Synergistic action of diacylglycerol and unsaturated fatty acid for protein kinase C activation: Its possible implications

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ABSTRACT Kinetic properties of the purified α, β, and γ subunits of protein kinase C (PKC) to respond to diacylglycerol, phosphatidylserine (PtdSer), and Ca2+ were reinvestigated in the presence of several fatty acids. Although responses of these enzyme subheadings to the lipids slightly differed from one another, the reaction velocity of these subunits was significantly enhanced by synergistic action of diacylglycerol and a cis-unsaturated fatty acid. Arachidonic, oleic, linoleic, linolenic, and docosahexaenoic acids were active in this role, whereas saturated fatty acids such as palmitic and stearic acids were inactive. Elaidic acid was also inactive. In the presence of both PtdSer and diacylglycerol, the cis-unsaturated fatty acids increased further an apparent affinity of PKC to Ca2+ and allowed the enzyme to exhibit almost full activation at nearly basal levels of Ca2+ concentration. The concentration of fatty acid giving rise to the maximum activation of enzyme was 20–50 μM. The result presented herein implies that the receptor-mediated release of unsaturated fatty acids from phospholipids may take part, in synergy with diacylglycerol, in the activation of PKC even when the Ca2+ concentration is low. A possibility arises, then, that the activation of PKC is an integral part of the signal-induced degradation cascade of various membrane phospholipids, which is initiated by the action of phospholipase C and phospholipase A2.

The hydrolysis of phosphatidylinositol, particularly its 4,5-bisphosphate, catalyzed by phospholipase C is generally accepted to be crucially important to initiate signal transduction for eliciting cellular responses (1–3). Recent studies have suggested that receptor-mediated hydrolysis of phosphatidylcholine may also be involved in transmembrane signaling (for a review, see refs. 4 and 5). In fact, it is becoming clear that both phospholipase A2 (6) and phospholipase D (7–11; see ref. 4 for additional references) are activated in a signal-dependent manner.

Early reports from this laboratory (12, 13) have described that diacylglycerol produced in membranes activates protein kinase C (PKC) in the presence of Ca2+. Diacylglycerol and phospholipids, especially phosphatidylserine (PtdSer). Kinetic analysis has shown that diacylglycerol increases an apparent affinity of the enzyme for Ca2+ and PtdSer and thereby activates PKC in the micromolar range of Ca2+ concentrations (13). Subsequent studies in several laboratories (14–21) have found that, in the absence of PtdSer, unsaturated fatty acids such as arachidonic and oleic acids may activate PKC to various degrees, most efficiently activating the γ subunit, and a potential role of unsaturated fatty acids as second messengers has been postulated. More recently, synergic action of fatty acids and diacylglycerol for the activation of PKC has been reported (refs. 22–25; also S. G. Chen and K. Mura- kami, personal communication). Studies on the interaction of fatty acids with diacylglycerol and Ca2+ have revealed, however, conflicting results. In some studies (18, 26) activation of PKC by fatty acid is independent of Ca2+ and in some others (17, 23) is dependent on this divalent cation. Diacylglycerol sometimes shows no effect on fatty acid activation of PKC (17), slightly modulates the effect of fatty acids (23), or strongly synergizes with fatty acids (22, 25). This inconsistency is probably due to the fact that most studies so far reported used a mixture of several PKC subtypes in different ratios, which were obtained from various tissues such as neutrophils (14), brain (15, 22, 23), spleen (22), and platelets (24). In addition, the enzyme was assayed under distinct conditions at different Ca2+ concentrations. It is clear that the response of PKC to fatty acids significantly varies with the subunit used (17–21, 25) and with the phosphatase acceptor used (21).

The studies described herein will show that the α, β, and γ subunits of PKC are all activated dramatically and reproducibly by the simultaneous addition of diacylglycerol and unsaturated fatty acid in the presence of PtdSer. This activation greatly varies with the Ca2+ concentration present, and the enzyme exhibits nearly full activity at the basal level of Ca2+ concentration. The kinetic properties of the response of these PKC subtypes to diacylglycerol and unsaturated fatty acids will be described in this paper, although the biochemical mechanism of this enzyme activation by this lipid–protein interaction is still not understood.

MATERIALS AND METHODS

PKC and Assay. The α, β, and γ subunits were purified from the rat brain cytosol as described (18), and all preparations used were practically pure. The enzyme activity was assayed routinely with calf thymus H1 histone as phosphate acceptor. The reaction mixture (0.25 ml) contained 20 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 10 μM (γ-32P)ATP (8 × 104 cpm/nmol), 50 μg of H1 histone, and other chemicals including CaCl2 (1 μM), PtdSer (8 μg/ml), diolein (0.8 μg/ml), and fatty acid (50 μM) unless otherwise indicated in each experiment. PtdSer and diacylglycerol were first mixed in chloroform and then dried under nitrogen. The residue was then sonicated in a buffer solution to prepare lipid vesicles as described (18). Fatty acid normally dissolved in ethanol was diluted with a buffer solution, sonicated under nitrogen, and added to the reaction mixture. The reaction was started by the addition of enzyme. After incubation for 3 min at 30°C, the acid-precipitable materials were collected on a nitrocellulose filter. The radioactivity was quantitated by Cerenkov counting with a scintillation spectrometer as described (18).

