Phosphorylation of GRK2 by Protein Kinase C Abolishes Its Inhibition by Calmodulin*

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G-protein-coupled receptor kinases (GRKs) are important regulators of G-protein-coupled receptor function. Two members of this family L, GRK2 and GRK5 L, have been shown to be substrates for protein kinase C (PKC). Whereas PKC-mediated phosphorylation results in inhibition of GRK5, it increases the activity of GRK2 toward its substrates probably through increased affinity for receptor-containing membranes. We show here that this increase in activity may be caused by relieving a tonic inhibition of GRK2 by calmodulin. In vitro, GRK2 was preferentially phosphorylated by PKC isoforms α, γ, and δ. Two-dimensional peptide mapping of PKCα-phosphorylated GRK2 showed a single site of phosphorylation, which was identified as serine 29 by HPLC-MS. A S29A mutant of GRK2 was not phosphorylated by PKC in vitro and showed no phorbol ester-stimulated phosphorylation when transfected into human embryonic kidney (HEK)293 cells. Serine 29 is located in the calmodulin-binding region of GRK2, and binding of calmodulin to GRK2 results in inhibition of kinase activity. This inhibition was almost completely abolished in vitro when GRK2 was phosphorylated by PKC. These data suggest that calmodulin may be an inhibitor of GRK2 whose effects can be abolished with PKC-mediated phosphorylation of GRK2.

Signaling via G-protein-coupled receptors (GPCRs) is subject to a variety of regulatory processes. One of the key regulatory mechanisms is the control of receptor function by G-protein-coupled receptor kinases (GRKs). These kinases phosphorylate the agonist-bound state of GPCRs, and this phosphorylation is often the initial step in homologous receptor desensitization, the loss of receptor responsiveness upon repeated or prolonged receptor stimulation (1, 2). To date, six members of the GRK family are known (3, 4). Of these, GRK2 (previously called β-adrenergic receptor kinase-1) as well as GRK3, GRK5, and GRK6 are widely expressed throughout the mammalian body whereas GRK1 (also known as rhodopsin kinase) and GRK4 have been found only in specific tissues.

All GRKs have a similar molecular architecture. A central catalytic domain is flanked by an N- and C-terminal domain. The function of the latter is to provide a membrane anchor, which seems to be essential for receptor phosphorylation (5). The C terminus of GRK1 is farnesylated, whereas GRK2 and GRK3 possess a pleckstrin homology domain implicated in phosphatidylinositol bisphosphate and G-protein βγ-subunit binding. GRK4 and GRK6 are palmitoylated, and GRK5 binds phospholipids through a poorly defined polybasic domain (3, 4). The function of the N terminus is less clear. It has been shown for GRK1 that antibodies against an epitope in the N terminus prevent phosphorylation of light-activated rhodopsin but not of a peptide substrate (6). However, the epitope against which the antibodies were directed is poorly conserved in the GRK family. Recent results have indicated that the N terminus of GRK2 and GRK3 contains an RGS (regulator of G-protein signaling) domain that specifically binds to and activates Gαq (7, 8). In addition, the extreme N terminus of GRK2 and GRK5 contains a binding site for Ca2+/calmodulin. Binding of Ca2+/calmodulin to this site causes an inhibition of GRK activity, and this may represent a mechanism for the regulation of GRKs (9–11).

In addition to GRKs, other kinases have been found to phosphorylate GPCRs. The most prominent kinases capable of phosphorylating receptors are the protein kinases A and C (PKC), which are activated by the second messengers generated after stimulation of these receptors. Recent studies have identified cross-talk between PKC and GRKs, which may provide another level of regulation of GRKs. PKC has been shown to phosphorylate GRK2 (12, 13) and GRK5 (14). Whereas phosphorylation of GRK5 by PKC attenuated its activity (14, 15), PKC-phosphorylated GRK2 caused significantly enhanced receptor phosphorylation and desensitization (12, 13). This enhancement was at least partially caused by a PKC-mediated stimulation of GRK2 translocation from the cytosol to the plasma membrane where the substrate receptors are located (13). The molecular mechanisms of this activation have, however, not been resolved. Hence, we have investigated the exact nature of the phosphorylation and regulation of GRK2 by PKC.

We report here that phosphorylation of GRK2 by PKC occurs in vitro, GRK2 was phosphorylated by PKC in vitro and showed no phorbol ester-stimulated phosphorylation when transfected into human embryonic kidney (HEK)293 cells. Serine 29 is located in the calmodulin-binding region of GRK2, and binding of calmodulin to GRK2 results in inhibition of kinase activity. This inhibition was almost completely abolished in vitro when GRK2 was phosphorylated by PKC. These data suggest that calmodulin may be an inhibitor of GRK2 whose effects can be abolished with PKC-mediated phosphorylation of GRK2.

