Catecholamines as well as phorbol esters can induce the phosphorylation and desensitization of the α1β-adrenergic receptor (α1βAR). In this study, phosphoamino acid analysis of the phosphorylated α1βAR revealed that both epinephrine- and phorbol ester-induced phosphorylation predominantly occurs at serine residues of the receptor. The findings obtained with receptor mutants in which portions of the C-tail were truncated or deleted indicated that a region of 21 amino acids (393–413) of the carboxyl terminus including seven serines contains the main phosphorylation sites involved in agonist- as well as phorbol ester-induced phosphorylation and desensitization of the α1βAR. To identify the serines involved in agonist- versus phorbol ester-dependent regulation of the receptor, two different strategies were adopted, the seven serines were either substituted with alanine or reintroduced into a mutant lacking all of them. Our findings indicate that Ser294 and Ser400 were phosphorylated following phorbol ester-induced activation of protein kinase C, whereas Ser404, Ser408, and Ser410 were phosphorylated upon stimulation of the α1βAR with epinephrine. The observation that overexpression of G protein-coupled kinase 2 (GRK2) could increase agonist-induced phosphorylation of Ser404, Ser408, and Ser410, strongly suggests that these serines are the phosphorylation sites of the α1βAR for kinases of the GRK family. Phorbol ester-induced phosphorylation of the Ser294 and Ser400 as well as GRK2-mediated phosphorylation of the Ser404, Ser408, and Ser410, resulted in the desensitization of α1βAR-mediated inositol phosphate response. This study provides generalities about the biochemical mechanisms underlying homologous and heterologous desensitization of G protein-coupled receptors linked to the activation of phospholipase C.

Desensitization is a general regulatory phenomenon of G protein-coupled receptors resulting in the attenuation of the receptor-mediated response. Two major patterns of desensitization referred to as homologous and heterologous desensitization can be distinguished. Homologous desensitization is defined as a rapid loss of responsiveness for a receptor repeatedly exposed to its specific agonist, whereas in heterologous desensitization stimulation of a receptor by an agonist can attenuate the response mediated by other receptors eliciting similar cellular effects (1).

In the G protein-coupled receptor family (2), receptor desensitization has been extensively characterized for rhodopsin mediating phototransduction in retinal rod cells and for the β2-adrenergic receptor (β2AR) which mediates catecholamine-induced stimulation of adenyl cyclase. The second messenger-dependent cAMP-dependent protein kinase can phosphorylate and desensitize the β2AR both in response to its agonist as well as to other agents increasing the cellular content of cAMP. On the other hand, a prominent role in homologous desensitization of rhodopsin and β2AR is played by the second messenger-independent rhodopsin kinase (3) and β-adrenergic receptor kinase (3ARK) (4), respectively. Once the receptor is occupied by the agonist, it is recognized by the kinase and becomes phosphorylated. The subsequent uncoupling of the receptor and G protein is then mediated by arrestin proteins, which specifically bind to the phosphorylated receptor (5, 6). Rhodopsin kinase and 3ARK are members of the newly discovered family of G protein-coupled receptor kinases (GRK) (7). These protein kinases have the unique ability to recognize and phosphorylate their G protein-coupled receptor substrates predominantly in their active (i.e. agonist-occupied) conformations. Recently, we have provided evidence that the α1βAR coupled to Gq-mediated activation of phospholipase C can be phosphorylated by at least two types of protein kinases, a protein kinase C (PKC) upon its stimulation by phorbol esters (8) and protein kinases belonging to the GRK family once the receptor is stimulated by phorbol esters (9) and protein kinases belonging to the GRK family once the receptor is stimulated by phorbol esters (9). The observation that agonist-induced phosphorylation of the α1βAR is insensitive to inhibitors of PKC suggests that a PKC is involved mainly in heterologous desensitization, whereas a GRK mediates agonist-induced desensitization (homologous) of the α1βAR (8).

