Autophosphorylation Suppresses Whereas Kinase Inhibition Augments the Translocation of Protein Kinase Cα in Response to Diacylglycerol

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We have seen that protein kinase Cα (PKCα) is transiently translocated to the plasma membrane by carbachol stimulation of neuroblastoma cells. This is induced by the Ca²⁺ increase, and PKCα does not respond to diacylglycerol (DAG). The unresponsiveness is dependent on structures in the catalytic domain of PKCα. This study was designed to investigate if and how the kinase activity and autophosphorylation are involved in regulating the translocation. PKCα enhanced green fluorescent protein translocation was studied in living neuroblastoma cells by confocal microscopy. Carbachol stimulation induced a transient translocation of PKCα to the plasma membrane and a sustained translocation of kinase-dead PKCα. In cells treated with the PKC inhibitor GF109203X, wild-type PKCα also showed a sustained translocation. The same effects were seen with PKCβ, PKCβII, and PKCδ. Only kinase-dead and not wild-type PKCα translocated in response to 1,2-di-octanoylglycerol. To examine whether autophosphorylation regulates relocation to the cytosol, the autophosphorylation sites in PKCα were mutated to glutamate, to mimic phosphorylation, or alanine, to mimic the non-phosphorylated protein. After stimulation with carbachol, glutamate mutants behaved like wild-type PKCα, whereas alanine mutants behaved like kinase-dead PKCα. When the alanine mutants were treated with 1,2-dioctanoylglycerol, all cells showed a sustained translocation of the protein. However, neither carbachol nor GF109203X had any major effects on the level of autophosphorylation, and GF109203X potentiated the translocation of the glutamate mutants. We, therefore, hypothesize that 1) autophosphorylation of PKCα limits its sensitivity to DAG and 2) that kinase inhibitors augment the DAG sensitivity of PKCα, perhaps by destabilizing the closed conformation.

The protein kinase C (PKC) isoforms constitute a family of closely related serine/threonine kinases that are involved in pathways regulating a large number of cellular processes such as proliferation, apoptosis, differentiation, migration, and neuronal signaling. The PKC family is generally subgrouped in classical, novel, and atypical isoforms based on structural similarities and sensitivity to different activators. The classical PKCs (PKCα, -β, -βII, and -γ) are regulated by both Ca²⁺ and by diacylglycerol (DAG), whereas the novel PKCs (PKCδ, -ε, -η, and -θ) are insensitive to Ca²⁺ and considered to be primarily regulated by DAG. Atypical PKCs (PKCε and -ζ) are neither affected by Ca²⁺ nor by DAG (1–3).

When classical or novel PKCs are activated, they frequently translocate to the plasma membrane. The translocation is primarily mediated via two classes of PKC domains, C1 and C2 domains. These domains are targeted to the membrane by DAG and Ca²⁺, respectively. Structural and mutational analyses of PKCα in vitro have led to the proposal of a model in which an initial Ca²⁺ increase leads to the interaction of the PKCα C2 domain with Ca²⁺ and a loose binding of the enzyme to lipids. This interaction leads to a disruption of binding of the C2 domain to the C1a domain, which thereby becomes available for DAG and can be inserted into the membrane (4, 5). According to this model the C1 domain is inaccessible for ligands unless the PKC structure has been at least partially loosened by the interaction of Ca²⁺ with the C2 domain. We have recently seen that the catalytic domain contributes to the inaccessibility of the C1 domain in the absence of Ca²⁺ (6). Thus, a tethering of the C1a domain by the C2 domain is conceivably also dependent on intramolecular bindings that involve the catalytic domain.

There are also other mechanisms that may contribute to the regulation of PKCα translocation. PKCα is frequently autophosphorylated on two C-terminal sites, the turn motif (Thr-638 in human PKCα) and the hydrophobic site (Ser-657 in human PKCα). The phosphorylation of these sites has been suggested to be part of the regular maturation process of PKC molecules. Newly synthesized PKC molecules are initially phosphorylated on a threonine residue in the activation loop, a step that is necessary for the catalytic activity of the enzyme. Thereafter, the autophosphorylation sites are phosphorylated, completing the maturation of the enzyme (7, 8). The phosphorylation of the turn motif has been shown to be crucial for PKCβII (9) but not for PKCα (10) activity, whereas phosphorylation of the hydrophobic site is not required for PKC activity (11, 12). The autophosphorylation has also been suggested to be of importance for the stability of the PKC molecule (13, 14).

