}$$

This equation describes the coupling capacity, $(k_1)r$, where V_{max} is the maximum adenylyl cyclase activity measured for saturating agonist concentrations, and V_{100} is the theoretical value when k_1 is infinite. The increase in EC_{50} and the decrease in V_{max} that occurs with 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_d and V_{100} values do not change upon desensitization, the ratio can be expressed as shown in Equation 2.

$$(k_1r)_D/(k_1r)_N = (V_{max})_D(EC_{50})_N/(V_{max})_N(EC_{50})_D \quad \text{(Eq. 2)}$$

The expression $(k_1r)_D/(k_1r)_N$ is defined as the fraction activity remaining and is quantitated using experimentally determined EC_{50} and V_{max}

TABLE I
Characterization of βARs expressed in HEK 293 cells

Membranes were prepared from naive HEK 293 cells expressing the wild type or mutated βARs. Basal adenylyl cyclase activity and the V_{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 adenylyl cyclase	V_{max} for epinephrine	EC_{50} for epinephrine	Receptor level	K_d for epinephrine
	pmol cAMP/min/mg	*pmol cAMP/min/mg*	*nM*	*fmol/mg*	*nM*
WT βAR	8.3 ± 1.2 (5)	75.5 ± 8.6 (5)	3.5 ± 0.3 (5)	3363 ± 118 (5)	339 ± 49 (4)
PKA^-	8.6 ± 0.8 (6)	119.9 ± 11.6 (6)	15.7 ± 1.2 (6)	2311 ± 220 (6)	503 ± 46 (3)
$GRK2^-$	7.3 ± 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 monoexponential decay. With several mechanisms contributing to desensitization and internalization, the data cannot be explained by a simple monoexponential decay. However it is useful to give a $t_{½}$ 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 $[^{32}P]H_3PO_4$ (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/βAR was collected and washed 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 M urea, 14.3 mM 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 phosphorscreen 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 primary 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 Adenylyl Cyclase for the Mutant and WTβARs—As we have previously shown, determination of the coupling efficiency for agonist activation of adenylyl cyclase requires the measurement of receptor levels, the low affinity K_d for agonist binding, and the EC_{50} for activation of adenylyl 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_d for epinephrine binding is shown in Fig. 1. We found no significant differences in the K_d values for the mutant receptors *versus* the WT. The mutant and WT cell lines displayed similar values for basal activity and the V_{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.

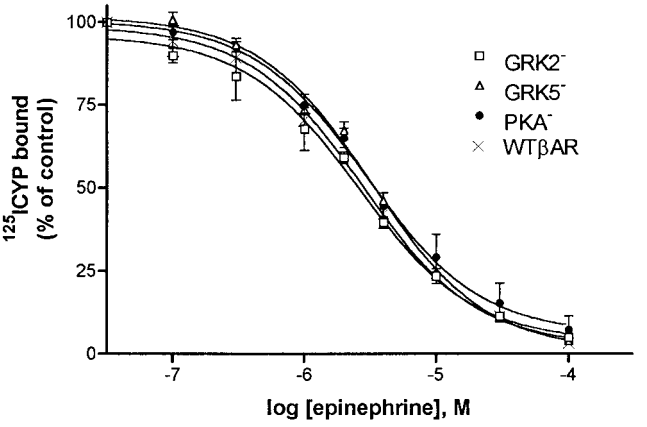

FIG. 1. **Epinephrine displacement of ^{125}ICYP binding in the WT and mutant βARs.** Membranes were prepared in parallel from untreated HEK 293 cells expressing the WTβAR (×), PKA^- (●), $GRK2^-$ (□), or the $GRK5^-$ (△). K_d values were determined by fitting the data to a one-component competition curve as described under "Experimental Procedures." The *lines* shown are theoretical curves calculated from the fitted parameters.