The sites involved in GRK-mediated phosphorylation have been unambiguously identified only for rhodopsin and more recently for the β2AR. Rhodopsin is phosphorylated in a light-dependent manner by rhodopsin kinase (GRK1). Both in vitro and in vivo studies have demonstrated that the sites for GRK1-mediated phosphorylation are represented by specific serines located in the distal part of the C terminus of rhodopsin (10–15), whereas for the β2AR the phosphorylation sites for 3ARK (GRK2) and GRK5 have been identified only in vitro (16). For few other G protein-coupled receptors, including α2AAR (17), N-formyl peptide receptor (18) and C5a receptor (19) potential phosphorylation sites for GRK2-mediated phosphorylation have been proposed on the basis of mutagenesis studies. The phosphorylation sites for the second messenger-depend-
ent PKC have been characterized only for a few G protein-coupled receptors, including rhodopsin (20), β2AR (21), and more recently for the GLP1 receptor (22), using site-directed mutagenesis. Recently, we have demonstrated that the sites for phorbol ester- as well as agonist-induced phosphorylation of the α1B-AR are located in the C-tail of the receptor as suggested by the fact that a truncated mutant (T368) lacking the last 147 amino acids was totally impaired in its ability to undergo phosphorylation (8). In this study, we have constructed a large number of receptor mutants to identify the phosphorylation sites of the C-tail involved in agonist- versus phorbol ester-induced desensitization of the α1B-AR. Our findings demonstrate that GRK and PKC can phosphorylate the α1B-AR on different serine residues of the C-tail, triggering desensitization of the receptor-mediated response.

EXPERIMENTAL PROCEDURES

Construction of Mutated α1B-ARs—The cDNA encoding the hamster α1B-AR (23) was mutated by a polymerase chain reaction mutagenesis technique using Taq DNA polymerase (Boehringer Mannheim). The mutated DNA fragments obtained were digested with BstHIII and Apal and cloned into the expression vector pRK5 (24) containing the α1B-AR cDNA. Recombinant clones were isolated and sequenced by cycle sequencing using Exo III/RsaI polymerase (Stratagene).

Cell Culture and Transfection—COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum and transfected with different DNA by the DEAE-dextran method. The cDNA encoding the bovine GRK2 (25) was subcloned in pcMV5 (26). For phosphorylation experiments, COS-7 cells (5 × 10⁶) grown in 100-mm dishes were grown in 100-mm dishes, and the transfected DNA was 1 µg million cells for the receptors and for GRK2 3 µg/million cells. The total amount of DNA transfected was kept constant (4 µg/million cells) under different conditions adding pCMV. For inositol phosphate determination, COS-7 cells (0.25 × 10⁶) grown in 20-mm dishes were transfected with 4 µg/million cells for the receptors and 0.6 µg of DNA/million cells for GRK2. The total amount of DNA transfected was kept constant (1 µg/million cells) under different conditions adding pCMV. COS-7 cells were harvested 48 h after their transfection.

Western Blot Analysis of GRK 2—The expression of bovine GRK2 in transfected COS-7 cells was assessed as previously described (9) using an antiserum raised against a glutathione S-transferase fusion protein encoded by residues 467–688 of rat GRK3 (27).

Ligand Binding—Membrane preparations from cells expressing the wild type or mutated α1B-AR and ligand binding assays using [125I]HEAT were performed as described previously (8). Prazosin (10⁻⁶ M) was used to determine nonspecific binding. [125I]HEAT concentration was 300 pM for saturation binding and 80 pM for competition binding analysis of epinephrine. Data were analyzed by nonlinear least-squares regression analysis (28).