There are also indications that autophosphorylation is a means to directly regulate PKC function. There are findings showing that kinase inhibition or mutation of an autophosphorylation site prolongs the plasma membrane localization of PKC (15, 16), and a recent paper demonstrates that only non-
phosphorylated PKCs activates phospholipase D1 (17). A regulatory role for autophosphorylation is further supported by several studies showing that autophosphorylation is influenced by a number of exogenous stimuli (18–20).

Thus, it is becoming clear that autophosphorylation of PKC can be a regulated event and that it has significant impact on PKC function. However, it is far from clear how autophosphorylation influences the properties of PKC. We have previously seen that the catalytic domain of PKCs limits the durability of its translocation upon carbachol stimulation and its sensitivity to DAG (6). This led us to raise the novel hypothesis that regulating the DAG sensitivity may be the mechanism whereby autophosphorylation influences PKC function. Our data demonstrate that this is indeed the case; autophosphorylation does act as a regulatory switch determining the DAG sensitivity of PKCα.

**EXPERIMENTAL PROCEDURES**

*Plasmids*—Expression vectors encoding full-length PKCα, PKCβI, PKCβII, PKCδ, and kinase-dead PKCδ, all fused to EGFP, have previously been described (21–23). PKCα, PKCβ, and PKCδ were mutagenized in the autophosphorylation sites using the QuickChange mutagenesis kit (Stratagene). The primers were designed to alter threonine or serine in the turn motif to glutamate (αT638E and δT710E) or alanine (αT638A and δS663A) and to alter serine in the hydrophobic site to glutamate (αS657E and δS729E) or alanine (αS657A and δS663A). Similarly, both autophosphorylation sites were mutated to glutamate (αEDM and δEDM) or alanine (αADM and δADM). The primers are listed in Table I. All constructs were sequenced to confirm that they contained the right mutation.

*Confocal Microscopy of PKC Translocation*—Cells were examined by confocal microscopy on the day after transfection. The coverslips were washed twice with buffer H (20 mM Hepes, 137 mM NaCl, 3.7 mM KCl, 1.2 mM MgSO4, 2.2 mM KH2PO4, 1.6 mM CaCl2, 10 mM glucose, pH 7.4) and mounted on the heated stage of a Nikon microscope. The cells were examined with a Bio-Rad Radiance 2000 confocal system using a 60× objective lens and emission filter 515HQ30. Images were captured every 5 or 10 s, and the fluorescence was detected with a CCD camera (Fujifilm). Band intensities were analyzed with Lab Science software (Fujifilm).

**RESULTS**

**Inhibition of the Kinase Activity Makes PKCa Respond with a Sustained Translocation to the Plasma Membrane after Stimulation with Carbachol**—We have previously seen that stimulation of SK-N-BE(2)C neuroblastoma cells with the acetylcholine analogue carbachol gives rise to a transient translocation of PKCa (6). The relocation to the cytosol was dependent on the presence of the catalytic domain of PKCa. The isolated regulatory domain of PKCa responded with a sustained translocation upon carbachol stimulation.

To explore whether inhibition of the catalytic activity of PKC influences the translocation of PKCa, SK-N-BE(2)C cells expressing PKCa-EGFP were treated with 2 μM GF109203X before stimulation with carbachol. The localization of PKCa-EGFP was thereafter examined by confocal microscopy. In cells pretreated with GF109203X for 30 s, there was an immediate and sustained translocation of PKCa-EGFP to the plasma membrane after stimulation with 1 mM carbachol (Figs. 1, B, E, and H). This contrasts the effect in control cells pretreated with Me6SO, in which stimulation with carbachol gave a transient membrane translocation of PKCa-EGFP (Fig. 1, A, D, and G).

