Unique Phosphorylation of Protein Kinase C-α in PC12 Cells Induces Resistance to Translocation and Down-regulation*

(Received for publication, July 31, 1996, and in revised form, September 19, 1996)

Andrea Gatti†‡§ and Phillip J. Robinson¶

From the †Endocrinology Unit, John Hunter Hospital, Hunter Region Mail Centre, New South Wales 2310, Australia and the ‡Department of Experimental Oncology, European Institute of Oncology, 20141 Milan, Italy

Cell exposure to phorbol ester stimulates translocation and activation of protein kinase C (PKC), ultimately followed by its down-regulation. Upon activation, PKC-α, the best studied isotype of the PKC family, undergoes changes in its phosphorylation state. With a two-dimensional immunoblot procedure we have previously shown the existence in PC12 cells of several multiply phosphorylated forms of PKC-α, whose number increases in response to phorbol esters (Gatti, A., Wang, X., and Robinson, P. J. (1996) Biochim. Biophys. Acta 1313, 111–118). Using the same experimental system, here we report that besides the predominant pool of 80-kDa PKC-α forms that respond to phorbol ester by translocating to the cell membranes and down-regulating, there is a small pool of cytosolic 82-kDa PKC-α forms that are characterized by a more acidic pI and by an unique resistance to phorbol ester-mediated translocation and down-regulation. The appearance of similarly slower migrating and more acidic PKC-α forms is reproduced upon in vitro autophosphorylation in the presence of phosphatidylserine and phorbol ester, but not in the presence of calcium. These results suggest that site-specific transphosphorylation or autophosphorylation of this kinase may regulate its subcellular localization and susceptibility to down-regulation.

Protein kinase C (PKC)† exists as a family of at least 12 isoforms with closely related structures (1). Members of this family are involved in a variety of cell responses to signals that target most signaling systems, including the nervous, endocrine, and immune systems (2). Moreover, in all of the examined cell types, PKC is the major, if not the only, intracellular target for tumor-promoting phorbol esters (3). All identified PKCs share a common functional requirement for phosphatidylserine (PS) and differ in their sensitivity to other activators (4). Conventional PKC isoforms (cPKCs) represent a group of four isoenzymes (PKC-α, -β, -βII, and -γ) that are sensitive to synergetic activation by 1,2-diacylglycerol and calcium. Under resting conditions most PKC-α is located in the cytosol and is inactive. The in situ dynamic equilibrium between membrane-associated and cytosolic forms of PKC is regulated to a large extent by the intracellular level of calcium and 1,2-diacylglycerol (5). Therefore, phorbol esters and several physiological stimuli cause translocation of the responsive PKCs to the cell membranes with subsequent kinase activation and phosphorylation of endogenous substrates (6).

Phorbol esters produce a plethora of biological effects, whose extraordinary diversity has been explained by the multiple expression of PKC isotypes and the isotype-specific tissue distribution and intracellular localization (4). An additional source of functional heterogeneity in the biological effects of phorbol ester may result from the multisite phosphorylation of PKC (7, 8). PKC is initially translated as an unphosphorylated and inactive particulate-bound form (primary product), which is converted to an activable cytosolic form (mature form) by at least three sequential stages of priming phosphorylations (9–12). Once the mature PKC has been formed, it is believed to be subjected to further autophosphorylation and transphosphorylation by various activators and protein kinases, respectively. We have previously reported that in vitro both the combinations of calcium plus PS (Ca²⁺/PS) and PS plus phorbol ester (PS/TPA) greatly stimulate the autophosphorylation of PKC but apparently on distinct sites (13, 14).

Additionally, the mature PKC-α isoform uniquely undergoes a mobility shift to a slower migrating form upon autophosphorylation induced by PS/TPA and not by Ca²⁺/PS. Interestingly, the unique autophosphorylation induced in vitro by phorbol ester in the absence of calcium correlates with an altered PKC substrate specificity and altered kinetics of substrate phosphorylation (15).