 Autoradiography—Two-dimensional phosphoamino acid analysis of the phosphorylated receptors. COS-7 cells expressing the different α1B-AR in the absence or presence of bovine GRK2 were grown in 100-mm dishes, equilibrated in phosphate-free DMEM for 2 h, and then incubated in the same buffer containing 32Pi. GRK2 were grown in 100-mm dishes, equilibrated in phosphate-free binding analysis of epinephrine. Data were analyzed by nonlinear least-

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Altogether, these results indicate that the serines involved in the phosphorylation of the \( \alpha_{1B} \) AR are located in two different regions of the C terminus; the first including amino acids 387–412 contains the majority of the sites for both agonist- and PMA-induced phosphorylation, whereas the second, located between residues 412 and 469, includes sites involved in part of PMA-induced phosphorylation of the \( \alpha_{1B} \) AR. A crucial role of serine residues located between amino acids 387 and 412 was confirmed by the results obtained with the deletion mutant D393–413 which was completely impaired in its ability to undergo receptor phosphorylation either upon stimulation with epinephrine or PMA (Fig. 3).

We next investigated the correlation between agonist-induced phosphorylation and desensitization of the inositol phosphate (IP) response mediated by the wild type and mutated \( \alpha_{1B} \) AR. In our previous work, we demonstrated that overexpression of GRK2 and GRK3 enhanced both agonist-induced phosphorylation and desensitization of the \( \alpha_{1B} \) AR (9). In agreement with our previous findings, overexpression of GRK2 caused an inhibition of 52\% of the \( \alpha_{1B} \) AR-mediated IP response as compared with that of the receptor expressed alone (Fig. 4). The inhibitory effect of GRK2 on the receptor-mediated IP increase for the T469 and T412 receptor mutants was similar to that observed for the wild type \( \alpha_{1B} \) AR (Fig. 4). In contrast, the IP response mediated by the T368, T387, and D393–413 receptors, which cannot undergo agonist-induced phosphorylation, was not significantly decreased by overexpression of bovine GRK2 (Fig. 4). This strongly suggests that the serines located between residues 393 and 413 are involved in both GRK-mediated phosphorylation and desensitization of the \( \alpha_{1B} \) AR.

In a second set of experiments, we assessed the correlation between PMA-induced phosphorylation and desensitization of the wild type and mutated receptors. Cells expressing the \( \alpha_{1B} \) AR or T368 receptor were treated with increasing concentrations of PMA for 10 min. Fig. 5 shows that at a concentration of \( 10^{-8} \) M PMA inhibited the \( \alpha_{1B} \) AR-mediated response of about 45\%, without any significant effect on the response mediated by the T368 mutant (Fig. 5A). However, at a concentration of \( 10^{-7} \) M PMA could also impair the T368-mediated response, even if at a smaller extent as compared with the wild type receptor (25\% versus 68\% of impairment for the T368 and \( \alpha_{1B} \) AR, respectively). Thus, high concentrations of PMA (\( >10^{-8} \) M) might inhibit the receptor-mediated IP response by a mechanism independent from receptor phosphorylation. This was confirmed by experiments exploring the effect of PMA on the IP response mediated by G\( \alpha_{q} \) expressed in COS-7 cells following its stimulation with AlF\( _4^- \). Fig. 5 shows that \( 10^{-7} \) M PMA could inhibit the G\( \alpha_{q} \)-mediated response of about 30\%, whereas no significant inhibition could be observed at a concentration of \( 10^{-8} \) M (Fig. 5A).

Taken together, these results suggest that PMA can impair the \( \alpha_{1B} \) AR-mediated response by at least two different mechanisms, depending on its concentration. The first occurring at low PMA concentrations (\( 10^{-8} \) M) might be truly mediated by receptor phosphorylation, because it was not observed with the T368 receptor mutant lacking all the phosphorylation sites.

**Fig. 2.** Topological model of the hamster \( \alpha_{1B} \) AR. Potential phosphorylation sites (serine residues) are indicated by solid circles. Arrows indicate the position of the stop codons introduced to construct the truncated mutants (T368, T387, T412, and T469) named after the number of the last encoded amino acid. The box includes the amino acids deleted in the D393–413 mutant.

