Identification, Partial Purification, and Characterization of a Novel Phospholipid-dependent and Fatty Acid-activated Protein Kinase From Human Platelets*

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A novel lipid-dependent protein kinase in human platelets was partially purified and characterized. This enzyme was calcium-independent and was selective for phosphatidic acid as a cofactor/activator with initial activation observed at approximately 2 mol % and peak activity achieved at 4 mol % phosphatidic acid. In the presence of phosphatidylserine, enzyme activation was observed with concentration of phosphatidic acid as low as 0.5 mol % with peak activity at 2 mol %. Other anionic phospholipids also activated the enzyme but to a lesser extent and with less potency. Enzyme activity was independent of diacylglycerol or phorbol esters and the enzyme did not bind 1H]phorbol dibutyrate. In a soluble protein kinase assay, the enzyme was activated by cis-unsaturated fatty acids with maximum activation occurring at 5-10 μM sodium olate. Western blot analysis showed that this enzyme did not cross-react immunologically with antibodies raised against the currently identified isoforms of protein kinase C. A number of additional biochemical criteria distinguished this enzyme from known isoforms of protein kinase C. These biochemical and immunologic data define a novel lipid-dependent protein kinase in human platelets. The role of this enzyme in signal transduction as a phosphatidic acid-activated enzyme and as a possible target for cis-unsaturated fatty acids is discussed.

Protein kinase C (PKC), is a family of phospholipid-dependent and diacylglycerol (DAG)-activated protein kinases that play key roles in signal transduction and cell regulation (1). PKC isoforms show distinct tissue distribution with most tissues containing one or more isoforms (1, 2). These isoforms have been grouped into three functional classes depending on their requirements for calcium and/or diacylglycerol. cPKC is composed of the calcium- and diacylglycerol-dependent isoforms PKC α, βI, and γ; nPKC consists of the diacylglycerol-dependent and calcium-independent isoforms PKC δ, ε, θ, and γ; and aPKC consists of the calcium- and diacylglycerol-independent isoforms PKC ζ and λ (1).

In human platelets, PKC has been shown to play a critical role in mediating secretion of platelet granules and induction of irreversible aggregation (3-7). Human platelets were shown to contain PKC α, βI, βII, δ, and γ and PKC ζ (8-11). The function of these individual isoforms in platelets and in other tissues remains poorly defined.

During the course of studying the regulation of platelet PKC isoforms by fatty acid, we identified a major calcium-independent protein kinase activity that was distinguished from other known calcium-independent isoforms of PKC. In this study, this calcium-independent fatty acid-activated enzyme was partially purified and characterized biochemically. This novel enzyme was found to be selectively dependent on phosphatidic acid for activation and potently activated by cis-unsaturated fatty acids with an EC₅₀ of approximately 5 μM. Immunologic and biochemical parameters distinguish this novel enzyme from known isoforms of PKC.

EXPERIMENTAL PROCEDURES

Materials

Histone III-S, myelin basic protein, lysophosphatidic acid, phorbol dibutyrate, oleic acid, and other fatty acids were obtained from Sigma. Phosphatidylserine, phosphatidylglycerol, phosphatidylethanolamine, and phosphatidic acid were obtained from Avanti Polar Lipids. [γ-³²P]ATP (specific activity 3000 Ci/mmol) and [³H]PDBu (specific activity 19.1 Ci/mmol) were from DuPont NEN. DEAE-Sephacel, AH-Sepharose, and phenyl-Sepharose were obtained from Pharmacia LKB Biotechnology Inc. Hydroxypatite was from Peptide International (Louisville, Kentucky). Antibodies to PKC isoforms have been described (2). Additional antibodies to PKC isoforms, δ, ε, θ, and γ were a generous gift from Dr. David Burns, Sphin Bioscience Inc. Antibodies 1 and 2, corresponding to amino acid residues 112-124 and 8-24 of PKC ε, and peptide ε, corresponding to residues 143-164 of PKC ε, were obtained from peptide synthesis facility of the Howard Hughes Medical Institute at Duke University.

