Differential Localization of Protein Kinase C δ by Phorbol Esters and Related Compounds Using a Fusion Protein with Green Fluorescent Protein*

(Qiming J. Wang‡, Dipak Bhattacharyya‡, Susan Garfield§, Kassoum Nacro¶, Victor E. Marquez¶, and Peter M. Blumberg¶)

Enzyme localization often plays a controlling role in determining its activity and specificity. Protein kinase C (PKC) has long been known to translocate in response to physiological stimuli as well as to exogenous ligands such as the phorbol esters. We report here that different phorbol derivatives and related ligands, selected for differences in chemical structure and profile of biological activity, induce distinct patterns of redistribution of PKC δ. Localization of a PKC δ-green fluorescent protein (GFP) fusion construct was monitored in living Chinese hamster ovary cells as a function of ligand, concentration, and time using confocal laser scanning microscopy. δ-PKC-GFP was expressed predominantly in the cytoplasm, with some in the nucleus and perinuclear region. Phorbol 12-myristate 13-acetate (PMA) induced plasma membrane translocation followed by slower nuclear membrane translocation. As the concentration of PMA increased, the proportion of nuclear to plasma membrane localization increased markedly. In contrast to PMA, bryostatin 1, a unique activator of PKC that induces a subset of PMA-mediated responses while antagonizing others, at all doses induced almost exclusively nuclear membrane translocation. Like PMA, the complete tumor promoter 12-deoxyphorbol 13-tetradecanoate induced plasma membrane and slower nuclear membrane translocation, whereas the inhibitor of tumor promotion 12-deoxyphorbol 13-phenylacetate, which differs only in its side chain, induced a distinctive distribution of PKC δ-GFP. Finally, the novel constrained diacylglycerol derivative B8-DL-B8 induced a slow Golgi localization. We speculate that differential control of PKC δ localization may provide an interesting strategy for producing ligands with differential biological consequences.

Protein kinase C comprises a family of serine/threonine protein kinases that play pivotal roles in signal transduction pathways affecting a broad range of cellular functions (1, 2). The 12 subspecies of protein kinase C identified so far define four subfamilies: the classical PKCs (α, β, I/BII, γ) are Ca2+-dependent and diacylglycerol (DAG)4-responsive; the novel PKCs (δ, ε, η, θ) are Ca2+-independent and DAG-responsive; the atypical PKCs (ζ and λ/i) are both Ca2+ and DAG-independent; and the PKC-μ/PKD subfamily (PKD1 and 2) possesses a number of unique structural features, including a nonhomologous kinase domain. Different PKC isoforms may fulfill both redundant and unique biological roles within cells (3), and the basis for this specificity is an area of active investigation.

The structure of PKC can be divided into two functionally distinct halves: an N-terminal regulatory domain and a C-terminal catalytic domain. There are two conserved regions, C1 and C2, in the regulatory domain of the classical PKCs. The twin C1 domains are zinc finger structures that interact with lipids and constitute the binding site for DAG and the phorbol esters (4). Structural analysis of the complex between the C1 domain of PKC δ and phorbol 13-acetate suggests that the C1 domain functions as a hydrophobic switch (5). The C1 domain displays a hydrophobic surface interrupted by a hydrophilic cleft. By inserting into the hydrophilic cleft, the phorbol ester or DAG provides a hydrophobic cap over this hydrophilic cleft, facilitating the association of the C1 domain with the lipid bilayer or other hydrophobic surfaces. The C2 domain binds Ca2+ and phosphati-

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‡ To whom correspondence should be addressed: Bldg. 37, Rm. 3A01, NCI, NIH, 37 Convent Dr., MSC 4255, Bethesda, MD 20892-4255, Tel.: 301-496-3189; Fax: 301-496-8709; E-mail: blumberg@dc37a.nci.nih.gov.

§ The abbreviations used are: DAG, diacylglycerol; GFP, green fluorescent protein; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; CHO, Chinese hamster ovary; B8-DL-B8, (DL)-{(1-hydroxy-3-methyl-4-[4-methyl-3-(methylthyl)pentylidene]-3-oxo-2-oxo-5-yl-methyl-4-methyl-3-(methylthyl)pentanoate.}

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because all of these agents induce similar levels of activation in vitro. In this report, utilizing a green fluorescent protein fusion construct with PKC δ, we explore the hypothesis that different ligands may induce different patterns of translocation of PKC.