Desensitization of the Mutant and WTβARs—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 adenylyl 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_{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 $t_{½}$ values for the apparent rates of desensitization. The $t_{½}$ values were determined by fitting the data to an equation for monoexponential 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^- relative to the WTβAR. Although there was a slightly reduced apparent rate and extent of desensitization for the $GRK2^-$ and the $GRK5^-$, the decrease was only significant for the $GRK5^-$ βAR.

Internalization of the WT and Mutant βARs—Internalization of the mutant and WTβARs 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-

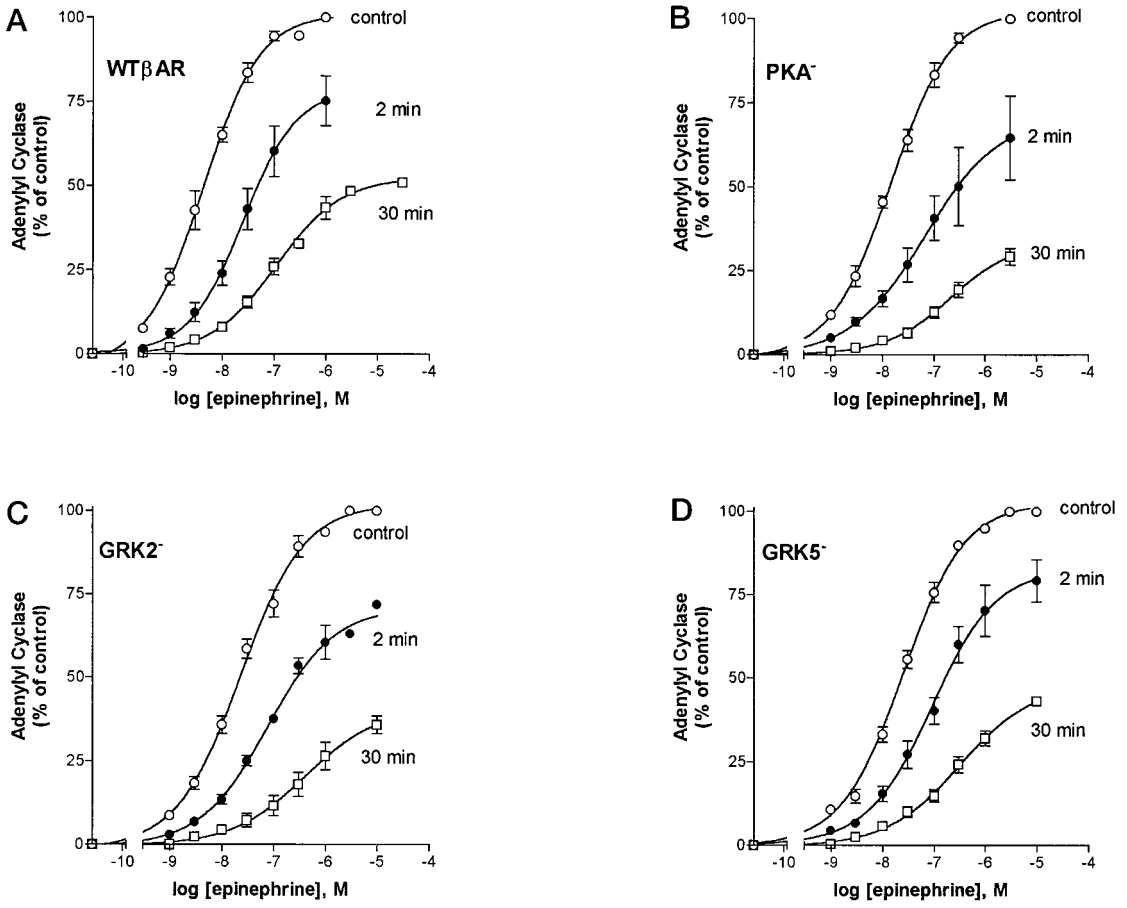

FIG. 2. **Epinephrine-induced desensitization of the WT and mutant βARs.** Cells expressing the WTβAR (*A*), PKA$^-$ (*B*), GRK2$^-$ (*C*), or GRK5$^-$ (*D*) were pretreated with either carrier (○) or with 10 μM epinephrine for 2 (●) or 30 (□) min. Membranes were prepared, and adenylyl cyclase was assayed in triplicate for each experiment with the various epinephrine concentrations as indicated for each experiment. The data are normalized relative to the V_{max} for epinephrine of the control sample (100%) for each experiment after subtraction of basal. Data shown are the mean ± S.E. of 3–5 experiments or the mean ± range of 2 experiments. The absence of error bars indicates data points that were the average of triplicate determinations from one experiment.

termined 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 βARs. The apparent rate of internalization of the WTβAR (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, $n = 3$) βAR mutants. The extent of internalization was also similar, with 80% ± 1.9 ($n = 9$) of the WTβAR 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 βARs in Response to 10 μM Epinephrine—Cells expressing the WT, GRK5$^-$, and PKA$^-$ βARs were labeled with ^{32}P for 3 h and subsequently treated with either carrier or 10 μM epinephrine for 1 min. Phosphorylation of the βAR 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. 5*A*, and the Western blot of the same membrane is seen in Fig. 5*B*. The time course of phosphorylation for the mutant βARs was similar to what we have previously reported for the WTβAR (8), with the peak at about 1 min, declining after 5 min (data not shown).