Methods

Preparation of Fresh Human Platelet Extract

Blood from healthy volunteers was suspended in acid citrate dextrose as anticoagulant, and platelets were separated as described (7). The final platelet pellet was suspended in homogenization buffer containing 20 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 2 mM EDTA, 10 mM EGTA, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, and 0.02% leupeptin, and then homogenized by sonication on ice. Triton X-100 was added to a final concentration of 0.2% (v/v), and the preparation was incubated on ice for 20 min with constant shaking. The extract was centrifuged at 160,000 × g for 1 h at 4°C. The clarified supernatant was used as the enzyme source in subsequent purification steps.

Purification of the Novel Ca²⁺-independent Kinase

DEAE-Sephacel Column Chromatography—The clarified supernatant was loaded on to a DEAE-Sephacel column equilibrated with buffer A (20 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, pH 7.4, 10 mM β-mercaptoethanol). The column was washed with three column volumes of buffer A containing 30 mM KCl. The enzyme was eluted on a linear gradient of KCl from 30 to 350 mM in 100 ml on a Pharmacia FPLC system. Fractons of 2 ml were collected and assayed for enzyme activity. The calcium-independent lipid-dependent activity eluted between 70 and 180 mM KCl.
Threonine-Sepharose Column Chromatography—Peak fractions from a DEAE-Sepharose column were pooled and diluted to 50 ml with buffer A and loaded onto a threonine-Sepharose column equilibrated with buffer A. The column was developed with a linear gradient of KCl from 0 to 1 M in 70 ml of buffer A, and enzyme activity eluted between 550 and 900 mM KCl.

Hydroxyapatite Column Chromatography—Active fractions from the threonine-Sepharose column were pooled, and the salt concentration was decreased by diluting the enzyme with 20 mM phosphate buffer, pH 7.4. The diluted enzyme was loaded onto a high resolution hydroxyapatite column equilibrated with 10 mM phosphate buffer, pH 7.4 containing 0.5 mM EDTA, 0.5 mM EGTA, 10% (v/v) glycerol and 10 mM β-mercaptoethanol. The enzyme was eluted with a linear gradient of potassium phosphate from 10–250 mM in 90 ml, and 1 ml fractions were collected.

Phenyl-Sepharose Column Chromatography—The gradient described for hydroxyapatite column separated the Ca²⁺-independent enzyme from Ca²⁺-dependent PKC isoenzymes. The active fractions containing peak activity of the Ca²⁺-independent protein kinase were pooled and diluted with buffer A. Solid KCl was added to achieve a final concentration of 1 M and loaded onto a 0.5 ml phenyl-Sepharose column equilibrated with buffer A containing 1 mM KCl. The enzyme was eluted by decreasing the salt gradient from 1 to 0 mM KCl in 15 ml. Fractions of 0.5 ml were collected and assayed for activity.

**Enzyme Assay**

Protein kinase C and the novel fatty acid-dependent kinase activities were assayed using mixed micellar and vesicle assays as described (12, 13). Other lipids were used at the indicated concentrations. EGTA (10 mM) was used when assaying the novel Ca²⁺-independent kinase. Fatty acid-dependent activities were measured using the vesicle assay. Fatty acids were delivered as sodium salts as described (14). Ca²⁺-independent activity represents the activity in presence of EGTA, whereas calcium-dependent activity represents the activity measured in presence of 400 μM CaCl₂ minus the activity measured in presence of EGTA.

[^H]PDBu Binding

[^H]PDBu binding was carried as described (15).

Western Blot Analysis

Western blotting with PKC isoenzyme-specific antibodies was carried out as described (2).

