Selective Regulation of Protein Kinase C Isoenzymes by Oleic Acid in Human Platelets*

Wasiuddin A. Khan, Gerard Blobe, Andrew Halpern, Wendy Taylor, William C. Wetsel‡, David Burns§, Carson Loomis§, and Yusuf A. Hannun‡

From the Departments of Medicine and Cell Biology, Duke University Medical Center, Durham, North Carolina 27710, the Laboratorv of Molecular and Integrated Neurosciences, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709, and §Sphinx Pharmaceuticals, Durham, North Carolina 27717

Cis-unsaturated fatty acids activate soluble protein kinase C (PKC) in vitro and in intact platelets. The following studies were conducted to determine the effects of oleate on individual isoenzymes of PKC in human platelets. Human platelets were found to contain predominantly PKC α, β1, and δ with minor immunoreactivity for PKC ε, η, and γ. In intact platelets, sodium oleate caused a time-dependent redistribution of PKC α, β1, and δ from cytosol to membrane fractions with little effects on PKC β2. In addition, other studies demonstrated the ability of oleate to induce translocation of predominantly PKC δ in intact platelets, sodium oleate caused a time-dependent redistribution of PKC α, β1, and δ with an EC50 of 50 μM whereas it fully activated 100% of Vmax purified calcium-independent PKC predominantly δ with an EC50 of 5 μM. The selective effects of oleate on PKC isoenzymes were investigated in platelet cytosol which contains endogenous PKC and its physiologic substrates. Under these conditions, oleate potently activated calcium-independent PKC causing the phosphorylation of the 40-kDa substrate. Activation of calcium-dependent isoforms occurred only at higher concentrations of oleate. Thus, oleate activates multiple isoenzymes of PKC with predominant effects on calcium-independent PKC.

Protein kinase C (PKC),1 a phospholipid-dependent and diacylglycerol (DAG)-activated protein kinase, is a key element in signal transduction and cell regulation (1, 2). Molecular cloning studies have shown that PKC is a family of closely related isoenzymes. These isoenzymes have been grouped into two classes depending on their requirements for calcium: PKC α, β1, and γ are calcium-dependent while PKC δ, ε, η, and θ are calcium-independent (2).

In vitro, PKC is also activated by cis-unsaturated fatty acids (3–8) in a mechanism distinct from that of DAG (9). In addition, other studies demonstrated the ability of cis-unsaturated fatty acids to activate PKC in intact platelets (10, 11).

Since agonist stimulation of intact platelets results in elevations in DAG and arachidonic acid to levels sufficient to activate PKC, arachidonic acid may function as a second messenger in platelet regulation through the activation of PKC. More recently, free fatty acids were found to activate preferentially soluble PKC and were unable to interact with membrane-bound enzyme in vitro or in platelet membranes. The above studies led us to postulate a two-compartment model for PKC activation whereby membrane-associated PKC is activated by DAGs and soluble PKC is activated by free fatty acids (12).

A major question arising from those studies relates to whether fatty acids and DAG interact with different isoforms of PKC. This was particularly important to establish with respect to the calcium-independent isoenzymes of PKC since little is known about their regulation by fatty acids. In this study, we evaluated the interaction of fatty acids with PKC isoenzymes by examining the ability of oleate to induce translocation of PKC isoenzymes in intact human platelets, and the ability of oleate to activate purified PKC isoenzymes from human platelets. We find that platelets contain multiple isoenzymes of PKC (α, β1, and δ). Sodium oleate causes translocation of predominantly PKC α, β1, and δ, although to a lower extent than PMA or thrombin. Oleate appears to potently activate protein kinase C δ in cytosol resulting in calcium-independent phosphorylation of a 40-kDa substrate. The implications of these results on the regulation of PKC isoenzymes by fatty acids are discussed.

EXPERIMENTAL PROCEDURES

Materials

[γ-32P]ATP and [32P]orthophosphate were from Du Pont-New England Nuclear. Histone type III-S and oleic acid were from Sigma. 1,2-Dioleoyl-sn-glycerol-3-phosphoserine was from Avanti Polar Lipids, Inc. sn-1,2-Dioleoylphosphatidylcholine as described elsewhere (13).

Methods

Purification of Protein Kinase C—Protein kinase C was purified from platelet cytosol to homogeneity, as described previously (14), to a specific activity of 2 × 106 nmol/min/mg.