Although distinct changes in the phosphorylation state have been correlated to activation and inactivation of PKC function (16–18), the relationship between the state of activation and the state of phosphorylation remains to be fully elucidated. In principle, a stimulus-dependent alteration in the balance between distinct PKC phosphoforms might be as critical for a cell response as the translocation of the enzyme itself. However, technical difficulties in detecting the individual phosphoforms of a given PKC isoform have severely hampered the analysis of the in vivo correspondence between changes in the ratio of differentially phosphorylated PKC forms and changes in the enzyme functionality. PKC-α is the most studied isotype among the PKC species and the only cPKC expressed in PC12 cells. With a two-dimensional immunoblot method we have recently described the in situ phosphorylation of PKC-α in response to PC12 cell exposure to phorbol ester (19). In the present paper, we use this methodological approach to examine the potential functional role of the unique phosphorylation of PKC-α previously observed in vitro (13, 14). We report that a group of immunoreactive PKC-α forms with highly acidic pI and retarded mobility on SDS-PAGE are resistant to phorbol ester-
PKC-α Down-regulation in PC12 Cells

phenylmethylsulfonyl fluoride, and 1 mM EDTA, pH 7.4. All subsequent steps were carried out at 4 °C. When cell homogenates were required, cells were lysed with 10 passes through a 26-gauge needle and centrifuged at 10,000 × g for 60 min, the supernatant was collected and designated as the soluble fraction. The pellet was resuspended in 20 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, 0.75 mM CaCl2, 1 mM phenylmethylsulfonyl fluoride, 40 μM leupeptin, and 1 mM EDTA-glucosidase on the outside. The resuspended pellet was dissociated by repeated aspirations through a plastic pipette tip, shaken for 30 min, and then centrifuged at 13,000 × g for 15 min. The resulting supernatant was designated the particulate fraction. Postnuclear cell homogenates and subcellular fractions from PC12 cells were loaded onto a pre-equilibrated diethylaminoethyl cellulose (DE52, Whatman, 2 ml) column to partially purify PKC, as described previously (19).

SDS-PAGE and Two-dimensional Electrophoresis—DE52 extracts were electrophoresed on 7.5% SDS-PAGE, as described previously (20). When indicated, DE52 samples were subjected to two-dimensional electrophoresis essentially as described previously (21) with the following modifications. Samples were diluted 1:1 with buffer for isoelectric focusing (9.5 M urea, 5% CHAPS, 5% N-mercaptoethanol, and Pharmacia Biotech, Inc. ampholines 1.2% pH 5–8, 0.8% pH 3–10), and additional solid urea was added to a final concentration of 1 mg/ml. Aliquots (50–100 μl) were loaded onto 7.5-cm-long isoelectric focusing gel tubes (0.2 cm, internal diameter) and resolved with non-equilibrium isoelectric focusing (7000 V-h) in the first dimension. The second dimension was run at 10% SDS-PAGE on a Bio-Rad minigel.

In Vitro Autophosphorylation of PKC—Rat brain PKC was purified to apparent homogeneity and subjected to phosphopeptide mapping with V8 protease as described previously (13, 15). Purified PKC was electrophoresed on 7.5% acrylamide gels to resolve PKC-α from PKC-β and γ, since PKC-α has been shown to migrate at a slightly higher molecular mass (81 versus 80 kDa) under these conditions (14). In vitro autophosphorylation of PKC-α was carried out and assessed as described previously (19).