**RESULTS**

Purification of a Novel Ca²⁺-independent and Lipid-dependent Protein Kinase—During previous studies on platelet protein kinase C, we detected a major calcium-dependent protein kinase. This Ca²⁺-independent enzyme was partially purified and separated from PKC isoenzymes by sequential column chromatography on DEAE-Sepharose, threonine-Sepharose, hydroxyapatite, and phenyl-Sepharose. Both Ca²⁺-dependent and Ca²⁺-independent phospholipid-dependent protein kinases co-chromatographed on DEAE-Sepharose and threonine-Sepharose (data not shown). Active fractions from threonine-Sepharose were loaded onto a high resolution hydroxyapatite column. Under the elution conditions, the Ca²⁺-independent activity eluted between 50 and 80 mM potassium phosphate, whereas Ca²⁺-dependent PKC activity eluted between 105–120 mM KPO₄ (Fig. 1A). The elution profile of Ca²⁺-dependent PKC isoenzymes in platelets is in agreement with previously reported studies (11). After resolving from Ca²⁺-dependent PKC, the Ca²⁺-independent kinase activity was further purified on a phenyl-Sepharose column. Fig. 1B shows the elution profile of Ca²⁺-independent kinase, which shows no detectable calcium-dependent activity. This procedure resulted in approximately 600-fold purification of this enzyme (Table I). In order to estimate the size of this novel Ca²⁺-independent kinase, the phenyl-purified enzyme was subjected to molecular sizing column chromatograph (Superose 12). The enzyme eluted between 70 and 90 kDa (data not shown).

In order to determine the relationship of this kinase to other PKC isoenzymes, immunoblotting of purified enzyme with PKC isoenzyme-specific antibodies was carried out. This kinase did not show any immunoreactivity with antibodies to PKC α, βI, βII, γ, δ, ε, ζ, η, θ (data not shown). Therefore, our data suggest that human platelets express a novel lipid-dependent Ca²⁺-independent kinase.

Initially, we compared the phospholipid requirement of the Ca²⁺-independent kinase with that of purified PKC α under identical assay conditions (Fig. 2). Under optimal assay conditions, activation of the novel kinase by phosphatidic acid was.

**FIG. 1.** Chromatographic separation of the novel Ca²⁺-independent kinase. Peak Ca²⁺-independent protein kinase activity after sequential purification on DEAE-Sepharose and threonine-Sepharose was subjected to further purification as described under "Experimental Procedures." A, calcium-independent protein kinase; B, calcium-dependent protein kinase. A, separation of Ca²⁺-independent protein kinase from Ca²⁺-dependent protein kinase on hydroxyapatite column. B, elution profile of Ca²⁺-independent enzyme activity on phenyl-Sepharose column.

**TABLE I**

Purification of the novel Ca²⁺-independent protein kinase from human platelets

<table>
<thead>
<tr>
<th>Total Protein (mg)</th>
<th>Specific Activity (pmol/min/mg)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell extract</td>
<td>96</td>
<td>16.6</td>
</tr>
<tr>
<td>DEAE-Sepharose</td>
<td>10.6</td>
<td>645</td>
</tr>
<tr>
<td>Threonine-Sepharose</td>
<td>1.33</td>
<td>2771</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>0.12</td>
<td>10,002</td>
</tr>
</tbody>
</table>

The enzyme activity of the first three steps was determined in the presence of 10 mM EGTA/PS/DiCI₂ only, whereas after hydroxyapatite the activity was determined in the presence of 10 mM EGTA/PS/DiCI₂ and 10 mM EDTA/PA.
requirement for PA for full activation of the enzyme raised the of this novel kinase by DAG or phorbol esters and the sufficient These results demonstrate that this novel kinase does not de-

insight into this question, we evaluated the possibility that PA (as cofactor for an unknown activator. In order to provide some insight into this question, we evaluated the possibility that PA may function as an activator in the presence of PS as a cofactor. Therefore, the dependence of the enzyme on PA was examined in the absence or presence of different concentrations of PS (Fig. 5). In the absence of PS, PA was sufficient to activate the enzyme although the dependence on PA demonstrated sigmoidal kinetics, suggesting the requirement for more than one molecule of PA. Since initial activation required at least 2 mol % PA (Fig. 5), this translates into the requirement for micelles containing at least 3 molecules of PA on average. With increasing concentrations of PS, there was a significant shift of the dependence on PA to the left such that at 10 mol % PS, the kinetics of PA activation were more hyperbolic with a Hill number of approximately 1, suggesting the requirement for 1 molecule of PA/micelle to achieve full activation of the enzyme. Therefore, this kinetic analysis raises the possibility that PA may function as an activator of the enzyme in the presence of PS.