Separation of Protein Kinase C Isoenzymes—PKC purified through phenyl-Sepharose was separated into two peaks upon chromatography on hydroxylapatite (15). The two peaks eluted at positions corresponding to rat brain types II (β) and III (α); this was also verified by Western blotting. In other studies, for the purification of calcium-independent PKC, the enzyme was purified by sequential chromatography on DEAE-Sepharose, Mono-Q, and threonine-Sepharose columns. The purified PKC was subjected to hydroxylapatite chromatography which resulted in three peaks of activity (see "Results").

Assay for Protein Kinase C—PKC activity was assayed using Triton
X-100/PS/DAG mixed micelles as described elsewhere (16, 17). The concentration of PS and DAG under standard mixed micellar conditions were 0.43 mM and 86 μM, respectively. No free fatty acids were detected by TLC in these lipid preparations (at least 1:1000). When sodium oleate was used as an activator, detergent and phospholipids were omitted. All reactions contained 200 μg/ml histone, 10 μM ATP, and 20 mM Tris-HCl, pH 7.5. In all assays, oleic acid was first neutralized in ethanol with sodium hydroxide and further diluted to appropriate concentrations in aqueous solutions.

Western Blotting—Western blotting of PKC isoenzymes using isoenzyme-specific antibodies was performed as previously described (18).

Preparation of δ-Specific Antipeptide Antibodies—For PKC δ, additional antisera were prepared as follows. A peptide corresponding to the carboxyl-terminal region of the published rat brain PKC δ sequence (19) CAPKGF$^{SFVNPK}$EQPLE was coupled to Limulus polyphemus hemocyanin with maleimidobenzoyl-N-hydroxysuccinimide ester by the method of Doolittle (20). Peptide-hemocyanin conjugates were purified by gel filtration (Sephadex G-25) and injected subcutaneously into the backs of New Zealand White rabbits. The first injection utilized complete Freund’s adjuvant, and three biweekly booster injections were in incomplete Freund’s adjuvant. The above peptides were coupled using sulfo-maleimidobenzoyl-N-hydroxysuccinimide ester to amino-agarose (15 mg peptide/ml gel). Residual reactive groups were blocked with mercaptoethanol. Crude antisera were applied to 1-ml peptide columns, which were washed with Tris base, and stored at -20 °C with azide. Homogenates from insect cells infected with recombinant human PKC and did not cross-react with any of the other PKC species (data not shown).

Preparation of Platelet Homogenate, Cytosol, and Membranes—Washed human platelets were prepared as described elsewhere (12). Intact platelets were treated with vehicle, thrombin, phorbol ester, or oleate, and then platelet membranes and cytosol were fractionated as previously described (12).

40-kDa Substrate Phosphorylation—Phosphorylation of 40-kDa substrate in platelet homogenates and cytosol was performed as described elsewhere (10).

All experiments were performed at least twice with similar results.

RESULTS AND DISCUSSION

Identification of PKC Isoenzymes in Platelets—Previous studies have indicated the presence of PKC α in platelets using Western blot analysis (21-24). PKC β has also been resolved by hydroxylapatite chromatography (23), and an immunoreactive species has been detected with PKC β antisera (21, 23); however, in one study it was suggested that a related species was present rather than β because of the associated calcium-independent activity (23). Little is known regarding the expression of calcium-independent isoenzymes in human platelets. Very recently the existence of a PKC δ migrating abnormally at 80 kDa has been reported (24), and Ware and co-workers demonstrated the existence of PKC δ using PCR amplification of message (25). Therefore, it became important to determine the full compliment of PKC isoenzymes present in platelets. For these studies, we utilized highly specific polyclonal antisera developed for each isoenzyme (18). Fig. 1 shows the immunoblot analysis of PKC isoenzymes in fresh platelet homogenate. The major calcium-dependent isoenzymes expressed were α, β, and γ. No immunoreactivity was detected for δ. The expression of α and β species is consistent with previous studies although these earlier studies did not distinguish between β1 and β2, which are alternatively spliced variants of the same gene (2). The absence of PKC γ is consistent with its exclusive localization to neural tissues.