Phosphorylation Sites of the \( \alpha_{1B} \)-Adrenergic Receptor
significance was analyzed by a paired Student's t test. 

compared with PMA-induced phosphorylation of the wild type receptors and of 30% for the T412, without any significant effect of PKC. In agreement with this finding, pretreatment of the cells expressing the S394–415A mutant with the specific GRK2 inhibitor RO-318220, completely abolished the residual phosphorylation observed for the T412 receptor expressed alone, which is considered as 100%. The IP response following 20 min of stimulation with epinephrine was 280 ± 35, 325 ± 45, 314 ± 51, 281 ± 38, 274 ± 32, and 309 ± 39 over basal for the α1B, T368, T387, T412, T469, and the Δ393–413, respectively. The results are the mean ± S.E. of three independent experiments.

The second, occurring at higher concentrations of PMA (>10⁻⁸ M), might involve phosphorylation of other signaling proteins acting downstream of the receptor, including Gqα.

On the basis of these observations, we compared the effect of a low concentration of PMA (10⁻⁸ M) on the IP response mediated by different receptor mutants expressed in COS-7 cells. After labeling with ³²P, cells were incubated for 15 min with no additions (−), 10⁻⁴ M epinephrine (E), or 10⁻⁷ M PMA (P). The phosphorylated receptors were immunoprecipitated with the antisera against the N terminus of the α1B, as described under “Experimental Procedures.” 0.25 pmol of wild type α1B, 0.12 pmol of T412, and 0.2 pmol of T469 receptor were loaded onto 10% SDS-polyacrylamide gel electrophoresis. Position of prestained molecular mass markers are indicated in kDa. The results are representative of three independent experiments. B, COS-7 cells expressing the wild type or mutated α1B were labeled with ³²P, and incubated for 15 min with no additions (basal), 10⁻⁴ M epinephrine (EPI), or 10⁻⁷ M PMA. The ³²P content of the phosphorylated receptors was quantified as indicated under “Experimental Procedures.” Epinephrine- and PMA-induced phosphorylation was calculated by subtracting the value of basal phosphorylation for each receptor. Results are the mean ± S.E. of three independent experiments. Statistical significance was analyzed by a paired Student's t test, *p < 0.05 as compared with PMA-induced phosphorylation of the wild type α1B.

The results of these experiments are shown in Fig. 3. For all the mutant receptors, phosphorylation at Ser394 and Ser400 was reduced compared with that mediated by the wild type expressed alone, while phosphorylation at Ser412 was unchanged. In agreement with this finding, pretreatment of the cells expressing the S394–415A mutant (Δ393–413) with the specific GRK2 inhibitor RO-318220, completely abolished the residual phosphorylation observed for the T412 receptor expressed alone, which is considered as 100%. The IP response following 20 min of stimulation with epinephrine was 280 ± 35, 325 ± 45, 314 ± 51, 281 ± 38, 274 ± 32, and 309 ± 39 over basal for the wild type, T368, T387, T412, T469, and the Δ393–413, respectively. The results are the mean ± S.E. of three independent experiments.

As indicated in Fig. 6, mutagenesis of either Ser394 or Ser400 caused a decrease of the PMA-induced receptor phosphorylation of 31 and 38%, respectively, without any significant effect on agonist-induced phosphorylation. On the other hand, substitution of either Ser400 or of the doublet Ser413-Ser415, resulted in a decrease of epinephrine-induced phosphorylation of 36 and 64%, respectively, without affecting PMA-induced receptor phosphorylation. Substitution of either Ser400 or of the doublet Ser413-Ser415 did not alter receptor phosphorylation. Finally, the substitution of all the serines located between residues 393 and 415 (S394–415A mutant) resulted in a complete loss of the agonist-induced phosphorylation and in a reduction of 76% of the PMA-induced phosphorylation.