Immunoblot—Proteins from SDS-PAGE and two-dimensional electrophoresis were transferred onto nitrocellulose membrane, as described previously (22). The nitrocellulose was blocked with phosphate-buffered saline containing 1% polyvinylpyrrolidone (Mw = 40,000) and the same washing buffer supplemented with 40 μM leupeptin. After cell lysis, subcellular fractions were obtained by ultracentrifugation at 100,000 × g for 60 min, the supernatant was collected and designated as the soluble fraction. The pellet was resuspended in 20 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, 0.75 mM CaCl2, 1 mM phenylmethylsulfonyl fluoride, 40 μM leupeptin, and 1 mM EDTA-glucosidase on the outside. The resuspended pellet was dissociated by repeated aspirations through a plastic pipette tip, shaken for 30 min, and then centrifuged at 13,000 × g for 15 min. The resulting supernatant was designated the particulate fraction. Postnuclear cell homogenates and subcellular fractions from PC12 cells were loaded onto a pre-equilibrated diethylaminoethyl cellulose (DE52, Whatman, 2 ml) column to partially purify PKC, as described previously (19).

RESULTS

Early Response of PKC-α Upon Cell Exposure to Phorbol Ester—In PC12 cells, as in all examined cell types, phorbol ester induces most PKC to redistribute from the soluble to the particulate fraction. We initially measured TPA-mediated translocation using SDS-PAGE and immunoblot analysis with the MC5 anti-PKC antibody (from Amersham Corp.). This antibody was previously shown in PC12 cells to specifically recognize the PKC-α, the only cPKC isotype in these cells (19). DE52 extracts of TPA-treated and untreated PC12 cells were probed with MC5, and most of the major immunoreactive PKC-α form was found to translocate from the soluble to the particulate fraction within 30 min of cell incubation with 100 nM TPA (indicated as the 80-kDa form, Fig. 1). However, a smaller proportion of the PKC-α corresponding to a slower migrating form accumulated in the soluble fraction upon cell exposure to phorbol ester (indicated as the 82-kDa form, Fig. 1). Note that the immunoreactive signals from both the 80- and 82-kDa forms were fully displaced if a mix of purified rat brain cPKCs was included in the incubation medium containing the primary antibody (data not shown), demonstrating the specificity of the immunodetection.

DE52 extracts of PC12 cells were then subjected to a two-dimensional immunoblot procedure and probed with the MC5 antibody. The expression of PKC-α in untreated cells was preferentially restricted to the cytosolic fraction where it consisted of two distinct pools of differentially migrating PKC-α forms, here designated pool 1 and pool 2 (Fig. 2, panels A and C). We previously demonstrated multiple phosphoforms of PKC-α within pool 1 after brief stimulation of intact cells with TPA (19). However, the appearance of pool 2 has not previously been reported and was revealed in this study by use of a broader range of ampholites in the preparation of isoelectrofocusing gels. The unequal isoelectric migration of the two pools appears to be the result of the difference in the relative degree of phosphorylation of the respective PKC-α forms, with the more acidic forms in pool 2 being apparently more phosphorylated than those of pool 1. Consistent with this observation, the more acidic forms are characterized by a slower electrophoretic mobility in the second dimension of the two-dimensional procedure.
that pool 2 of PKC-α two-dimensional immunoblot with the same antibody showed DE52 extracts from soluble (20 μg of protein) and particulate (50 μg of protein) fractions were diluted in buffer for the first dimension and resolved by isoelectric focusing (7000 V-h). The second dimension in 10% SDS-PAGE was terminated shortly before the dye (bromphenol blue) migrated off the end of the gel. Proteins were electrophoretically transferred onto nitrocellulose membrane, and immunorecognition was carried out with the MC5 anti-PKC-α antibody with ECL detection. Only the relevant sections of the films are shown. Open arrows indicate two differentially migrating pools of PKC-α forms, designated as pool 1 and pool 2. The migration of molecular mass markers is indicated on the right in kilodaltons. Results shown are representative of three independent experiments.