EXPERIMENTAL PROCEDURES

Materials—PMA, 12-deoxyphorbol 13-phenylacetate, and 12-deoxyphorbol 13-tetradecanoate were obtained from LC Services Corp. (Woburn, MA). Bryostatin 1 was from the Drug Chemistry and Synthesis Branch, NCI. The constrained DAG lactone (1L-3(Z)-1,(hydroxymethyl)-4-(4-methyl-3-(methylethyl)pentylidene)-3-oxo-2-oxolan-yl)methyl4-methyl-3-(methylethyl)pentanoate (referred to in the text as B8-DL-B8) was synthesized as will be described elsewhere.2

CELL CULTURE—CHO-K1 cells (CCL 61) were obtained from the American Type Culture Collection (Manassas, VA). The cells were cultured at 37 °C in Dulbecco's modified Eagle's medium supplemented with 4500 mg/liter glucose, 4 mM l-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin (Advanced Biotechnologies Inc., Columbia, MD), and 10% fetal bovine serum (Life Technologies, Inc.) in a humidified atmosphere containing 5% CO2.

RESULTS

Characterization of the GFP Fusion Protein with PKC δ—After the cDNA construct had been verified by sequencing, the pEGFP plasmid encoding δ-PKC-GFP was transiently transfected into CHO-K1 cells. As a control, the pEGFP plasmid without the δ-PKC insert was transfected in parallel. The immunoreactivity of the transfected cells was examined by Western blotting. As illustrated in Fig. 1A, GFP and δ-PKC-GFP were identified by a monoclonal anti-GFP antibody as specific bands with the expected molecular sizes, 26 and 105 kDa,
Localization of Protein Kinase C δ

In order to explore the specificity of GFP—nuclear membrane translocation as a maximal ratio of plasma membrane and PMA (10 nM, 100 nM, 1 μM) of 12-deoxyphorbol 13-phenylacetate group confers anti-

respectively. A polyclonal anti-PKC δ antibody likewise recognized the δ-PKC-GFP as a 105-kDa protein but did not recognize the 26-kDa band corresponding to GFP. A low level of proteolysis was also evident as reflected in lower molecular weight bands in the δ-PKC-GFP expressing cells. To determine whether degradation of δ-PKC-GFP was induced over the time period of our incubations (0–40 min) in the presence of ligand, we compared by Western blotting total cell lysates of δ-PKC-GFP expressing cells incubated for 40 min in the presence or absence of 1 μM PMA. No increase in degradation was observed (Fig. 1A). Finally, in CHO cells expressing GFP alone, we saw no translocation in the presence of 1 μM PMA (Fig. 1B); all GFP remained in the cytoplasm and nucleus. The patterns of translocation that we describe below for the δ-PKC-GFP therefore appear to reflect the intact fusion protein.

The Effect of PKC Activators on the Translocation of δ-PKC-GFP—In order to explore the specificity of δ-PKC-GFP translocation in response to different ligands, we examined a series of compounds that had been shown previously to differ substantially in their biological activities. PMA is the archetypal phorbol ester tumor promoter, whereas bryostatin 1 is a partial protein kinase C antagonist. Among 12-deoxyphorbol 13-monoesters, we compared 12-deoxyphorbol 13-tetradecanoate with 12-deoxyphorbol 13-phenylacetate, since a long ester chain such as tetradecanoate confers promoting activity, whereas a more hydrophilic phenylacetate group confers anti-promoting activity. Finally, we examined one of the novel constrained DAG analogs which has been found to have high potency for PKC as well as a novel pattern of selectivity.²

In the absence of stimuli, approximately 88% of δ-PKC-GFP was present in the cytoplasm, and 15% in the nucleus. When a single dose of the complete tumor promoter PMA was added, δ-PKC-GFP slowly translocated to the plasma and nuclear membrane (open symbols for 1 μM PMA in Fig 2A). The extent and timing of the translocation depended on the concentration of PMA (Fig. 2, B and C). At 10 nM PMA little translocation was seen to either compartment. At 100 nM PMA, there was predominantly plasma membrane translocation, reaching a peak at 10 min, with a slower and more limited nuclear membrane localization at 20–30 min. At 1 μM PMA, the translocation to the plasma membrane was more rapid, peaking by 5 min, and was greater than that at 100 nM PMA (Fig. 2B). Translocation to the nuclear membrane was slower but more extensive. Between 10 and 15 min it exceeded the level of translocation to the plasma membrane and continued to rise, while the level of plasma membrane translocation decreased. Fig. 2C shows the maximal plasma and nuclear membrane translocation as a function of dose of PMA. Over the dose range examined (10 nM to 10 μM), the extent of maximal translocation to both compartments increased as a function of dose. At doses below 300 nM, the plasma membrane localization was predominant, whereas at higher doses the nuclear membrane localization was predominant.