**Regulation by cis-Unsaturated Fatty Acids**—Sodium oleate activated this novel Ca\(^{2+}\)-independent protein kinase in a dose-dependent manner, with maximum activation occurring at 5–10 \(\mu M\) (Fig. 6A). The concentration of sodium oleate needed for maximal activation is approximately 5–10-fold lower than that required by Ca\(^{2+}\)-dependent PKC isoenzymes.

Since the novel Ca\(^{2+}\)-independent kinase appears to be activated potently by free fatty acids, other fatty acids were studied for their ability to activate this kinase. All the fatty acids were used at 25 \(\mu M\). As shown in Fig. 6B, the cis-unsaturated fatty acids were more effective activators of this enzyme, whereas saturated fatty acids and fatty acid methyl esters were less effective.
Fatty Acid-activated Protein Kinase

Fig. 4. Lack of regulation by DAG or phorbol esters. A, cofactor requirement of novel Ca\(^{2+}\)-independent protein kinase (□) and PKC α (●). Enzyme activities were determined in a mixed micellar assay containing 5 mol% PA or 10 mol% PS with or without 2.5 mol% Dic1&... B, [\(^{3}H\)]PDBu binding of the Ca\(^{2+}\)-independent protein kinase (○) and PKC α (●).

Fig. 5. Kinetic analysis of the interaction of PA and PS. Enzyme activity was determined in the presence of the indicated concentrations of PA in the presence of 0 (○), 5 (●), or 10 (▲) mol% PS.

Lack of Synergy of Sodium Oleate with DAG to Activate the Novel Kinase—PKC α, β, and γ have been shown to be synergistically activated by cis-ununsaturated fatty acids and DAG (15–17). The synergistic activation of the novel kinase by sodium oleate and DAG was studied, and these responses were compared with PKC α (Fig. 7A). While PKC α showed a dose-dependent synergistic activation by DAG and sodium oleate (data not shown), the Ca\(^{2+}\)-independent kinase did not show any such synergy between fatty acid and DAG (Fig. 7A). These results support the contention that this kinase is a novel kinase sensitive to fatty acid and insensitive to DAG.

Effect of PA on Sodium Oleate-induced Activity—Because this novel kinase exhibited strong sensitivity to sodium oleate and PA, it was of interest to determine whether PA or fatty acids interact at an identical site and whether they show any synergistic response. Fig. 7B shows activation by fatty acid in the presence of several concentrations of PA. PA progressively inhibited the fatty acid-induced activation of the enzyme. These data suggest that PA either competes for oleate binding at an identical site or, if interacting at a different site, it causes steric hindrance, and, as a consequence, oleate is unable to activate the enzyme.

Inhibition by Sphingosine and H\(_2\)S—Sphingosine at low concentrations inhibits PKC isoenzymes by interacting at the regulatory sites (18), whereas H\(_2\)S inhibits kinase activity by interacting at the catalytic site (19). The inhibition of Ca\(^{2+}\)-independent kinase by sphingosine and H\(_2\)S was studied and compared with PKC α (Fig. 8). Both compounds inhibited the activities of PKC α and the novel kinase in a similar fashion.
FIG. 7. Interaction of oleate with DAG and PA. A, synergistic activation of the novel Ca²⁺-independent protein kinase (○) and PKC α (□) by sodium oleate and DiC₁₈:₁. Enzyme activity was measured at 3 μM DiC₁₈:₁ in the presence of 10 μM sodium oleate. B, effect of PA on sodium oleate-induced activation of the novel Ca²⁺-independent protein kinase. Enzyme activity was determined in the presence of the indicated concentrations of PA and varying concentrations of sodium oleate. ○, 0 μg/ml PA; ●, 1 μg/ml PA; Δ, 10 μg/ml PA; △, 40 μg/ml PA.

with H₇ showing moderate selectivity for the calcium-independent enzyme.

**Substrate Dependence**—PKC isoenzymes have been shown previously to exhibit different substrate specificities. Therefore, the substrate specificity of this novel kinase was examined. PA induced histone III-S phosphorylation by the novel kinase, which was 2–3-fold higher than that achieved by PKC (Fig. 9A). The K₅₀ for histone III-S was 32 μg/ml for PA and 12 μg/ml for PS-induced activity.