These findings identify, on one hand, Ser394 and Ser400 as sites of PMA-dependent phosphorylation and, on the other, Ser400 and Ser413 as sites of agonist-induced phosphorylation of the α1B. The fact that the mutagenesis of all the serines between amino acids 393 and 415 (S394–415A mutant) did not completely abolish PMA-induced receptor phosphorylation suggests the existence of additional sites of phosphorylation for PKC. In agreement with this finding, pretreatment of the cells expressing the S394–415A mutant with the specific PKC inhibitor RO-318220, completely abolished the residual agonist- as well as PMA-induced receptor phosphorylation (results not shown).

As mentioned above, the T412 truncated mutant, but not the T469, displayed a 30% decrease of the PMA-induced receptor phosphorylation (Fig. 3). This suggests that a PMA-dependent phosphorylation site is located between amino acids 413 and 469 of the α1B. To identify the residue which contributes to this residual phosphorylation, all the serines and threonines of the S394–415A mutant located between amino acids 413 and 469 were mutated either individually or in different combinations as indicated in Fig. 6.
Overexpression of GRK2 significantly increased epinephrine-induced phosphorylation of the $\alpha_{1B}$AR, as well as the S404i, S408i, S410i, and S408i/S410i, all of which had increased phosphorylation compared to the wild type $\alpha_{1B}$AR. The role of these serines in epinephrine-induced phosphorylation is additive as demonstrated by the fact that for the mutant S408i/S410i and S404i/S408i/S410i, the epinephrine-induced phosphorylation was 66 and 94% of that of the wild type $\alpha_{1B}$AR. The figure shows that the mutation of one serine or threonine can be compensated by the phosphorylation of another.

To further characterize the role of individual serines in the phosphorylation of the $\alpha_{1B}$AR, serines 394, 398, 400, 404, 408, 410, 413, and 415 were reintroduced individually or in combination in the S394–415A mutant, which lacks all of them. As shown in Fig. 6, the epinephrine- and PMA-induced phosphorylation of the S394–415A mutant was 12 and 24% of that of the wild type $\alpha_{1B}$AR, respectively. Reintroduction of Ser394i (S394i) or Ser400i (S400i) restored the PMA-, but not agonist-induced phosphorylation of the S394–415A mutant to 64 and 71% of that of the wild type $\alpha_{1B}$AR, respectively. The effect of Ser394i and Ser400i seemed to be additive as demonstrated by the fact that reintroduction of both (S394i/S400i) almost completely restored the PMA-dependent phosphorylation of the S394–415A mutant to 64 and 94% of that of the wild type $\alpha_{1B}$AR. The effect of individual serines in the phosphorylation of the $\alpha_{1B}$AR is additive as demonstrated by the fact that for the mutant S408i/S410i and S404i/S408i/S410i, the epinephrine-induced phosphorylation was 66 and 94% of that of the wild type $\alpha_{1B}$AR.
Phosphorylation of the receptors expressed alone. Ser400 (S400i) was insensitive to the overexpression of GRK2.

Involvement of Ser394 and Ser400 in PMA-induced Desensitization of the α1BAR—To assess whether the PMA-induced phosphorylation of Ser394 and Ser404 induced by PMA could impair the α1BAR-mediated IP response, cells expressing wild type α1BAR or different mutated receptors were treated with low concentration of PMA (10^{-8} M) for 10 min. As shown in Fig. 9, in cells treated with PMA (10^{-8} M) the IP response mediated by the α1BAR was inhibited by 45%. On the other hand, that mediated by the S394–415A mutant, which was impaired in its ability to undergo PMA-induced phosphorylation, was inhibited by 15%. The individual or combined reintroduction of serines 394 or 400 in the S394–415A mutant could restore the inhibitory effect of PMA on the receptor-mediated IP response (Fig. 9). On the other hand, the reintroduction of Ser394, Ser408, and Ser410 did not confer any sensitivity of the receptor mutant to PMA (Fig. 9). The fact that the IP response mediated by the S394–415A mutant was still impaired by 15% by PMA as compared with that of the T368 mutant is in agreement with phosphorylation of Ser394 and Ser404 induced by PMA could impair the α1BAR-mediated IP response by 52% as compared with that of the receptor expressed alone, without any significant effect on the response mediated by the T368 and S394–415A receptor mutants, which were impaired in agonist-induced phosphorylation. However, overexpression of GRK2 could reduce the IP response mediated by the S404i, S408i, and S410i mutants of about 30–35% and that mediated by the S404i/S408i/S410i receptor of 49%. On the other hand, the IP response mediated by receptors carrying Ser394 (S394i) and Ser400i (S400i) was insensitive to the overexpression of GRK2. Altogether, these findings provide strong evidence that Ser394, Ser408, and Ser410 confer to the α1BAR the ability to undergo both GRK2-mediated phosphorylation and desensitization.