DISCUSSION

Our experiments demonstrate that mutant βARs 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 βAR. 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 βAR.

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

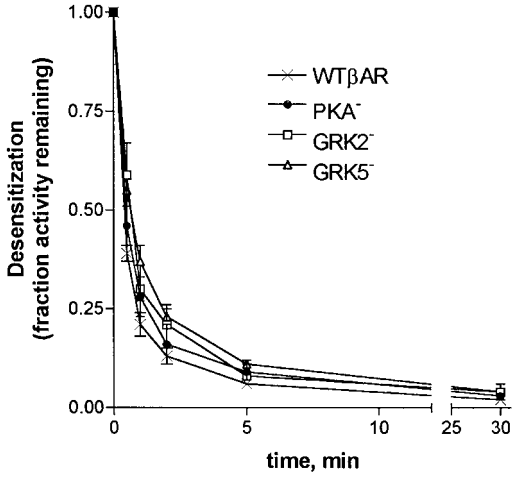

FIG. 3. **Time course of epinephrine-induced desensitization.** Cells expressing the WTβAR (×), the PKA⁻ (●), the GRK2⁻ (□), or the GRK5⁻ (△) were pretreated with 10 μM epinephrine from 0.5 to 30 min, and membranes were prepared and assayed as described in Fig. 2. The extent of desensitization induced 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).

TABLE II
Characterization of 10 μM epinephrine-induced desensitization of βARs expressed in HEK 293 cells

HEK 293 cells expressing the WT or mutant βARs were pretreated with 10 μM epinephrine for various times from 0 to 30 min. Membranes were prepared and assayed for epinephrine-stimulated adenylyl cyclase activity as described under "Experimental Procedures." The extent of desensitization expressed as fraction activity remaining was calculated as described under "Experimental Procedures." The table shows the extent of desensitization after 2 or 30 min of 10 μM epinephrine pretreatment calculated from the data in Fig. 2. The apparent rate of desensitization was calculated from the data in Fig. 3 using the equation for monoexponential decay. The table shows the mean ± S.E., where $n \geq 3$ or ± range, where $n = 2$. The number of determination (n) is in parentheses.

Cell line	Time of pretreatment		
	2 min	30 min	$t_{1/2}$
	fraction activity remaining		*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 ± 0.02 (3)	0.56 ± 0.07 (3)
GRK5⁻	0.21 ± 0.02 (5)[a]	0.042 ± 0.004 (5)[a]	0.65 ± 0.06 (4)[b]

[a] 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, $p < 0.05$.