EXPERIMENTAL PROCEDURES

Materials—Crude phosphatidylinositol (type II-S from soybean) and calmodulin (from bovine testes, activity at least 40,000 units/mg protein) were from Sigma. The GRK2 antiserum has been described previously (13).
PKC Relieves GRK2 Inhibition by Calmodulin

Cell Culture—Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics in a humidified incubator with 7% CO₂. HEK293 cells were transfected with a modified calcium phosphate method (16). COS cells were transfected using DEAE-dextran (17). Transiently transfected cells were analyzed 48 h after transfection.

Mutagenesis—The construction of the cDNA coding for GST fused to human GRK2 (552–689) has already been described (13). cDNAs coding for the N terminus (amino acids 1–185) or the pleckstrin homology domain (amino acids 552–656) of human GRK2 were amplified from the full-length cDNA by PCR with Pfu polymerase (Stratagene) and cloned into the vector pGEX1T (Amersham Pharmacia Biotech) for expression as GST fusion proteins (18). Point mutations on human GRK2 were introduced by the method of Kunkel (19). All constructs were verified by automated sequencing.

Determination of PKC and GRK2 Activity—PKC activity was determined using a soluble peptide substrate, [Ser³²P]PKCα-(19–31) as described (20). GRK2 activity was usually measured by using urea-treated rod outer segments (50 pmol of rhodopsin) as the substrate in the presence of 50 μmol purified G-protein βγ-subunits as described previously (24). Incubation was carried out at 30 °C for 8 min. ³²P incorporation into rhodopsin was determined by SDS-polyacrylamide gel electrophoresis of the reaction mixture, excision of the rhodopsin band, and Cerenkov counting or quantification with a phosphorimager. To assess phosphorylation of soluble substrates by GRK2, 16 pmol of a GST fusion protein of the C terminus of the parathyroid hormone receptor were incubated with GRK2 for 30 min at 30 °C. For investigation of the effects of calmodulin and/or PKC on GRK2 activity, GRK2 was preincubated with 70 units of calmodulin and/or 25 pmol of PKC for 30 min at 30 °C in the dark before adding rod outer segments. PKC activity was activated with 10 μM PMA.

Phosphorylation of Recombinant Proteins by PKC—Approximately 3 pmol of purified PKC were incubated with the indicated amounts of recombinant proteins (GRK2 or GRK2 fragments) in the presence of 4 μg of crude phosphatidylcholine vesicles in 20 mM Tris/HCl, pH 7.2, 2 mM EDTA, 8 mM MgCl₂, 50 mM CaCl₂, 1 mM PMA, 100 μM [γ³²P]ATP (<500 cpm/μmol) for 45 min at 30 °C. Proteins were resolved by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. To assess phosphorylation of soluble substrates by PKC, 16 pmol of a GST fusion protein of the C terminus of the parathyroid hormone receptor were incubated with PKC for 30 min at 30 °C in the dark before adding rod outer segments. PKC was activated with 10 μM PMA.

RESULTS

PKC Isoform Specificity for GRK2 and GRK5—It has been previously shown that crude preparations of PKC purified from rat or bovine brain are capable of phosphorylating GRK2 and GRK5 (12–14). To examine which of the PKC isoforms were responsible for phosphorylation of GRKs, we analyzed PKC preparations from baculovirus-infected Sf9 cells for their ability to phosphorylate GRK2 and GRK5. The activity of these fractions was calibrated with histone H1 as a substrate. Equal amounts of the various PKC isoforms were then used to phosphorylate purified GRK2 and GRK5 (Fig. 1). Whereas GRK5 was a good substrate for all PKC isoforms tested except for PKCα, GRK2 was only significantly phosphorylated by PKCs α, γ, and δ. The radioactivity incorporated into GRKs in the absence of PKC (Fig. 1, A and B, right lanes) is caused by autophosphorylation. Because GRK2 is selectively phosphorylated by only certain PKC isoforms, whereas, with the exception of PKCe, GRK5 serves as substrate for all PKCs, these data suggest that the two types of phosphorylation might be different. Because of their higher activity toward GRK2, all subsequent experiments were done with recombinant PKCs or PKCo.