Among the calcium-independent isoenzymes, PKC δ was the predominant species with very faint staining for PKC ε and PKC ζ (Fig. 1). Modest staining was observed with antisera to human PKC δ2. Thus, human platelets appear to contain at least three calcium-dependent isoenzymes and at least one calcium-independent isoenzyme, underscoring the complexity of signal transduction pathways in platelets involving PKC.

Differential Translocation of PKC Isoenzymes in Response to Thrombin, Phorbol Ester, and Oleate—Activation of cellular PKC has been associated with translocation of enzyme from cytosol to membrane fractions in various cell systems (2, 26). Translocation may involve an increase in affinity of PKC to membrane such that it becomes resistant to extraction in calcium chelator-containing buffers. Additionally, membrane proteins that bind activated PKC in the presence of phospholipids have been postulated to play a role in translocation and activation of PKC (27, 28). In human platelets, PKC was found to translocate in response to activation by phorbol ester with results showing that PMA causes preferential translocation of PKC δ but not α (21, 24) although another study found that PMA causes selective translocation of PKC α (22).

In order to investigate the activation of individual PKC isoenzymes by oleate in intact platelets, the effects of oleate on translocation of calcium-dependent and independent PKC activity were investigated and compared to the effects of PMA. Treatment of platelets with 100 nM PMA for 2 min resulted in substantial loss (74%) of calcium-dependent PKC from cytosol and a marked increase (293%) in the membrane-bound form. On the other hand, oleate caused a 34% decrease in cytosolic calcium-dependent PKC activity and a modest (35%) increase in the membrane-associated form.

Calcium-dependent PKC activity also demonstrated translocation in response to PMA and oleate. PMA caused a substantial (65%) decrease in cytosolic calcium-dependent activity and a small increase (35%) in membrane-associated activity. The relatively small effect on membrane association of calcium-independent activity may be related to rapid pro-

\[ \text{D. Burns, unpublished observations.} \]
teolysis and down regulation of this activity in response to phorbol esters. Sodium oleate caused a 16% decrease in cytosolic calcium-independent activity and a 15% increase in membrane-associated activity. These studies demonstrate that phorbol esters cause translocation of calcium-dependent as well as calcium-independent forms of PKC in human platelets. Similarly, oleate causes modest translocation of calcium-dependent and calcium-independent activity.

To determine if PMA and oleate cause differential isoenzyme translocation in platelets, immunoblot analysis of cytosolic and membrane isoenzymes of PKC was carried out in platelets treated with PMA or oleate, and the results were compared with thrombin treated platelets. For these studies, platelets were treated with 100 nM PMA, 10 nM γ-thrombin, or 50 μM oleate. Platelets were then fractionated into cytosolic and membrane compartments at 0.5, 3, and 10 min. PKC α decreased in cytosol in response to platelet activation by PMA, γ-thrombin, or oleate (Fig. 2A). This was accompanied by increases in membrane-associated isoenzyme. PKC βI also showed a dramatic decrease in cytosol in response to PMA and γ-thrombin with corresponding increases in the membrane form (Fig. 2B). However, with oleate, there was less of a decrease in the cytosol and only modest changes in the membrane-associated form (Fig. 2B). On the other hand, PKC βII showed similar changes with PMA, γ-thrombin, and oleate with significant decreases in the cytosolic form at 3 and 10 min after treatment with these agents and a significant increase in membrane form especially at 3 min (Fig. 2C). PKC δ showed dramatic decreases in cytosol following treatment with PMA, γ-thrombin, or oleate (Fig. 2D) with modest increases in membrane-associated form detected primarily at 3 min after treatment. For PKC α and δ, the decrease in cytosolic immunostaining was more dramatic than the increase in membrane immunostaining while for PKC βI and PKC βII the decrease in cytosolic immunoreactivity was accompanied by reciprocal changes in membrane reactivity (Fig. 2). These results suggest that PKC α and PKC δ may undergo prompt proteolytic degradation (down-regulation) either in the cytosolic compartment or once they have translocated.

Significantly, it appears that oleate causes more pronounced translocation of PKC α, βII, and δ with very little effect on PKC βI. These results suggest that PKC βI may be a poor target for activation by fatty acids.