[b] The apparent rates of desensitization of the WTβAR and the GRK5⁻ mutant were compared using an unpaired t test and found to be significantly different, $p < 0.05$.

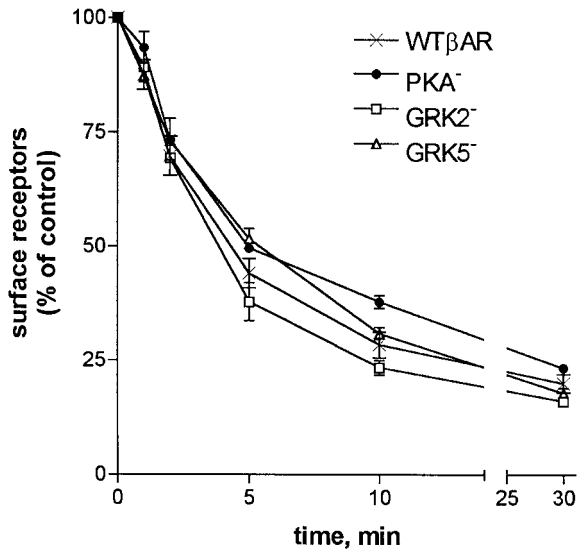

FIG. 4. **Receptor internalization in response to epinephrine.** Cells expressing the WTβAR (×), the PKA⁻ (●), the GRK2⁻ (□), or the GRK5⁻ mutant (△) were pretreated with either carrier or 10 μM epinephrine for 1–30 min, and surface receptor number was measured in triplicate for each time point using 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).

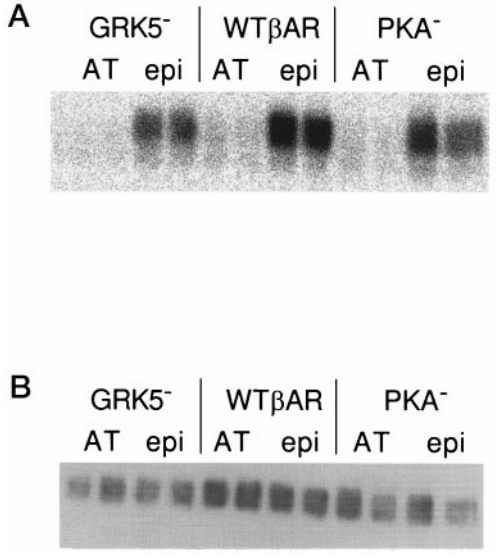

FIG. 5. **Phosphorylation of the WTβAR, GRK5⁻βAR, and the PKA⁻βAR.** Cells expressing the various receptors were labeled for 3 h with ^{32}P and pretreated for 1 min with either AT carrier or 10 μM epinephrine (*epi*) in duplicate. The βAR was solubilized, purified, resolved by SDS-PAGE, and transferred 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.

located in the proximal portion of the receptor carboxyl terminus are involved in GRK phosphorylations *in vivo*. All 11 of the serine/threonine residues found in the receptor carboxyl terminus, from amino acid 355 to 413, have been implicated as possible sites of GRK phosphorylation. Decreased desensitization and phosphorylation was reported for a mutant βAR containing substitutions at all 11 carboxyl-terminal serine/threonine sites (5, 6). A mutagenesis study in which only serines 355, 356, and 364 and threonine 360 were substituted for either glycine or alanine suggested that the *in vivo* site(s) of GRK phosphorylation may be among these residues (20). This study demonstrated that substitution of the four residues resulted in a mutant βAR almost completely defective in the rapid agonist-induced desensitization, internalization, and phosphorylation. The authors speculated that the presence of desensitization, partial phosphorylation, and internalization observed in the βAR containing substitutions at all 11 residues resulted from relief of a conformational inhibition present in the mutant with only four substitutions (20). Their data, however, are consistent

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 these studies, *in vitro* 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 vitro* and *in vivo* phosphorylations of the βAR.