**FIG. 2.** Two-dimensional immunoblot with MC5 anti-PKC-α antibody. Prior to lysis, intact PC12 cells were incubated as indicated with or without 100 nM TPA for 30 min. EGTA-extracted subcellular fractions were then subjected to DE52 chromatography. Aliquots of DE52 extracts from soluble (20 μg of protein) and particulate (50 μg of protein) fractions were diluted in buffer for the first dimension and resolved by isoelectric focusing (7000 V-h). The second dimension in 10% SDS-PAGE was terminated shortly before the dye (bromphenol blue) migrated off the end of the gel. Proteins were electrophoretically transferred onto nitrocellulose membrane, and immunorecognition was carried out with the MC5 anti-PKC-α antibody with ECL detection. Only the relevant sections of the films are shown. Open arrows indicate two differentially migrating pools of PKC-α forms, designated as pool 1 and pool 2. The migration of molecular mass markers is indicated on the right in kilodaltons. Results shown are representative of three independent experiments.

**FIG. 3.** Two-dimensional immunoblot with the anti-PKC-α antibody from Transduction Laboratories. Conditions are the same as in Fig. 2, except for the different PKC-α antibody.

was incubated together with a mix of purified cPKCs, confirming the specificity in the immunorecognition of both pools (data not shown).

**PKC-α Response Upon Prolonged Cell Exposure to Phorbol Ester**—A prolonged phorbol ester treatment of cells mediates association of PKC with cell membranes followed by both catalytic inactivation (23, 24) and proteolytic degradation of the kinase itself (25). In order to assess whether the lack of a detectable response of pool 2 to phorbol ester was extended beyond the acute phase of treatment, PC12 cells were exposed to differential down-regulation, and the recovery of PKC-α was assessed by immunoblot with the MC5 antibody. The standard immunoblot analysis of DE52 extracts from cells treated with an increasing TPA concentration for 2.5 h revealed that 1 μM TPA caused a substantial loss of the 80-kDa form without altering the recovery of the 82-kDa species (Fig. 4, panel A). A two-dimensional immunoblot with the same antibody showed that pool 2 of PKC-α is insensitive to 2.5-h exposure to 1 μM TPA, despite the loss of pool 1 (Fig. 4, panel B). Additional one- and two-dimensional immunoblot analyses of samples deriving from a more prolonged cell exposure to phorbol ester (15 h with 1 μM TPA) gave similar results (data not shown), thus indicating that pool 2 consists of truly resistant forms of PKC-α.

**Autophosphorylation of PKC-α in Vitro**—To determine whether in vitro PKC-α autophosphorylation produces patterns that may explain the unique two-dimensional profile of pool 2 observed in intact cells, purified cPKC was autophosphorylated in the presence of various activators, after which the extracted PKC-α was subjected to phosphopeptide mapping. We have previously reported that autophosphorylation of PKC-α in the presence of PS plus calcium (Ca^{2+}/PS) is not associated with any detectable shift in its electrophoretic mobility, while autophosphorylation in the presence of PS plus phorbol ester (PS/TPA) promotes the conversion into an apparently higher molecular weight form (14). Here, phosphopeptide mapping with V8 protease of these differentially migrating forms revealed that PKC-α autophosphorylation produced two distinct patterns (Fig. 5, lanes 1–3), suggesting that the sites of autophosphorylation may be switched by the availability of calcium. In the presence of calcium, the phosphopeptide maps of PKC-α were similar to those of PKC-β/γ (Fig. 5, lanes 5 and 6). However, PS/TPA in the absence of calcium produced very different phosphopeptide maps of PKC-β/γ (lane 4) without any concomitant mobility shift of the respective undigested proteins (14).

We finally examined whether highly acidic PKC-α forms (equivalent to pool 2) are detectable upon in vitro treatment of purified rat brain PKC with its activators. Although both combinations of PS/TPA and Ca^{2+}/PS apparently promote PKC-α autophosphorylation (as visualized by the acidic shift in the two-dimensional profile, Fig. 6), only PS/TPA produced detectable pool 2 (Fig. 6, panel C). To confirm the identity of pool 2, samples from in vitro autophosphorylation of purified cPKC (with PS/TPA) and from in situ treated PC12 cells (30 min with 100 nM TPA) were mixed and subjected to the same two-dimensional immunoblot procedure. The PKC-α forms of pool 2 from PC12 cells were found to co-migrate with the highly acidic variants resulting from the PS/TPA-mediated autophosphorylation of rat brain PKC-α (data not shown).