The effect of GF109203X led us to hypothesize that the kinase activity of PKCs suppresses its plasma membrane localization and leads to a relocation of the protein to the cytosol. To further investigate the role of the kinase activity, the translocation of a kinase-dead PKCa-EGFP, i.e. PKCa with a mutation of Lys-368 in the ATP-binding site to an arginine, was investigated. This PKCa mutant responded with a translocation that was sustained upon carbachol stimulation (Fig. 1, C, F, and I). To estimate the time course of the translocation, the fluorescence intensity of EGFP in the cytosol was quantified.
contain any plasma membrane or nuclear components during the entire time course of the experiment. The analysis confirmed that PKCaWT-EGFP pretreated with GF109203X and that the kinase inactive variant PKCaKD-EGFP responded with a sustained translocation after carbachol stimulation, whereas carbachol induced a transient translocation of PKCaWT-EGFP to the plasma membrane in cells pretreated with Me2SO (Fig. 1, A and D).

**Treatment with the PKC Inhibitor GF109203X after a Period of Stimulation with Carbachol Induces a Relocation of PKCa to the Plasma Membrane**—Pretreatment with GF109203X suppressed the relocation of PKCa-EGFP to the cytosol after a carbachol-stimulated plasma membrane translocation. We next wanted to investigate whether GF109203X also could reverse the relocation to the cytosol once it had occurred. To do this, SK-N-BE(2)C cells expressing PKCa-EGFP were stimulated with carbachol, and after the translocation, when PKCa-EGFP had returned to the cytosol, 2 μM GF109203X was added (Fig. 2). Stimulation with carbachol led to a translocation of PKCa-EGFP to the membrane within 10 s (Fig. 2, A and B), and when the protein had returned to the cytosol after 90 s (Fig. 2C), GF109203X was added to the cells. This induced a re-translocation of PKCa-EGFP to the plasma membrane, where it also remained throughout the rest of the experiment (Fig. 2D).

**Inhibition of PKCβI, PKCβII, and PKCδ Kinase Activity Leads to a Sustained Translocation of the Proteins to the Plasma Membrane after Stimulation with Carbachol**—We have previously seen that carbachol induces a transient translocation to the plasma membrane of PKCβII and to a lesser extent of PKCδ and a sustained translocation of PKCe in neuroblastoma cells (6). To investigate if kinase inhibition also influences the translocation of other PKC isoforms, SK-N-BE(2)C cells expressing PKCβI, PKCβII, or PKCδ fused to EGFP were treated with 2 μM GF109203X and thereafter stimulated with carbachol. The localization of EGFP-tagged PKC isoforms was thereafter followed by confocal microscopy (Fig. 3). As seen before, carbachol elicited a transient translocation of PKCβI and PKCβII, and this response was not altered by pretreatment with vehicle, Me2SO. On the other hand, exposure to GF109203X made both PKCβ isoforms respond with a sustained translocation after carbachol stimulation, although in a few cells a small amount of PKCβII relocated to the cytoplasm. The novel isoform PKCδ showed a weaker response to carbachol.
PKC stimulation, and some cells did not respond at all. However, treatment with GF109203X before stimulation with carbachol potentiated the magnitude of the PKCα translocation.

To further investigate the contribution of kinase inhibition to the response, the carbachol-stimulated translocation of kinase-dead PKCβI, PKCβII, and PKCδ was also analyzed. The kinase-dead PKCβI, PKCβII, and PKCδ all responded to carbachol and translocated to the plasma membrane and remained there throughout the experiment. Although full-length PKCδ sometimes did not respond to carbachol, kinase-dead PKCδ translocated in all cells. The results were supported by a quantification of the fluorescence intensity of EGFP in the cytoplasm. Thus, a potentiation and prolonging of a translocation by kinase inhibition is an effect that is common for several PKC isoforms.

**Stimulation with Carbachol Leads to a Sustained Translocation of Non-phosphorylated PKCa**—The potentiating effect of kinase inhibition on PKC translocation has previously been observed (15, 16), and this has raised the hypothesis that autophosphorylation of PKC would be a negative feed-back mechanism and that PKCα responds with a sustained translocation to the plasma membrane after stimulation with carbachol. Therefore, we have set out to investigate if the effects obtained by kinase inhibition on PKC translocation has previously been observed and that PKCα responds with a sustained translocation to the plasma membrane after stimulation with carbachol. Thus, autophosphorylation of the hydrophobic site and the turn motif conceivably contributes to the transient nature of PKCα translocation. However, mutation of the autophosphorylation sites to glutamate mutants, sometimes returned to the cytosol after stimulation with carbachol. Thus, autophosphorylation of PKC does not make PKCα resistant to translocation signals. Wild-type PKCa and the glutamate mutants (PKCaT638E, PKCaS657E, and PKCaEDM, the mutant with both sites mutated) all either responded with a transient translocation to the plasma membrane or did not respond at all. There were some variations in terms of the proportion of cells in which PKCa translocated, but a sustained translocation was not observed except for two cells expressing PKCaS657E.