As shown in Fig. 8, overexpression of GRK2 in COS-7 cells decreased the α1BAR-mediated IP response by 52% as compared with that of the receptor expressed alone. Since the level of epinephrine-induced phosphorylation of the S404i/S408i/S410i mutant was comparable to that of the wild type α1BAR either in the absence or presence of GRK2, it is likely that Ser104, Ser287, and Ser310 represent the only sites for agonist-induced phosphorylation which can be mediated by GRK2.

FIG. 8. Effect of GRK2 on the IP response mediated by wild type and mutated α1BAR. COS-7 cells were transfected with the DNA encoding the wild type or mutated α1BAR alone or in combination with the DNA encoding GRK2. The experimental conditions are as in Fig. 4. The results are the mean ± S.E. of three independent experiments. Statistical significance was analyzed by a paired Student’s t test. *p < 0.05 as compared with the GRK2-mediated desensitization of the α1BAR. §p < 0.05 as compared with the GRK2-mediated desensitization of the S404i, S408i, and S410i receptors.
the previous observation that the Ser394–415A mutant displayed a small level of PMA-induced phosphorylation (Fig. 6). Altogether these findings provide strong evidence that Ser394 and Ser400 mediate a large portion of the PMA-dependent phosphorylation and desensitization of the α1B AR.

**Effect of Different Mutations on the Basal Level of Phosphorylation of the α1B AR**—In the absence of any stimuli, the α1B AR expressed in COS-7 cells, HEK293 (9), or Rat-1 cells (8) displays a basal level of phosphorylation which is approximately similar in the different cell systems expressing the recombinant receptor. Treatment of cells with the PKC inhibitor RO318220 did not significantly decrease the basal level of receptor phosphorylation (8). On the other hand, for several of the α1B AR mutants described in this study the basal level of phosphorylation was clearly lower than that of the wild type α1B AR (Fig. 6).

In general, mutations of one or two serines included in the sequence 393–413 resulted in a decrease of basal phosphorylation ranging from 13 to 30% as compared with the wild type α1B AR (Fig. 6). On the other hand, mutation of all the serines (S394–415A mutant) (Fig. 6) or their removal in mutants T368, T387, and Δ393–413 (Fig. 3) dramatically reduced the basal level of receptor phosphorylation. The reintroduction of any of the serines included in the sequence 393–413 into the S394–415A mutant lacking all of them could restore basal phosphorylation to levels ranging from 20 to 64% that of the wild type α1B AR (Fig. 6).

Altogether, these findings indicate that in the α1B AR different serines of the C-tail might be phosphorylated under basal conditions. However, there seems to be no clear relationship between the role of distinct serines in maintaining basal phosphorylation and their involvement in agonist- or phorbol ester-induced phosphorylation of the receptor. In addition, whether the basal phosphorylation of the α1B AR has any functional correlate remains to be explored. In our experiments, the mutation of different serines did not result in any significant change in either basal or agonist-induced IP response of the mutated receptor as compared with the wild type under the experimental conditions used in this study (results not shown).