Bryostatin 1 generated a distinct pattern of δ-PKC-GFP translocation under our conditions. Compared with PMA, bryostatin 1 induced prominent nuclear membrane translocation with only transient and minimal plasma membrane translocation (Fig. 3A). In addition, there seemed to be a more apparent perinuclear localization of δ-PKC-GFP in the bryostatin 1 treated cells compared with the PMA-treated cells. The lack of plasma membrane localization was independent of the dose of bryostatin 1 over the range examined (10 nM to 10 μM) (Fig. 3, B and C). The rates of translocation induced by bryostatin 1 and PMA cannot be directly compared because of the different patterns of localization. Thus, whereas 100 nM PMA initiated a considerably earlier response than did 100 nM bryostatin 1, the PKC translocated to the plasma membrane and the nuclear membrane, respectively. If comparison was limited to nuclear translocation, the rates of translocation in response to bryostatin 1 and PMA were more similar, and indeed 1 μM bryostatin 1 induced somewhat more rapid nuclear translocation than did 1 μM PMA, as reflected by the slopes of the two nuclear membrane translocation curves. At the onset of bryostatin 1 induced translocation (5–10 min after treatment of 1 μM bryostatin in Fig. 3A), some patchy cytoplasmic distribution of δ-PKC-GFP was also observed. This colocalized neither with endoplasmic reticulum (visualized with rhodamine 6G chloride) nor with mitochondria (visualized with MitoTracker Red CMXROS).

The pair of analogs 12-deoxyphorbol 13-phenylacetate and 12-deoxyphorbol 13-tetradecanoate differ substantially in the pattern of translocation of δ-PKC-GFP that they induce, consistent with their very different behavior as tumor promoters. Like PMA, the tumor-promoting analog 12-deoxyphorbol 13-tetradecanoate (1 μM) caused translocation first to the plasma
membrane and then to the nuclear membrane (Fig. 4A). In contrast, the inhibitor of tumor promotion 12-deoxyphorbol 13-phenylacetate (1 μM) predominantly induced nuclear membrane localization with little plasma membrane localization. In addition, there was a patchy pattern of fluorescence appearing throughout the cytoplasm after ligand addition (Fig. 4B). 12-Deoxyphorbol 13-phenylacetate and 12-deoxyphorbol 13-tetradecanoate were also examined at concentrations of 10 nM, 100 nM, 1 μM, and 3 μM. The difference in their patterns of translocation remained the same, although the rate and the extent of maximal membrane translocation increased as a function of dose (data not shown). In an effort to determine the basis for the punctate staining upon treatment with 12-deoxyphorbol 13-phenylacetate, we stained the cells with fluoroprobes for the Golgi network, endoplasmic reticulum, mitochondria, and lysosomes, and assessed colocalization with the δ-PKC-GFP; in no case did the markers colocalize.

B8-DL-B8 is a constrained DAG analog that was shown previously to be only 15-fold less potent than phorbol 12,13-dibutyrate in in vitro experiments (21). B8-DL-B8 at 1 μM induced a strong perinuclear localization with some nuclear membrane localization, but less plasma membrane localization (Fig. 5A). Relative to PMA, the constrained diacylglycerol induced slow translocation, achieving a stable pattern by 40 min of treatment.

Colocalization of δ-PKC-GFP to Golgi after the Treatment with DAG Analog—In order to identify the perinuclear compartment to which δ-PKC-GFP localized after the treatment with the DAG analog, cells were pretreated with a red fluorescent probe BODIPY TR ceramide to visualize the Golgi network. Fig. 5B, panel ii, illustrates a field of cells treated with 1 μM B8-DL-B8 and stained with the BODIPY TR ceramide, showing the red-stained Golgi network. In this field, two of the cells expressed the transfected δ-PKC-GFP, displaying perinuclear localization in the presence of B8-DL-B8 (Fig. 5B, panel i). Superposition of the images showed the marked colocalization of δ-PKC-GFP and the Golgi network in the perinuclear region (Fig. 5B, panel iii). As a control, we confirmed that BODIPY TR ceramide (2 μg/ml) by itself did not affect the distribution of δ-PKC-GFP (data not shown).