On the other hand, in 100% of the cells that expressed the kinase-dead construct, PKCαKD, carbachol induced a sustained translocation of the protein to the plasma membrane. The same effect was also seen for PKCaADM, a mutant where both autophosphorylation sites were mutated to alanine and, thus, mimicked the non-phosphorylated PKCa. PKCa mutation to alanine either in turn motif, PKCaT638A, or hydrophobic site, PKCaS657A, consistently responded to carbachol, and translocated to the plasma membrane after stimulation. Contrasting the glutamate mutants and wild-type PKCa, a sustained translocation of these mutants was frequently observed, but unlike PKCaADM these mutants, sometimes returned to the cytosol after stimulation with carbachol. Thus, autophosphorylation of the hydrophobic site and the turn motif conceivably contributes to the transient nature of PKCα translocation. However, mutation of the autophosphorylation sites to glutamate does not make PKCα resistant to translocation signals.

**Mutation of the Autophosphorylation Sites in PKCδ and PKCe Do Not Alter Their Sensitivity to Carbachol**—We have previously seen that PKCe responds with a sustained translocation and that PKCδ responds with a weak and transient translocation upon carbachol stimulation (6). To see if the effect of autophosphorylation influences the responsiveness of these isoforms as well, we created vectors encoding PKCδ with both autophosphorylation sites mutated to alanine (PKCδADM) and

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**Fig. 3. Effects of kinase inhibition on carbachol-stimulated translocation of PKCβI, PKCβII, and PKCδ.** SK-N-BE(2)C cells transfected with vectors encoding PKCβI WT (A), PKCβII WT (B), and PKCδ WT (C) were treated with 2 μM GF109203X (GFX) or vehicle Me2SO (DMSO), for 30 s before stimulation with 1 mM carbachol. Cells were also transfected with vectors encoding PKCβI KD (D), PKCβII KD (E), and PKCδ KD (F) and subsequently stimulated with 1 mM carbachol. Fluorescence intensity of EGFP in the cytosol for the different cells was quantified. Data represent the means ± S.E. for 4–14 cells in 1–4 different experiments and are expressed as the percentage of the levels in unstimulated cells. *Arrows* indicate the addition of GF109203X or Me2SO (DMSO), and *arrowheads* indicate the addition of carbachol.
PKCα with both sites mutated to glutamate (PKCαEDM). However, the mutations did not augment the weak response of PKCδ or suppress the sustained PKCε response (data not shown). Thus, in contrast to PKCα, the sensitivity of PKCδ and PKCε to carbachol-generated second messengers is not regulated by autophosphorylation.

Neither Carbachol nor GF109203X Has Major Effects on the Amount of Autophosphorylated PKCα—The previous experiments could have the implication that autophosphorylation of PKCα upon its activation at the plasma membrane is a mechanism to cause its relocation to the cytosol and thereby to turn off the PKC signal. If this hypothesis is true, it would be expected that the levels of autophosphorylation would increase after carbachol stimulation and decrease by treatment with GF109203X. We, therefore, analyzed by Western blots the levels of phosphorylation of the autophosphorylation sites were analyzed by Western blotting using antibodies against the hydrophobic site (E) and turn motif (F). The blots are representative for two independent experiments.
either the hydrophobic site (Fig. 5A) or the turn motif (Fig. 5B). There was a slight tendency to increased phosphorylation of the hydrophobic site. The same result was obtained when the phosphorylation of overexpressed PKCα-EGFP was analyzed (data not shown). Thus, carbachol does not increase the auto-

Similarity, we wanted to investigate whether treatment with GF109203X affected the phosphorylation. Treatment of cells with 2 μM GF109203X did not decrease the phosphorylation of the hydrophobic site (Fig. 5C). Phosphorylation of the turn motif (Fig. 5D) was elevated in the presence of Me2SO, but it was clear that GF109203X did not influence the phosphorylation. The same pattern of GF109203X effect was seen when analyzing phosphorylation of PKCα-EGFP (data not shown).