Identification of the PKC Phosphorylation Site on GRK2—We had shown previously that GRK2 could be phosphorylated by rat brain PKC preparations to a stoichiometry of about one, suggesting a single phosphorylation site on GRK2. To verify the existence of a single phosphorylation site, we performed two-dimensional phosphopeptide mapping of GRK2. GRK2 was phosphorylated in the presence of [γ³²P]ATP with PKCo, digested with endopeptidase LysC, and the peptides resolved by two-dimensional electrophoresis. Only a single radiolabeled spot derived from GRK2 was identified, indicating the presence of a single phosphorylation site (Fig. 2).

To map this phosphorylation site, we performed HPLC-MS analysis of the LysC-digested peptides. The peptide containing the transferred phosphate was detected online with a radiodetector, and its mass was directly analyzed by electrospray-MS (Fig. 3). The major peak of radioactivity was identified as (AT)PARARKS based on the mass of the peptide and the fragmentation spectrum. This region corresponds to residues 22–30 of human GRK2 and identifies Ser²⁹ as the single phosphorylation site for PKC in GRK2.

To confirm this finding, a synthetic peptide comprising residues 7–34 of human GRK2 was synthesized, phosphorylated with PKCo, digested with endopeptidase LysC, and subjected to two-dimensional peptide mapping as described above. The resulting phophopeptide pattern was identical to the one obtained with full-length GRK2, as shown in separate or overlay analyses (Fig. 4). These data corroborate the correct identification of the phosphorylation site.

Ser²⁹ Is the PKC Phosphorylation Site in Vitro and in Vivo—To verify that Ser²⁹ is indeed the site phosphorylated by PKC, we constructed a mutant of human GRK2 in which this residue was replaced with alanine (S29A). This mutant was expressed in Sf9 cells using the baculovirus system and purified to homogeneity (Fig. 5). Purified S29A GRK2 was able to phosphorylate rhodopsin in a manner similar to the wild-type protein although its specific activity was lower (data not shown). As expected, PKCe was unable to phosphorylate the S29A GRK2, in contrast to wild-type GRK2 (Fig. 5). These data confirm that Ser²⁹ is indeed the only phosphorylation site for PKC in GRK2.

Phosphorylation of Ser²⁹ in GRK2 Abolishes the Inhibitory Effect of Calmodulin—We and others have shown previously that phosphorylation of GRK2 with PKC purified from bovine...
PKC Relieves GRK2 Inhibition by Calmodulin

Calmodulin inhibited the activity of bovine GRK2 by more than 75% as measured by the GRK2-mediated phosphorylation of light-activated rhodopsin (Fig. 6, right). This inhibition was completely reversed when active PKC was present in the assay (Fig. 6, right). These data show that PKC activates GRK2 by relieving the Ca\(^{2+}\)/calmodulin-mediated inhibition.

Mutation of Ser\(^{29}\) Abolishes PKC-mediated Activation of GRK2 in Intact Cells—Finally we wanted to verify that Ser\(^{29}\) represents the functionally relevant PKC phosphorylation site in intact cells. To achieve this, HEK293 cells were transiently transfected with wild-type or S29A mutant GRK2. When the cells were stimulated with PMA, cells transfected with wild-type GRK2 showed a roughly 2-fold increase in cytoplasmic GRK activity (Fig. 7, left). In the case of the S29A mutant, this stimulation of GRK activity by PMA was almost completely abolished (Fig. 7, right).

The residual effects of PMA seen under these conditions are probably caused by endogenous wild-type GRK2. These results indicate that PKC-mediated phosphorylation of GRK2 at Ser\(^{29}\) does indeed cause its activation in intact cells by relieving a constitutive inhibition through calmodulin or a related protein.

**DISCUSSION**

The function and activity of G-protein-coupled receptors is controlled by several classes of proteins via a multitude of mechanisms. A general feature of these mechanisms is that they are interrelated and that they appear to be controlled at several different levels. A particularly intriguing example of this complex control is the inhibition of receptor activity by GRKs, which is itself subject to control by PKC. Whereas GRK5 activity toward receptors was decreased following phosphorylation by PKC (14), GRK2 activity toward small soluble receptor substrates was also decreased by PKC, but its activity versus membrane-bound receptors was increased (12, 13). These data suggested that PKC might activate GRK2 by increasing its association with membrane-bound receptors (13).

PKC phosphorylation studies utilizing a protein comprised of the C terminus of GRK2 fused to GST had initially suggested the presence of a C-terminal phosphorylation site in GRK2 (13). In the present study, using intact GRK2 and purified recombinant PKC isoforms, we have mapped the PKC phosphorylation site in GRK2 unequivocally to serine 29 in the N terminus, and we can exclude phosphorylation of the GRK2 C terminus by PKC. We have further shown that PKC phosphorylation of GRK2 does not directly stimulate the activity of the isolated GRK2 in vitro.