A peptide derived from the pseudosubstrate region of PKC ε has been shown to be an efficient substrate for Ca²⁺-dependent and Ca²⁺-independent PKC isoenzymes. Therefore, we used peptide ε along with other pseudosubstrate peptides to assay the activity of the novel kinase. As shown in Fig. 9B, the pseudosubstrate peptides derived either from PKC ε or PKC ζ were phosphorylated by the novel kinase. Moreover, myelin basic protein and histone I were efficiently phosphorylated by the novel kinase.

**Mg²⁺ and ATP Dependence Values**—The Mg²⁺ and ATP-dependent activation of Ca²⁺-independent kinase was also studied. The K₅₀ values of ATP and MgCl₂ were 15 μM and 2.4 mM, respectively (data not shown). The requirement for millimolar Mg²⁺ suggests the presence of a regulatory site for Mg²⁺ in addition to the requirement for a MgATP substrate as has been shown for PKC previously (20).

FIG. 8. Inhibition of the novel Ca²⁺-independent protein kinase activity by sphingosine and H₇. Ca²⁺-independent protein kinase (○) or PKC α (●) activities were measured in the presence of the indicated concentrations of sphingosine (A) or H₇ (B). The activities are expressed as percent of maximal activation achieved in the presence of EGTA/PS/DiC₁₈:₁ or Ca²⁺/PS/DiC₁₈:₁ for the novel kinase and PKC α, respectively.

**Relationship to other Ca²⁺-independent PKC Isoenzymes**—The preceding results demonstrate that this platelet kinase shows novel biochemical characteristics. To gain further understanding about the relationship of this novel Ca²⁺-independent kinase to other Ca²⁺-independent PKC isoenzymes, baculovirus-expressed semipurified PKC isoenzymes ε, ζ, and η were assayed for their ability to phosphorylate histone III-S, pseudosubstrate ε peptide, and protamine sulfate, respectively. The data shown in Fig. 10 demonstrate that PKC ε, ζ, and η are not activated by PA. Taken together, our data demonstrate that this enzyme is a novel Ca²⁺-independent lipid-dependent protein kinase.

**DISCUSSION**

This study identifies a novel and major protein kinase from human platelets whose histone phosphorylating activity exceeds that of PKC α, PKC βI, or PKC βII, the major isoforms of PKC in platelets. This enzyme is distinct from the calcium-dependent isoforms of PKC (the cPKC subfamily) since its activity is not dependent on calcium. Moreover, this enzyme is distinct from the nPKC family of isoenzymes (PKC δ, ε, and η) since it lacks responsiveness to diacylglycerol or phorbol esters and since PKC δ and PKC ε are activated best by PI and PS, respectively (21–25). Finally, this enzyme is also distinguished from PKC ζ (the only characterized member of the nPKC subfamily) by the following criteria. 1) It does not cross-react im-
EGTA

PS

Protein kinase. A, immunologically with PKC (specific antibodies, 2) it is activated
expressed and partially purified PKC isoenzymes were assayed for their
distinct substrate profile since PKC
primarily of protamine sulfate but not histone
activation in presence of indicated cofactors. The substrates used for
by PA better than PS as opposed to PKC
and pseudosubstrate peptide
FIG. 9. Substrate specificity of the novel Ca\textsuperscript{2+}-independent pro-
tein kinase. A, histone-dependent activity in the presence of 10 mol %
PS (○) or 5 mol % PA (■). B, phosphorylation of other substrates. □, EGTA; ■, EGTA/PS/DAG\textsubscript{cat}.

FIG. 10. Cofactor requirement of PKC \(\delta\), \(\epsilon\), and \(\zeta\). Baculovirus-
expressed and partially purified PKC isoenzymes were assayed for their
activation in presence of indicated cofactors. The substrates used for
activity determination were histone III-S, pseudosubstrate peptide \(\zeta\), and pseudosubstrate peptide \(\zeta\), for PKC \(\delta\) (■), \(\epsilon\) (□), and \(\zeta\) (□).

based on the PKC \(\epsilon\) pseudosubstrate site. Immunologic criteria
also distinguish this isoenzyme from the other calcium-depend-
ent and calcium-independent isoenzymes.