We also investigated the autophosphorylation of kinase-dead PKCα and the PKCα variants with autophosphorylation sites mutated to alanine (Fig. 5, E and F). As expected, both kinase-dead PKCα and the alanine double mutant were devoid of phosphate on the two autophosphorylation sites, whereas wild-type PKCα was phosphorylated on both sites. The S657A mutant was at least partially phosphorylated on Thr-638, whereas the Thr-638 was phosphorylated on neither site, confirming previous results with PKCαII, which shows phosphorylation of the turn motif as a prerequisite for phosphorylation of the hydrophobic site (9).

PKCs with Autophosphorylation Sites Mutated to Glutamate Responds to Carbachol with a Sustained Translocation when Pretreated with GF109203X—The Western blot analyses demonstrated that GF109203X does not influence the autophosphorylation of PKCα, suggesting that an inhibition of autophosphorylation is not the mechanism through which GF109203X potentiates the translocation of PKCα. To further confirm that the GF109203X effect is independent of the phosphorylation status of the turn motif and the hydrophobic site, we investigated the effect of GF109203X on the translocation of PKCα with both sites mutated to glutamate. Stimulation with carba-

Fig. 6. Pretreatment with GF109203X makes PKCαEDM respond with a sustained translocation after carbachol stimulation. SK-N-EB(2)/C cells transfected with vectors encoding PKCαEDM were stimulated with carbachol (cch) with no pretreatment (A, D, and G) or pretreated with Me2SO (DMSO; B, E, and H) or 2 μM GF109203X (C, F, and I) before stimulation with carbachol. The localization of the protein was monitored by confocal microscopy. Images show unstimulated cells (A–C) and cells 30 s (D–F) and 4 min (G–I) after the addition of carbachol. The results are representative of 3–6 separate experiments.

Inhibition of the Catalytic Activity Makes PKCα Sensitive to Diacylglycerol—The previously mentioned experiments indicate that autophosphorylation does not make PKCα resistant to receptor-stimulated translocation in general. We, therefore, hypothesized that kinase inhibitors and reduced autophospho-
Autophosphorylation Limits the Sensitivity of PKCα to DAG

This study reports a novel role for PKCα autophosphorylation as a regulatory switch determining the DAG sensitivity of PKCα. We, therefore, propose a model in which the V5 domain participates in intramolecular interactions that maintain an inaccessibility of the C1a domain of PKCα to DAG. The interaction is dependent on autophosphorylation and can also be disrupted by direct interactions with PKC inhibitors such as GF109203X.

Our initial experiments demonstrated that inhibition of PKCα either with application of GF109203X or by a kinase-inactivating mutation of the ATP-binding site resulted in a sustained translocation upon carbachol stimulation. The results may be explained by a model in which an activation of PKCα results in autophosphorylation, which in turn induces a relocation of PKCα to the cytosol. This could function as a negative feedback signal to turn off the activated PKCα. Such a hypothesis is supported by papers which have demonstrated that PKC inhibitors prolong the agonist-induced membrane localization of classical PKC isoforms (15, 16). Our data demonstrate that the phenomenon is common for several PKC isoforms. Both PKCα as well as the PKCβ isoforms are similarly affected by kinase inhibition, and there was also a similar, albeit smaller, effect for PKCδ.

If the hypothesis is true, it would be expected that mutation of the C-terminal autophosphorylation sites to alanine, mimicking a dephosphorylated state, would abolish the relocation of PKC to the cytosol during agonist stimulation. This is indeed what we found in our experiments. It would also be expected that mutation of the sites to glutamate, mimicking the phospho-orylation of the C-terminal sites and insensitivity to a receptor-stimulated translocation signal. It is, therefore, clear that autophosphorylation is not a mechanism for desensitization of the PKC translocation.