**DISCUSSION**

In this study we provide strong evidences that GRK and PKC can phosphorylate different subsets of serines in the C-tail of the α1B AR. Using site-directed mutagenesis, we have identified a first cluster of two serines (Ser394 and Ser400) phosphorylated following PMA-induced activation of PKC from a second cluster of three serines (Ser404, Ser408, and Ser410) phosphorylated upon agonist-induced stimulation of the α1B AR. The phosphorylation of these two clusters of serines by PKC and GRK2, respectively, resulted in desensitization of the α1B AR.

**Phosphorylation of Ser404, Ser408, and Ser410 by GRK2 Is Involved in Agonist-Induced Desensitization of the α1B AR**—We recently demonstrated the involvement of GRK2 and GRK3 in agonist-induced phosphorylation and desensitization of the α1B AR (9). In this study, we have identified Ser404, Ser408, and Ser410 as the phosphorylation sites involved in GRK2-induced desensitization (Fig. 6).

This was mainly demonstrated by two observations. First, receptor phosphorylation was reduced 36 and 64% by the mutation of Ser404 alone and by that of Ser408 and Ser410 in combination, respectively (Fig. 6). Second, the reintroduction of all the three serines in a receptor mutant lacking all of them (S394–415A mutant) could almost completely restore agonist-induced phosphorylation of the receptor. Since the extent of receptor phosphorylation correlated with the number of serines reintroduced, it seems that Ser404, Ser408, and Ser410 can be phosphorylated independently following agonist stimulation of the receptor. Overexpression of GRK2 was able to specifically increase agonist-induced phosphorylation of those receptor mutants carrying Ser404, Ser408, and Ser410 individually or in different combinations (Fig. 7). This argues for a nonsequential mechanism of receptor phosphorylation by GRK2.

The fact that Ser404, Ser408, and Ser410 are in proximity of a pair of acidic amino acids (Fig. 2) is in agreement with the notion that both GRK1 and GRK2 are acidotrophic kinases (29). Interestingly, rhodopsin and the β2AR possess a doublet of negatively charged residues located on the proximal side of the residue phosphorylated by GRK1 and GRK2, respectively. The N-formyl peptide receptor (for which the GRK2 phosphorylation sites have been deduced from in vitro studies using a fusion protein corresponding to the whole C-tail) as well as the α2δ2 AR (for which the sites for GRK2 phosphorylation have been proposed on the basis of mutagenesis studies) also possess a doublet of acidic amino acids in proximity to the phosphorylated residues (17, 18). Moreover, substitution of the two acidic residues with alanine seems to strongly reduce GRK2-induced phosphorylation of the N-formyl peptide receptor (18).

For rhodopsin (12), the N-formyl peptide receptor (18) as well as the C5a receptor (19), GRK-mediated phosphorylation seems to proceed sequentially. On the contrary, our findings suggest that GRK2 can phosphorylate Ser404, Ser408, and Ser410 of the α1B AR independently (Fig. 6). Similar results were reported for the α2δ AR, for which phosphorylation of four consecutive serines in the third intracellular loop seems to occur nonsequentially as demonstrated by the fact that mutation to alanine of any single serine reduced the extent of phosphorylation by about 25% (17). Thus, it appears that the sequential or nonsequential nature of GRK phosphorylation can vary depending on the receptor substrate and could be dictated by the structural or conformational properties of the phosphorylation site.

Our findings provide strong evidence that GRK2-mediated phosphorylation of Ser404, Ser408, and Ser410 might trigger the desensitization of the α1B AR. This is supported by the fact that the combined mutation of these three serines (S394–415A mutant) impaired the receptor’s ability to undergo GRK2-mediated receptor desensitization (Fig. 8). On the other hand, the reintroduction of any of these serines in the S394–415A mutant, lacking all of them, partially restored the GRK2-induced receptor desensitization (Fig. 8). Indeed, the presence of one of the three serines is sufficient to confer to the receptor some ability to undergo partial desensitization.