Since this enzyme is dependent on lipids for activation, it
may belong to an extended family of lipid-dependent protein
kinases. Whether it belongs specifically to the PKC family must
await sequence analysis (and formal definition of what defines
a member of the PKC family). In this context, other phospho-
lipid-dependent protein kinases have been described. Elias and
co-workers (27, 28) identified and purified a phosphatidyglyc-
erol-dependent protein kinase from spleen and from murine
leukemia cells. This 27-kDa kinase, designated as PK-P, was
equally activated by phosphatidylinositol, but poorly activated
by PA (29). In another study Bocckino et al. (30) detected PA-
dependent protein phosphorylation in soluble extracts of differ-
et tissues. This activity showed a mixed dependence on cal-
cium depending on substrates and did not phosphorylate
histone III-S as an exogenous substrate (30). This suggests that
the activity detected by Bocckino et al. is distinct from the
PA-dependent kinase described in this study. Overall, however,
evidence is emerging for the existence of a superfamily of phos-
holipid-dependent protein kinases.

The main biochemical features of this enzyme relate to its
requirement for phosphatidic acid, as well as its activation by
cis-unsaturated fatty acids. The selective dependence on PA of
this enzyme distinguishes it from other isoenzymes such as
PKC \(\alpha\), which shows no dependence on PA. This raises a major
question of whether PA serves as a cofactor for this enzyme
with an as yet unidentified activator (in analogy with the role
of PS as a cofactor for the other isoenzymes of PKC with DAG
serving as a specific activator). On the other hand, PA may
serve as an activator of this enzyme. This latter possibility is
supported by the kinetic analysis (Fig 5), where PS serves as a
cofactor and PA acts as a monomeric activator in analogy with
the kinetic profile of activation of cPKCs by PA and DAG. Ob-
viously, implicating PA as a physiologic activator requires ad-
ditional studies at a cellular level and the use of specific inhibi-
tors. Nonetheless, these studies raise the interesting possibility
that PA, generated during activation of platelets (31, 32) or
other cell types either through diacylglycerol kinase or through
the action of phospholipase \(P\), may specifically activate this
enzyme. According to this hypothesis, this PA-activated protein
kinase may play an important role in mediating the effects of
PA, including mitogenesis in a number of cell systems.

The dependence of this enzyme on cis-unsaturated fatty acids
has two major implications. First, fatty acids may serve as
physiologic activators of this enzyme. In previous studies, we
have shown that cis-unsaturated fatty acids activate the PKC
family of isoenzymes preferentially in the cytosol of human platelets suggesting a two-compartment model for regulation of
PKC, such that phospholipids and diacylglycerol activate mem-
brane-bound PKC whereas fatty acids (EC\textsubscript{50} ~ 5 \mu M) may specifically activate this enzyme. This is of particular interest because the dose dependence on fatty acids demonstrates that this enzyme is more sensitive to fatty acids (EC\textsubscript{50} ~ 5 \mu M) than PKC \(\alpha\) and other isoenzymes of PKC (EC\textsubscript{50} ~ 50 \mu M). These concentrations may be more readily
achievable in vivo following activation of phospholipase \(A\), and
would suggest that this enzyme is preferentially targeted over
isoenzymes of PKC. The second implication of the biochemical
activation of this enzyme by fatty acids relates to the mecha-
nism of action of fatty acids in activating PKC. This novel
enzyme is not dependent on DAG; therefore, the ability of fatty
acids to activate this enzyme provides strong evidence that
fatty acids interact with the enzyme at a site distinct from that
of DAG as has been suggested in a previous biochemical anal-
ysis (15). Moreover, the observed synergy of activation of PKC α by DAG and fatty acids presumably occurs by interaction of these two lipid mediators at distinct sites.

In conclusion, these studies expand the family of lipid-activated protein kinases with the identification of a novel protein kinase activated specifically by phosphatidic acid and cis-un satu rated fatty acids. This specificity suggests a distinct role for this enzyme in platelet physiology, possibly as a target for endogenously-generated PA. Further studies are required to elucidate the physiologic functions of this enzyme.

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REFERENCES