**DISCUSSION**

The central aim of this study was to investigate whether cell exposure to a single PKC activator (phorbol ester) may induce differential responses in distinct PKC-α phosphoforms. We have recently shown a phorbol ester-stimulated increase in the
PKC-α Down-regulation in PC12 Cells

The majority of PKC-α cells, presumably resulting from a combination of autophosphorylation and transphosphorylation events (19). Since the majority of PKC-α in a cell at any given time consists of a mature form (of ~80 kDa), these phosphorylation events do not appear to be related to the multistage priming phosphorylation reported by others (9–11). In the present study, the use of a broader range in the pH gradient of the first dimension of the two-dimensional immunoblot allowed the detection of additional PKC-α forms (here termed pool 2) from intact PC12 cells. These forms of PKC-α are more acidic than the previously recognized forms corresponding to pool 2 from intact PC12 cells. The possibility that the species corresponding to pool 2 and 82-kDa forms consists of an isotype other than PKC-α is not likely because of the lack of any such comigrating PKC form in PC12 cells (19). Taken together, the above observations suggest that the 82-kDa forms constitute pool 2 and that this consists of bona fide PKC-α. This is further substantiated by our previous finding that upon PS/TPA-mediated autophosphorylation, the mature PKC-α is converted to a slower migrating form (15).

Our results suggest that the autophosphorylated PKC-α, rather than the transphosphorylated form, is likely to represent pool 2 from intact PC12 cells. Evidence to support this proposal derives from the following observations. Firstly, the in vitro treatment of purified PKC with its activators (PS/TPA) induces the appearance of slower migrating and highly acidic PKC-α forms that co-migrate in two-dimensional immunoblots with those representing pool 2 from intact PC12 cells. The appearance of slower migrating PKC-α species, when compared with the mature 80-kDa form, is a rather unique event, since the only other reported mobility shift of PKC-α occurs during the priming process from the 76-kDa precursor to the activable 80-kDa protein kinase. Note that the 80-kDa protein species reported in our study was indeed mature PKC-α, since it was reduced to an inactive 76 kDa form by in vitro treatment with phosphatases.2 Further, among other cPKCs the change in gel mobility and in phosphopeptide map reported here is restricted to the PKC-α, indicating a high degree of specificity of the effect. Despite the above considerations, the possibility that in intact cells phorbol ester activates a protein kinase responsible for the conversion of pool 1 PKC-α into pool 2 by transphosphorylation cannot be completely ruled out yet.

Why do the 82-kDa PKC-α forms of pool 2 remain in the cytosol if phorbol ester mediates membrane translocation of most PKC-α forms and down-regulation of the entire membrane-associated pool? According to the simplest interpretation, a unique site-specific autophosphorylation of PKC-α induced by the exogenous activators reduces the overall protein lipophilicity and interferes with its association with cell membranes, thus preventing the membrane-driven process of down-regulation. Such an autophosphorylation of PKC-α may induce conformational changes and mask the phospholipid binding site. Alternatively, the unique autophosphorylation may promote PKC binding to an unidentified binding protein that prevents translocation. A direct correlation between an increased phosphorylation of PKC and its dissociation from the membrane has been described in several reports. The autophosphorylated form of PKC is reported to be preferentially retained in a high speed supernatant rather than to associate with PS vesicles (26). In cell-free experiments, autophosphorylation protected PKC from PS-induced proteolysis (27), and the

\[ A. \text{ Gatti, unpublished results.} \]
addition of Mg/ATP dissociated PKC from membranes presumably following autophosphorylation (28). Therefore, autophosphorylation may play a key role in regulating the biological function of PKC.