**Phorbol Ester-induced Desensitization of the α1B AR Involves Multiple Mechanisms Including Phosphorylation of Ser394 and Ser400**—Our finding identify Ser394 and Ser400 as the main phosphorylation sites of the α1B AR for PKC. This was demonstrated by the observation that mutation of either Ser394 or Ser400 reduced PMA-induced receptor phosphorylation, whereas the reintroduction of Ser394 and Ser400 in the S394–415A mutant, lacking both of them, could restore receptor phosphorylation to the levels observed in the wild type α1B AR (Fig. 6).

A large number of proteins have been shown to be phosphorylated by PKC (30). Among the sites phosphorylated by PKC, the most recurrent motif is (S/T/X)(K/R) (30), where X indicates any amino acid. Of the two PMA phosphorylation sites identified in the C-tail of the α1B AR, Ser400 is a classical phosphorylation site (SRK) for PKC (Fig. 2), while Ser394 is included in the motif RXXXXXX, which is a less common consensus site.

In addition to Ser394 and Ser400, a third site seems to be phosphorylated upon stimulation of cells expressing the α1B AR with PMA. This is demonstrated by two findings. First, the Ser394–415A mutant lacking both Ser394 and Ser400 retained
a small level of PMA-induced phosphorylation, which was 24% that of the wild type α1BAR. Second, the truncated receptor T412, in which both Ser394 and Ser400 are conserved, displayed a 30% decrease of PMA-induced phosphorylation. However, using site-directed mutagenesis, we were unable to identify the amino acid residues involved in this residual PKC-mediated phosphorylation of the α1BAR.

The cellular response mediated by most G protein-coupled receptors, linked to the activation of phospholipase C, has been shown to desensitize rapidly following phorbol ester-induced activation of PKC. Moreover, for many receptors, including the α1BAR (8), the M3-mAchR (31), the receptor of thrombin (32), the CCK receptor (31), and the NK2 receptor (33), the activation of PKC by phorbol ester can increase receptor phosphorylation. However, a clear demonstration for a direct causal relationship between PKC-mediated phosphorylation and desensitization is still missing for many G protein-coupled receptors.

In this study we provide evidence that PKC-mediated desensitization of the α1BAR results, at least in part, from receptor phosphorylation. This was demonstrated by three main observations: (a) the individual mutation of both Ser394 and Ser400 partially impaired PMA-induced desensitization, (b) the re-introduction of either Ser394 or Ser400 in the S394–415A mutant lacking both of them could restore PMA-induced receptor desensitization, and (c) the S394–415A mutant still retained its ability to undergo PMA-induced phosphorylation and desensitization, even if at a smaller extent as compared with the wild type α1BAR. Similar evidence for a causal relationship between PKC-induced phosphorylation and desensitization has been provided for the 5-HT1A (34) and NK2 (33) receptors.

However, our findings indicate that, in addition to receptor phosphorylation, other mechanisms might underlie PMA-induced desensitization of the α1BAR. Low concentrations of PMA (10−8 M) could exclusively desensitize the wild type α1BAR-mediated IP response (Fig. 5A). On the other hand, concentrations higher then 10−8 M could impair the IP response mediated by the Ts68 receptor, which could not undergo phosphorylation, as well as by Gqα upon its stimulation with AIF4 (Fig. 5A). Thus, PKC-mediated desensitization of G protein-coupled receptor linked to phospholipase C might involve phosphorylation of both receptors and other signaling proteins including Gqα. Our findings are in agreement with recent work showing that PKC-mediated desensitization of the receptor for platelet-activating factor involves both phosphorylation of the receptor and that of the phospholipase C β3 (35).

Conclusions—Our study has several implications. First it provides strong evidence that both GRK2- and PKC-mediated desensitization of the α1BAR might result from the phosphorylation of distinct serines in the C-tail of the receptor. Second, it contributes to the elucidation of the consensus sites for the phosphorylation mediated by kinases of the GRK family. Finally, it might provide generalities about the biochemical mechanisms involved in homologous and heterologous regulation of G protein-coupled receptors linked to the activation of phospholipase C.

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