Our data instead point to other important regulatory functions of autophosphorylation. Both kinase-inactivated PKCα and PKCα with the autophosphorylation sites mutated to alanine responded to exogenous application of DOG. This contrasts the reaction of wild-type PKCα, which is insensitive to DOG. We propose that this difference explains why the former mutants display a sustained translocation upon carbachol stimulation, whereas the translocation of wild-type PKCα is transient. We have previously seen that after the addition of carbachol there is a rapid and transient increase in intracellular Ca2+ levels, but the DAG levels accumulate gradually and remain elevated for at least several minutes (6). We, therefore, propose that autophosphorylation of the C-terminal sites serves as a regulatory switch determining whether PKCα is sensitive to DAG or not. It is in this context interesting to note that several stimuli and extracellular factors influence the amount of classical PKC isoforms that are autophosphorylated (14, 18–20). Our data, therefore, highlight the possibility that regulated phosphorylation of the hydrophobic site and the turn motif serves to alter the DAG sensitivity of PKCα and possibly also of other classical isoforms.

The question remains as how phosphorylation of the C-terminal sites alters the DAG sensitivity of PKCα. Previous studies demonstrate that DAG primarily binds the C1α (24) domain and that this interaction is suppressed by an intramolecular interaction between the C2 and the C1α domain in the inactive PKCα (4, 5). Our previous study (6) showed...
that the catalytic domain is necessary to maintain the inaccessibility of the C1a domain, and this study indicates that the autophosphorylation sites are necessary for the effect. It is in this context interesting to note the proposed model for the intramolecular interactions in PKCβ between a receptor for activated C-kinase-binding site and a pseudo-receptor for activated C-kinase motif (25, 26). The interaction is putatively mediated via the V5 and the C2 domain. Because the autophosphorylation sites are located in the V5 domain of PKCa, our data further support a model in which the V5 domain participates in intramolecular interactions that maintain the PKC molecule in a closed conformation. The strength of these interactions may be influenced by the presence of phosphate on the autophosphorylation sites.

Besides influencing the translocation response, phosphorylation of the turn motif and the hydrophobic site have also been suggested to increase the stability of PKC and to prevent it from accumulating in a detergent-insoluble fraction (9, 12). Several studies indicate that the down-regulation of PKC that follows upon prolonged activation of the enzyme is mediated via an initial dephosphorylation of the autophosphorylation sites (14, 27, 28), perhaps followed by a ubiquitination of the enzyme. However, there are also studies showing that primarily the fully phosphorylated PKC molecule is degraded without an initial dephosphorylation (29). Our experiments show that the mutant with both autophosphorylation sites mutated to alanine had a similar localization pattern as wild-type PKCa, demonstrating that the propensity of the non-phosphorylated variants to accumulate in detergent-insoluble fractions does not translate into an apparent alteration of its localization in the cell. Furthermore, when analyzing the expression of the alanine mutants, we could not detect any degradation products of PKCa. This indicates that the rate of degradation of non-phosphorylated PKCa is slow in the neuroblastoma cells. The kinase-dead PKCa variant was, as expected, not autophosphorylated, which may explain its DOG sensitivity and sustained response to carbachol stimulation. However, the inhibitor GF109203X potentiated the translocation response, but it did not reduce the autophosphorylation. Furthermore, it prolonged the translocation of PKCs with the autophosphorylation sites mutated to glutamate, and it could reverse the relocation of PKCs to the cytosol once it had occurred (Fig. 2). It is, therefore, likely that GF109203X enhances the translocation of PKCs through other means than by suppression of the autophosphorylation of PKCa. We speculate that the inhibitor, by interacting with the ATP-binding site, destabilizes the closed conformation of PKC and thereby exposes the C1a domain and makes it available for interaction with DAG.

Both exogenous application of PKC inhibitor and overexpression of a kinase-inactivated PKC mutant are widely used to suppress PKC activity in order to investigate the role for PKC in different cellular processes. Our data clearly show that both methods not only block PKC activity but also have other effects on the protein, such as increasing the amount of PKC molecules, albeit most likely catalytically inactive enzymes, at the plasma membrane. Given the fact that PKC has been shown to exert several effects independently of its kinase activity (31, 30–34), the use of inhibitors or dominant-negative variants may actually potentiate these PKC effects instead.
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