Our results demonstrate a biological role for PKC-α phosphorylation in the protection of a pool of this isotype from translocation and down-regulation. Pool 1 and pool 2 respond in a conventional and unique manner to cell treatment with phorbol ester, respectively. This indicates the existence of a substantial degree of functional heterogeneity within a single PKC isotype. After previously reporting that the substrate specificity of PKC is partly regulated by the same agents that control its phosphorylation state (15), we have shown here that site-specific phosphorylation regulates subcellular localization and susceptibility to down-regulation. Therefore, it is conceivable that a heterogenous population of PKC phosphoforms in vivo exerts a wide range of actions, the effects of which represent an important source of functional diversity.

Acknowledgments—We thank Dr. Xin Wang for help with purification of PKC and Luigi D’Offizi for expert assistance in preparation of the figures.

REFERENCES
1. Tanaka, C., and Nishizuka, Y. (1994) Annu. Rev. Neurosci. 17, 551–567
2. Nishizuka, Y. (1986) Science 233, 305–312
3. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U., and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847–7851
4. Nishizuka, Y. (1988) Nature 334, 661–665
5. Trilivas, I., and Brown, J. H. (1989) J. Biol. Chem. 264, 3102–3107
6. Kraft, A. S., and Anderson, W. B. (1983) Nature 301, 621–623
7. Huang, K.-P., Chan, K.-F. J., Singh, T. J., Nakabayashi, H., and Huang, F. L. (1986) J. Biol. Chem. 261, 12134–12140
8. Flint, A., Paladini, R., and Koshland, D. (1990) Science 249, 408–411
9. Borner, C., Filippuzzi, I., Wartmann, M., Eppenberger, U., and Fabbro, D. (1989) J. Biol. Chem. 264, 13902–13909
10. Pears, C. Stabel, S., Cazaubon, S., and Parker, P. (1992) Biochem. J. 283, 515–518
11. Cazaubon, S. M., and Parker, P. J. (1993) J. Biol. Chem. 268, 17559–17563
12. Keranen, L., Dutli, R., and Newton, A. (1995) Curr. Biol. 5, 1394–1403
13. House, C., Robinson, P. J., and Kemp, B. (1989) FEBS Lett. 249, 243–247
14. Robinson, P. J. (1991) Mol. Neurobiol. 5, 87–142
15. Robinson, P. J. (1992) J. Biol. Chem. 267, 21637–21644
16. Mitchell, F., Marais, R., and Parker, P. (1989) Biochem. J. 261, 131–136
17. Molina, C., and Ashendel, C. (1991) Cancer Res. 51, 4624–4630
18. Ohno, S., Mizuno, K., Adachi, Y., Hata, A., Akita, Y., Akimoto, K., Osada, S., Hirai, S., and Suzuki, K. (1994) J. Biol. Chem. 269, 17495–17501
19. Gatti, A., Wang, X., and Robinson, P. J. (1996) Biochim. Biophys. Acta 1313/2, 111–118
20. Laemmli, U. (1970) Nature 227, 680–685
21. O’Farrel, P. (1985) J. Biol. Chem. 250, 4007–4021
22. Towbin, H., Staehlin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
23. Rodriguez-Pena, A., and Rozengurt, E. (1984) Biochim. Biophys. Res. Commun. 120, 1053–1059
24. Inagaki, M., Hagiwara, M., Saitoh, M., and Hidaka, H. (1986) FEBS Lett. 202, 277–280
25. Young, S., Parker, P., Ulbrich, A., and Stabel, S. (1987) Biochem. J. 244, 775–779
26. Mochly-Rosen, D., and Koshland, D., Jr. (1987) J. Biol. Chem. 262, 2291–2297
27. Orr, J. W., Keranen, L. M., and Newton, A. C. (1992) J. Biol. Chem. 267, 15263–15266
28. Wolofsky, M., Cuatrecasas, P., and Sahyoun, N. (1985) J. Biol. Chem. 260, 15718–15722