**Purification and Characterization of Calcium-dependent Isoforms of PKC; Activation by Oleate**—To examine the regulation of PKC isoenzymes from platelets and their sensitivity to oleic acid, it became important to resolve PKC isoenzymes from human platelets. Initially, the calcium-dependent isoforms were resolved and characterized. Protein kinase C isoenzymes α, β (βI, βII), and γ can be separated on hydroxylapatite chromatography based on their selective elution at different salt concentrations (15). Therefore, PKC purified from fresh platelets was subjected to high resolution hydroxylapatite chromatography. Two peaks of calcium/PS/DiC16:1-dependent activity were separated (data not shown). These peaks correspond to the elution profile of rat brain PKC α and PKC β. To further characterize these peaks, Western blot analysis was carried out using various antibodies. Peak I reacted with βI and βII but not with α. Peak II reacted with α antisera and very faintly with βI (data not shown).

The cofactor requirements of these isoenzymes were studied next. Because of our current inability to resolve PKC βI from PKC βII, these two isoforms were studied combined. Both PKC α and PKC β showed total dependence on phospholipid and calcium for optimal activity (Fig. 3, A and B). In contrast, Tsukuda et al. (23) found that the first peak of platelet PKC on hydroxylapatite was predominantly calcium-independent. This may be due to inclusion of additional isoforms of PKC (see below).

**FIG. 2. Translocation of PKC isoenzymes.** Platelets were treated with either 100 nM PMA (PMA), 10 nM γ-thrombin (γ-Thr) or 50 μM sodium oleate (OA) for 0, 0.5, 3, or 10 min. Platelet cytosol and membrane fractions were then obtained and probed for individual PKC isoenzymes by Western blotting using isoenzyme-specific antibodies; A, PKC α; B, PKC βI; C, PKC βII; and D, PKC δ. The immunostained band shown here is the 80-kDa band.
Selective Regulation of Protein Kinase C

Fig. 3. Cofactor requirement of PKC α and β. Hydroxylapatite-purified PKC α (A) and PKC β (B) were assayed in the presence or absence of calcium (Ca), phosphatidylserine (PS), and/or dioleoylglycerol (DON) as indicated. C, dependence of PKC α and β on dioleoylglycerol. For these studies, PKC was assayed using mixed micelles of Triton X-100/phosphatidylserine in the presence of the indicated concentrations of dioleoylglycerol and in the presence of 100 μM free calcium as described under "Methods."

To investigate the diacylglycerol dependence of PKC α and PKC β, a mixed micellar assay was employed since in this assay PKC shows a strict requirement for DAG (16). Under those conditions, both isoforms of PKC demonstrated nearly identical dependence on dioleoylglycerol with peak activity reached at 0.8 mol% DiC18:1 (Fig. 3C).

The ability of oleate to activate the calcium-dependent isoenzymes of platelet PKC was investigated next. Sodium oleate activated PKC α and PKC β in a calcium-dependent manner (Fig. 4) with 50% activation obtained at a concentration of 50 μM as determined by double-reciprocal plots (data not shown). The level of stimulation by calcium/oleate was approximately 50% of that achieved by calcium/PS/DiC18:1.

Therefore, oleate was capable of equally activating the different calcium-dependent isoforms of PKC in human platelets.

Purification and Characterization of Calcium-independent Isoforms of PKC; Activation by Oleate—In multiple studies, we have detected calcium-independent activity of PKC in platelet cytosol and partially-purified preparations; however, with further purification, only calcium-dependent isoforms were isolated and resolved (as above). To target the calcium-independent activity, a Mono-Q chromatography step was introduced before the threonine-Sepharose column, and purified PKC was fractionated on hydroxylapatite chromatography. Under these conditions, calcium-independent activity was clearly retained and, in fact, was the predominant peak of PKC activity (Fig. 5). Calcium-dependent activity eluted at slightly higher salt concentrations and was resolved as two peaks corresponding to PKC β1 plus β2 and PKC α, respectively (Fig. 5). Western blotting data showed that the first peak reacted with antisera to PKC α, peak II reacted with antisera to PKC β1 and β2, and the third peak reacted with antisera to PKC α (data not shown).

Since hydroxylapatite chromatography allowed the separation of calcium-independent activity from other calcium-dependent isoenzymes, these fractions were further characterized for their cofactor dependence and for activation by fatty acids. PKC δ demonstrated phospholipid and DAG-dependent activity in a vesicle assay using histone as a substrate; and activity was independent of calcium (Fig. 6A). Under identical conditions, the enzyme was fully activated by oleate which was also independent of calcium (Fig. 6A).