Elucidation of the multiple factors that account for the diversity of biological effects depending on PKC isofrom, on cell type, and on activating ligand forms a central focus in the PKC field. Differences in substrate preference of isoforms clearly contribute to this diversity (22). Thus, for example, the γ-chain of the PceRI receptor (23) and the elongation factor EEF-1α (24) have been identified as somewhat selective substrates for protein kinase C δ. The role of interacting proteins to drive localization to a distinctive microenvironment, enhancing phosphorylation of those substrates in that microenvironment, represents another mechanism. The presence of PKC-anchoring proteins, such as RICKs and RACKs, has been demonstrated in cardiac myocytes (11). Terrian and co-workers (25) have identified a region of PKC ε that interacts with the actin cytoskeleton and affects its biological activity. Jaken and collaborators (26, 27) have described a series of binding proteins for PKCs that are also PKC substrates.

Differential subcellular localization has been demonstrated using in vitro approaches such as light and electron microscopic immunocytchemistry in tissues and cultured cells (11, 28, 29). Moreover, using GFP-tagged PKCs, several studies have shown unique patterns of translocation of PKCs in living cells in response to different physiological activators. Oancea and Meyer (30) have described that the coordination between calcium spikes and diacylglycerol signals tightly controls the extent and timing of cPKC activation using GFP-tagged PKC γ. Distinctive patterns of translocation of GFP-tagged PKC δ have been reported in response to treatment with ATP, TPA, and H2O2, in CHO-K1 cells (14). Saito and collaborators (15) have also shown that fatty acids affect the translocation of PKC-γ and -ε differentially, and these two isoforms show different fatty acid specificity. In this study, we have explored the issue of how different ligands, which all interact with the C1 domain of PKC but which vary in structure or in pattern of induced biological response, affect the translocation of PKC δ.

GFP-PKC fusions, including those with PKC δ, show similar levels of activation and a similar extent of translocation to that of PKC alone (12, 14, 15). Consistent with these findings, under our assay conditions δ-PKC-GFP was expressed in transiently
Localization of Protein Kinase C δ

transfected CHO cells at a reasonable level and showed the expected molecular size. The fusion protein was adequately stable in the absence of ligand and represented the major population of fluorescent donor in the cell. Over the short time course examined, the construct was also stable in the presence of ligand. Contrasting with the homogenous distribution of green fluorescent protein alone, the δ-PKC-GFP showed differential distribution in the cytoplasm, Golgi, and nucleoplasm. In addition, δ-PKC-GFP was translocated to the cell membranes in response to various PKC activators whereas GFP alone did not respond.

The mechanism of bryostatin 1 is of great interest both because of its clinical relevance and because of the complexity of its interactions with PKC. The naturally occurring bryostatins are novel antineoplastic compounds that are currently under clinical trials for several malignancies (31). Like PMA, bryostatin 1 activates the classical and novel PKCs through high affinity binding to the C1 domain of PKC (32). Consistent with its action as an activator of PKC, bryostatin 1 induces many biological responses typical of the phorbol esters, such as induction of proliferation in Swiss 3T3 cells (33) and activation of neutrophils (34). On the other hand, in the majority of systems bryostatin 1 antagonizes phorbol ester responses, such as differentiation of the human promyelocytic leukemia cell line HL-60 (35) and proliferation of human T lymphocytes (36).

Several mechanisms may account for the differential behavior of bryostatin 1. An enhanced rate of PKC down-regulation has been demonstrated in several systems and for several isoforms. On the other hand, in both mouse keratinocytes and in NIH3T3 cells bryostatin 1 has been shown to display biphasic induction of biological responses, paralleling degradation of PKC δ at low doses and protection of PKC δ at high doses (37, 38). Since PKC δ has been shown to be antiproliferative in these systems, whereas some other PKCs such as PKC ε have been shown to stimulate proliferation, protection of PKC δ provides an attractive model for the antagonistic action of bryostatin 1 in some systems. It is clear, however, that it cannot explain the ability of bryostatin 1 to acutely inhibit phorbol ester action. For example, in C3H10T1/2 cells, 30-min treatment with bryostatin 1 was able to inhibit by 85% the release of arachidonic acid induced by phorbol ester (39). Differential localization of PKC could provide such a mechanism.