Still another possibility to consider is that the numerous substitutions we have made in the GRK2 and GRK5 mutants have in some way altered the specificity of GRK phosphorylation through de-localized effects. Although we may have eliminated one or more sites of GRK phosphorylation, the amino acid changes in the mutants here may allow inappropriate GRK phosphorylation at other sites. However, de-localized effects are unlikely because of the similar functional properties of the WTβAR and mutant receptors. The desensitization, internalization, and agonist binding affinity of the mutant receptors were similar to those of the WTβAR, and only modest reductions in coupling efficiency were observed.

The similar desensitization we observed for the PKA$^-$ and the WTβAR (Fig. 2*B* and Fig. 3) was also unexpected, since it has been suggested that receptor phosphorylation by PKA is necessary to achieve maximum desensitization in response to high agonist concentrations. Hausdorff *et al.* (5) reported that a mutant βAR containing alanine substitutions for the serines of the two consensus PKA sites showed decreased desensitization upon exposure to high concentrations of isoproterenol relative to the wild type receptor in Chinese hamster fibroblast cells. Similarly, Moffett *et al.* (24) found that a mutant βAR with alanine substitutions for the serines of the carboxyl-terminal PKA site was subject to less desensitization than the wild type receptor in mouse Ltk$^-$ cells. In contrast, our results indicate that PKA-mediated receptor phosphorylation is not required for maximum desensitization in response to high agonist exposure. These results agree with studies we performed with cyc$^-$ and kin$^-$ mutants of the S49 wild type lymphoma cell line in which we found no alteration in the extent of agonist-induced homologous desensitization relative to the wild type (25). Internalization of the PKA$^-$βAR was also similar to that of the WTβAR. We speculate that our results may be caused by redundancy of PKA-mediated phosphorylation/desensitization with the GRK/β-arrestin/internalization pathway as has been previously suggested (26). Alternatively there may be cell-specific factors that explain these discrepancies.

To conclude that the sites phosphorylated by GRK2 and GRK5 *in vivo* do not correspond with the sites identified *in vitro* by Fredericks *et al.* (7) requires that GRK2 and GRK5 are expressed in HEK 293 cells. That GRK2 is present in HEK 293 cells has been shown by Menard *et al.* (10) using Western blot analysis and by other investigators using reverse transcription-coupled PCR as well as Western blots.[2] GRK5 is either absent or expressed at low levels in HEK 293 cells using reverse transcription-PCR.[2] The levels of GRK expression needed *in vivo* to mediate receptor phosphorylation are unknown. The absence or low expression of GRK5 in HEK 293 cells makes it difficult to determine the functional significance *in vivo* of the sites phosphorylated by GRK5 *in vitro*. However, since GRK2 expression in HEK 293 cells has been shown, the work presented here conclusively demonstrates the lack of *in vivo* functional significance for the six serine/threonine residues identified *in vitro* as sites of GRK2 and/or GRK5 phosphorylation.

Identification of the βAR sites phosphorylated *in vivo* is important for a more complete understanding of the complex processes of desensitization. With the exception of rhodopsin, G protein-coupled receptors have been notoriously refractory to chemical analysis of phosphorylation sites *in vivo* due to their extremely low concentrations in the cell. This approach, however, may ultimately be required for resolution of the molecular actions of the GRKs, PKA, and other protein kinases that have been implicated in the regulation of the βAR.