The extent of activation by oleate was consistently 90–110% of activation achieved with PS/DiC18:1. Moreover, oleate was

Fig. 4. Activation of calcium-dependent isoenzymes of PKC by sodium oleate, PKC α (A) and PKC β (B) were assayed as described under "Methods" with the indicated concentrations of oleate in the presence of either 100 μM free calcium or 10 mM EGTA.
Selective Regulation of Protein Kinase C

This study documents the existence of multiple isoforms of protein kinase C in human platelets with at least three distinct calcium-dependent isoforms (PKC α, β1, and γ) and at least one major calcium-independent isoenzyme (PKC δ). Additional faint immunostaining of PKC ε, z, and η was also detected by Western blot analysis.

Little is known concerning the translocation of PKC isoforms in response to physiologic activators of platelets. This study demonstrates the ability of thrombin to induce translocation of the four major isoforms of PKC detected in human platelets. This translocation is qualitatively similar to that induced by PMA thus suggesting that thrombin is able to activate all isoforms of PKC in intact human platelets. The translocation of calcium-dependent isoforms has not been studied in platelets with the exception of one study demonstrating the translocation of PKC γ in response to PMA (24). In our studies we only detected very faint immunostaining for PKC ε and translocation could not be evaluated. However, PKC δ was prominently decreased in cytosol with a corresponding increase in the membrane in response to PMA, thrombin, or oleate. This was further corroborated by the demonstration of translocation of calcium-independent activity of PKC in platelets.

Although fatty acids have been shown to activate PKC in multiple studies in vitro, little information is available on the ability of fatty acids to activate and translocate PKC in intact cell systems. In hepatocytes, it was shown that fatty acids are able to induce translocation of PKC β and α from cytosol to membrane with much more predominant translocation of β when compared to α as determined by hydroxylapatite fractionation but not by Western blotting (29). In human platelets, we find that oleate induces significant translocation of PKC α, β1, and δ, but not of β2. Therefore, oleate may activate and translocate calcium-dependent as well as calcium-independent isoforms of PKC. PKC δ appears to be the least responsive isoenzyme to oleate.

A major finding of this study is the selective activation of calcium-independent PKC by fatty acids with an EC50 of 5 μM as compared to an EC50 of 50 μM for activation of calcium-dependent isoforms. This selective activation was verified using purified isoforms as well as in a reconstituted cytosolic in vitro model using endogenous PKC and substrates. Moreover, the extent of activation of calcium-independent PKC by oleate (~100% of Vmax with PS/DiC18:1) was significantly greater than the extent of activation of calcium-de-
tion is proposed whereby fatty acids (especially arachidonate) have been shown to inhibit platelet responses to agonist stimulation. Enzymes of PKC and it may play an important role in platelet potency for oleate in activating calcium-dependent PKC is a result of the indicated concentrations of sodium oleate in the presence of 10 mM EGTA (calcium-independent PKC) or 100 µM free calcium (total PKC activity). Calcium-dependent PKC activity was calculated as the difference between total PKC activity and calcium-independent activity.

These results have two major implications. First, the biological consequences of PKC activation by fatty acids are not firmly established (primarily due to the lack of selective inhibitors of fatty acid activation of PKC). The biological consequences of PKC activation by fatty acids are not firmly established (primarily due to the lack of selective inhibitors of fatty acid activation of PKC). However, two lines of evidence support a potential role for fatty acids in inhibiting platelet responses. 1) PKC activation has been shown to desensitize platelets and inhibit further platelet activation by thrombin and other agonists (30) and 2) low concentrations of fatty acids have been shown to inhibit platelet responses to agonist stimulation (31, 32). Thus, endogenously generated free fatty acids and calcium-independent PKC may selectively participate in negative feedback during platelet activation.

Acknowledgments—We thank Marsha Haigood for expert secretarial assistance and Cindy Mooman for technical assistance.

REFERENCES


1 Additional studies show that it is unlikely that the decreased potency for oleate in activating calcium-dependent PKC is a result of formation of complexes between calcium and oleate. In particular, increasing concentrations of calcium did not inhibit activation of calcium-dependent PKC by oleate (W. Khan and Y. Hannun, unpublished observations).