Here, using GFP-tagged PKC δ, we were able to show that bryostatin 1 and PMA induce distinct patterns of subcellular translocation. Bryostatin 1 caused a localization of δ-PKC-GFP predominantly to the nuclear membrane at all doses used, compared with the consecutive plasma membrane to nuclear membrane localization induced by PMA. Since high concentrations of PMA also caused substantial nuclear membrane localization at later times, we wanted to see if the difference in translocation was merely a reflection of noncomparable doses of the two compounds. Our results showed that the strong nuclear membrane localization induced by bryostatin 1 persisted at lower concentrations, although the magnitude was reduced. In contrast, PMA at lower concentrations induced a more prominent plasma membrane translocation with only minimal nuclear membrane translocation at a later stage (after 20 min of stimulation at 100 nM PMA).

This differential pattern of PKC δ localization dependent on the specific dose of PMA is an important basic finding. Examples of complicated dose response relations for PMA have been described (40, 41), including very steep dose-response relations for tumor promotion in mouse skin (42). In the absence of other possibilities, a plausible interpretation was an effect due to different isoforms with different affinities and functions. The present results argue that different efficacies of response, reflecting the differential localizations, also deserves consideration.

The comparison between the two 12-deoxyphorbol 13-monoesters is striking. These two derivatives have similar high affinity for PKC. The promoting derivative 12-deoxyphorbol 13-tetradecanoate closely resembles PMA, another effective tumor promoter, in its pattern of translocation. In contrast, 12-deoxyphorbol 13-phenylacetate, which is antihyperplastic and antipromoting, caused intracellular patches of δ-PKC-GFP as well as less defined but prominent nuclear membrane localization. Our co-localization studies fail to identify the 12-deoxyphorbol 13-phenylacetate-promoted patchy fluorescence with the intracellular organelles, such as mitochondria or lysosomes. It is possible that 12-deoxyphorbol 13-phenylacetate-activated δ-PKC-GFP is aggregated or is associated with a set of substrates or anchoring proteins that are in the cytoplasm.

The difference between these two derivatives emphasizes the
A crucial role played by the hydrophobic regions of the PKC ligands in determining their activities. These findings fit with our emerging understanding of the interaction of ligands with the C1 domain (21). The binding site represents a hydrophilic cleft in the otherwise hydrophobic face of the C1 domain (5). The phorbol ester fits into this cleft and, by rendering this surface more hydrophobic, causes it to function as a hydrophobic switch, stabilizing its interaction with the plasma membrane. Three factors contribute to the hydrophobic character of the ligand complex: 1) filling of the hydrophilic cleft itself by the phorbol ester, 2) hydrophobic interactions between the ligand and the hydrophobic residues lining the edges of the cleft, and 3) hydrophobic interactions between the side chains of the ligand and the lipid bilayer or proteins associated with it. The 12-deoxyphorbol derivatives aptly illustrate the important role played by these latter effects. In terms of drug design, understanding the specifics of how different hydrophobic domains can drive different localization patterns remains an important objective.

The constrained DAG analog is one of the novel ligands to emerge from an on-going extensive synthetic effort to clarify the structural basis for ligand-PKC interactions. Its $K_d$ for binding to PKC $\alpha$ in vitro is 2.9 nM, compared with a value for phorbol 12,13-dibutyrate of 0.2 nM. For inhibition of epidermal growth factor binding to NIH3T3 cells, its $ED_{50}$ is 170 nM, compared with 20 nM for phorbol 12,13-dibutyrate. Finally, it shows a 50% inhibitory concentration for growth of the K-562, COLO 205, and HS 578T tumor cell lines of $2 \times 10^{-8}$ M. Comparison of its relative affinities for PKC $\alpha$ and for $\beta_2$-chimerin reveals that the constrained DAG shows modest selectivity for $\beta_2$-chimerin (43). Here, we show for the first time that the constrained DAG analog translocated $\delta$-PKC-GFP to a perinuclear region colocalizing with the Golgi and to nuclear membrane. This pattern was thus distinct from that induced by the other ligands.

The different patterns of localization of PKC $\delta$ as revealed with the GFP fusion constructs indicate yet another degree of heterogeneity of PKC response to ligands. These results emphasize again the inadequacy of current in vitro assays for assessing the full depth of PKC structure function relationships. Pressing objectives are to clarify the mechanistic basis for the different patterns of localization as well as to determine the degree to which the differences in localization in fact drive different biology.

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