On the other hand, our previous experiments clearly indicated that phosphorylation of GRK2 with PKC preparations obtained from bovine brain caused an ~2.5-fold increase in the activity of GRK2 on rhodopsin (12, 13). Surprisingly, when we tried to reproduce these findings with PKC\(\alpha\) or PKC\(\delta\) purified from SH9 cells, we obtained no stimulation. Instead, we observed a slight nonsignificant reduction of GRK2 activity after PKC phosphorylation (Fig. 6, left).

This discrepancy between our data suggests that the brain PKC preparations might have contained additional proteins that influence the activity of GRK2. One such protein might be calmodulin. Calmodulin has been shown to attenuate GRK2 activity, and the calmodulin binding site encompasses Ser\(^{29}\) of GRK2 (11). To investigate whether calmodulin could in fact be the postulated “missing protein,” we examined the influence of calmodulin on GRK2 activity.

**Fig. 2.** Two-dimensional phosphopeptide mapping of PKC\(\alpha\)-phosphorylated GRK2. GRK2 was phosphorylated in vitro by PKC\(\alpha\) and cleaved with LysC. The resulting peptide mixture was separated by two-dimensional phosphopeptide mapping, and the phosphopeptides were visualized by autoradiography. A, PKC-phosphorylated GRK2; B, autophosphorylated GRK2; C, mixture of PKC-phosphorylated and autophosphorylated GRK2.

**Fig. 3.** HPLC-MS analysis of PKC-phosphorylated GRK2. 200 ng of purified GRK2 were phosphorylated in vitro with PKC\(\alpha\) and 20 μM [\(^{32}\)P]ATP. The labeled protein was digested with LysC, and the resulting peptides analyzed by HPLC coupled to a radiodetector and an API 100 quadrupole mass spectrometer. The upper panel shows the total ion current and the middle panel represents the detection profile of the radiocounter. The delay between radiodetection and MS was ~4.6 min as indicated by the two lines. The lower panel shows the mass spectrum of the peak at 32.1 min. Despite weak signals, sequence information of the peptide with m/z 572.4 could be generated by front-end fragmentation. The peak at 58.6 min (middle panel) contains partially digested protein.
incorporation of phosphate into rhodopsin (13). Similarly, Chuang et al. (12) also observed activation of GRK2 after phosphorylation with a PKC preparation purified from bovine brain. As shown here, we were unable to reproduce these results using PKC isozymes purified from baculovirus-infected Sf9 cells. These discrepancies raised the possibility that the GRK2 activation observed after phosphorylation with PKC might be the result of additional protein(s) present in the brain PKC preparations, but not in the PKCs purified from Sf9 cells. One candidate for such a protein was calmodulin, which has previously been shown to inhibit GRK2 activity (10, 11). Indeed,
purified recombinant GRK2 was inhibited by calmodulin, and this inhibition could be fully reversed when GRK2 was phosphorylated by PKC. We and others have previously reported that PKC activation of GRK2 also occurs in intact cells (12, 13). Here, we show that removal of the PKC phosphorylation site on GRK2 also abolishes GRK2 activation through PKC. This indicates that a pool of GRK2 in the cell may be tonically inhibited by a complex with calmodulin or a related protein from which it is released through PKC phosphorylation.

Whereas our experiments offer a mechanistic explanation for the phenomenon of GRK2 activation by PKC, they also raise several questions. First, how does calmodulin inhibit GRK2, and how is this inhibition altered following phosphorylation of GRK2 by PKC? It is conceivable that calmodulin competes with the agonist-activated receptor for GRK2. This suggestion is supported by findings obtained with GRK1 (rhodopsin kinase) and rhodopsin. An antiserum directed against amino acids 17–34 of GRK1 was able to inhibit phosphorylation of light-activated rhodopsin but not of a peptide substrate (6). Interestingly, this region of GRK1 is also able to bind rhodopsin, as is the corresponding region of GRK5 (11). Furthermore, N-terminal truncation of GRK2 and 5 impairs their ability to phosphorylate rhodopsin (27). These data indicate that the N terminus of GRKs might be involved in binding to the receptor substrates. The sequence homology of the calmodulin-binding sites in GRK2 and GRK5 is, however, quite low.