REFERENCES

1. Perkins, J. P., Hausdorff, W. P., and Lefkowitz, R. J. (1991) in *The β-Adrenergic Receptors* (Perkins, J. P., ed) pp. 73–124, Humana Press Inc., Totowa, NJ
2. Freedman, N. J., and Lefkowitz, R. J. (1996) *Recent Prog. Horm. Res.* **51,** 319–351
3. Clark, R. B., Friedman, J., Dixon, R. A. F., and Strader, C. D. (1989) *Mol. Pharmacol.* **36,** 343–348
4. Yuan, N., Friedman, J., Whaley, B. S., and Clark, R. B. (1994) *J. Biol. Chem.* **269,** 23032–23038
5. Hausdorff, W. P., Bouvier, M., Dowd, B. F., Irons, G. P., Caron, M. G., and Lefkowitz, R. J. (1989) *J. Biol. Chem.* **264,** 12657–12665
6. Bouvier, M., Hausdorff, W. P., De Blasi, A., O'Dowd, B. F., Kobilka, B. F., Caron, M. G., and Lefkowitz, R. J. (1988) *Nature* **333,** 370–373
7. Fredericks, Z. L., Pitcher, J. A., and Lefkowitz, R. J. (1996) *J. Biol. Chem.* **271,** 13796–13803
8. January, B., Seibold, A., Whaley, B., Hipkin, R. W., Lin, D., Schonbrunn, A., Barber, R., and Clark, R. B. (1997) *J. Biol. Chem.* **272,** 23871–23879
9. Ferguson, S. S. G., Menard, L., Barak, L. S., Koch, W. J., Colapietro, A.-M., and Caron, M. G. (1995) *J. Biol. Chem.* **270,** 24782–24789
10. Menard, L., Ferguson, S. S. G., Zhang, J., Lin, F.-T., Lefkowitz, R. J., Caron, M. G., and Barak, L. S. (1997) *Mol. Pharmacol.* **51,** 800–808
11. Barovsky, K., and Brooker, G. (1980) *J. Cyclic Nucleotide Res.* **6,** 297–307
12. Hoyer, D. E., Reynolds, E., and Molinoff, P. B. (1984) *Mol. Pharmacol.* **25,** 209–218
13. Slowiejko, D. M., Levey, A. I., and Fisher, S. K. (1994) *J. Neurochem.* **62,** 1795–1803
14. Salomon, Y., Londos, C., and Rodbell, M. (1974) *Anal. Biochem.* **58,** 541–548
15. Clark, R. B., Friedman, J., Johnson, J. A., and Kunkel, M. W. *FASEB J.* **1**, 289–297
16. Johnson, J. A., Clark, R. B., Friedman, J., Dixon, R. A. F., and Strader, C. D. (1990) *Mol. Pharmacol.* **38,** 289–293
17. Clark, R. B., Kunkel, M. W., Friedman, J., Goka, T. J., and Johnson, J. A. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 1442–1446
18. Deleted in proof
19. Whaley, B. S., Yuan, N., Barber, R., and Clark, R. B. (1995) *Mol. Pharmacol.* **45,** 481–489
20. Hausdorff, W. P., Campbell, P. T., Ostrowski, J., Yu, S. S., Caron, M. G., and Lefkowitz, R. J. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 2979–2983
21. Ohguro, H., Van Hooser, J. P., Milam, A. H., and Palczewski, K. (1995) *J. Biol. Chem.* **270,** 14259–14262
22. Wilden, U., and Kuhn, H. (1982) *Biochemistry* **21,** 3014–3022
23. Aton, B. R., Litman, J. J., and Jackson, M. L. (1984) *Biochemistry* **23,** 1737–1741
24. Moffett, S., Adam, L., Bonin, H., Loisel, T. P., Bouvier, M., and Mouillac, B. (1996) *J. Biol. Chem.* **271,** 21490–21497
25. Green, D. A., Friedman, J., and Clark, R. B. (1981) *J. Cyclic Nucleotide Res.* **7,** 161–172
26. Lohse, M. J., Benovic, J. L., Caron, M. G., and Lefkowitz, R. J. (1990) *J. Biol. Chem.* **265,** 3202–3209

[2] M. Ascoli and R. Premont, personal communication.