The proximal C terminus of GRK2 (residues 457–546 of 689) has been implicated in receptor binding as well (28). This suggests that both C- and N-terminal regions of GRK are involved in receptor association. The C terminus has been shown to interact with light-activated rhodopsin by itself, suggesting that it may be sensitive to the conformational changes occurring in G-protein-coupled receptors upon activation (28). In contrast, truncation of the N terminus does not abolish the ability of GRKs to be activated by agonist-occupied receptors (27). It therefore appears that the N terminus is not involved in sensing the activated state of the receptor but may contribute some weak interaction that is required for efficient substrate recognition. Evidently, additional experiments are necessary to prove this hypothesis.

Another question is how phosphorylation of GRK2 by PKC affects its regulation by Ca\(^{2+}\)/calmodulin. Such phosphorylation might be because of a reduction in direct binding affinity (measured to be around 40 nM, see Ref. 11), but it might also be more complex and involve other regions of the GRKs. (A second calmodulin binding domain has been mapped to residues 466–689 of GRK2, Ref. 11.) Such a more complex interaction is suggested by the fact that phosphorylation of GRK2 by PKC (as well as of GRK5) has a direct inhibitory effect on the activity of the catalytic core region of GRKs if, instead of a membrane-bound receptor, a soluble substrate was phosphorylated. This was either a peptide (13, 14) or the soluble C terminus of the parathyroid hormone receptor (data not shown).

It is remarkable that GRK2 seems to possess a special versatility for regulating the activity of G\(_{\alpha}\)-coupled receptors. It is inhibited by Ca\(^{2+}\)/calmodulin and thereby regulated by G\(_{\alpha}\)-mediated alterations in intracellular free Ca\(^{2+}\)-levels. This inhibition is released through PKC activity, which can also be controlled via G\(_{\alpha}\). In addition, GRK2 contains an RGS domain, which leads to fairly selective inhibition of G\(_{\alpha}\)-mediated signal transduction pathways (7, 8). The reasons for this specialization of GRK2 are at the moment completely unclear, in particular because GRK2 was initially thought to be particularly active toward the G\(_{\alpha}\)-coupled \(\beta\)-adrenergic receptors.

Taken together, our data delineate a previously unknown mechanism of regulation of G-protein-coupled receptors, which is exerted by an interplay of PKC and Ca\(^{2+}\)/calmodulin in regulating the activity of GRK2. These observations are a further step in the complex machinery regulating the function of these receptors.

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REFERENCES

1. Lohse, M. J. (1993) Biochim. Biophys. Acta 1179, 171–188
2. Krupnick, J. G., and Benovic, J. L. (1998) Annu. Rev. Pharmacol. Toxicol. 38, 289–319
3. Lohse, M. J., Krasel, C., Winstel, R., and Mayor, F., Jr. (1996) Kidney Int. 49, 1047–1052
4. Pitcher, J. A., Freedman, N. J., and Lefkowitz, R. J. (1998) Annu. Rev. Biochem. 67, 653–692
5. Pitcher, J. A., Inglese, J., Higgins, J. B., Arriza, J. L., Casey, P. J., Kim, C., Benovic, J. L., Kwatra, M. M., Caron, M. G., and Lefkowitz, R. J. (1992) Science 257, 1264–1267
6. Palczewski, K., Buczko, J., Leiboda, L., Crabb, J. W., and Polans, A. S. (1995) J. Biol. Chem. 269, 6004–6013
7. Carman, C. V., Parent, J.-L., Day, P. W., Pronin, A. N., Sternweis, P. M., Wedegaertner, P. B., Gilman, A. G., Benovic, J. L., and Kozasa, T. (1999) J. Biol. Chem. 274, 34483–34492
8. Sallée, M., Mariggiò, S., D’Urbano, E., Iacovelli, L., and De Blasi, A. (2000) Mol. Pharmacol. 57, 826–831
9. Chuang, T. T., Paolucci, L., and De Blasi, A. (1996) J. Biol. Chem. 271, 26961–26966
10. Pronin, A. N., Satlapa, D. K., Slepk, V. Z., and Benovic, J. L. (1997) J. Biol. Chem. 272, 18273–18280
11. LeVine, T., LeVine III, H., and De Blasi, A. (1995) J. Biol. Chem. 270, 18660–18665
12. Mischak, H., Seitz, T., Janosch, P., Eulitz, M., Steen, H., Schellerer, M., Philipp, A., and Kolch, W. (1996) Mol. Pharmacol. 49, 35–55
12. Kuznetz, M. G., Arceus, L. B., Bahador, A., Mischak, H., Goodnight, J., Mushinski, J. F., and Blumberg, P. M. (1993) Mol. Pharmacol. 44, 298–307
13. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