Abbreviations: PKC, protein kinase C; PtdSer, phosphatidylserine; GAP-43, growth-associated protein 43.

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One unit of PKC was defined as the amount of enzyme catalyzing the incorporation of 1 nmol of $^{32}$P into H1 histone per min under the conditions described above.

**Chemicals.** $[^{32}P]ATP$ (carrier-free) was a product of New England Nuclear. Calf thymus H1 histone was prepared by the method of Hashimoto et al. (27). Growth-associated protein 43 (GAP-43; also called F1, B-50, and neuromodulin), a major target protein of PKC in the presynaptic nerve endings (28, 29), was partially purified from bovine brain by the method of Masure et al. (30). Bovine myelin basic protein was obtained from Sigma. Phospholipids, diacylglycerol, and all fatty acids were purchased from Serdary Research Laboratories (London, ON, Canada). An authentic sample of docosahexaenoic acid was donated by N. G. Bazan. Phorbol 12-myristate 13-acetate was obtained from Sigma. Other chemicals were purchased from commercial sources.

**Other Methods.** SDS/PAGE analysis of proteins and protein determinations were made as described (18).

**RESULTS**

**Activation by Diacylglycerol.** It was confirmed that the α, β, and γ subspecies of PKC were all activated by diacylglycerol in the presence of PtdSer in the micromolar range of Ca$^{2+}$ (Fig. 1A, B, and C). In the absence of diacylglycerol, PtdSer activated the enzyme only at unusually high concentrations of Ca$^{2+}$, around 0.1 mM. Diacylglycerol alone was inactive.

**Synergistic Action of Diacylglycerol and Arachidonic Acid.** Although there was some kinetic variation, the diacylglycerol-dependent activation of the PKC subspecies was greatly enhanced by the addition of arachidonic acid (Fig. 1D, E, and F). Arachidonic acid alone showed some stimulatory effect, particularly on the γ subspecies as reported (17–20), and the stimulation of this subspecies appeared to be independent of PtdSer at low Ca$^{2+}$ concentrations (Figs. 1F and 2C).

It is worth noting that the synergistic action of diacylglycerol and arachidonic acid was predominant at lower Ca$^{2+}$ concentrations, and all enzyme subspecies tested exhibited almost full activation at nearly basal levels of Ca$^{2+}$ (Fig. 1D, E, and F). Similar results were obtained with phorbol 12-myristate 13-acetate instead of diacylglycerol. The concentration of arachidonic acid giving rise to the maximum effect was slightly high, in the 10 μM range (Fig. 2). However, the kinetic properties varied slightly with the phosphate acceptor protein used (see below). In the absence of PtdSer, arachidonic acid plus diacylglycerol activated significantly all three enzyme subspecies. However, this activation did not depend on the Ca$^{2+}$ concentration (Fig. 1D, E, and F). Arachidonic acid did not activate the enzyme simply by mimicking the action of PtdSer. The effect of arachidonic acid was blocked by the addition of bovine serum albumin to the reaction mixture.

**Diacylglycerol Concentration.** Although diacylglycerol alone was inactive, this neutral lipid greatly increased an apparent affinity of PKC for Ca$^{2+}$ as well as for PtdSer and thereby rendered the enzyme active at lower concentrations of Ca$^{2+}$ as described (13). The addition of arachidonic acid to the reaction mixture did not appear to decrease the diacylglycerol concentration necessary for the enzyme activation (Fig. 3). Arachidonic acid enhanced the reaction velocity over a wide range of PtdSer but did not affect an apparent affinity of enzyme for PtdSer (Fig. 4).

**Fatty Acid Specificity.** The synergistic action of diacylglycerol and arachidonic acid on the activation of PKC described above was observed also for many other naturally occurring cis-unsaturated fatty acids such as oleic, linoleic, linolenic, and docosahexaenoic acids (Fig. 5). Saturated fatty acids such as palmitic and stearic acids were practically inactive. Elaidic acid was inactive for all enzyme subspecies tested. At a low concentration of Ca$^{2+}$, 1 μM, the stimulatory effect of the simultaneous presence of diacylglycerol and unsaturated

**Fig. 1.** Activation of PKC subspecies by 50 μM arachidonic acid with various concentrations of Ca$^{2+}$ in the absence of PtdSer and diacylglycerol (×) or in the presence of PtdSer (●), diacylglycerol (○), or both (●). Each PKC subspecies (=0.15 unit) was assayed with H1 histone as a model substrate under the standard conditions described except that various concentrations of CaCl$_2$ were added in the absence (A, B, and C) or presence (D, E, and F) of 50 μM arachidonic acid. (A and D) The α subspecies. (B and E) The β subspecies. (C and F) The γ subspecies. EGTA (5 mM) instead of CaCl$_2$ was added to the reaction mixtures indicated by arrows.

**Fig. 2.** Activation of PKC subspecies by arachidonic acid at various concentrations with 1 μM Ca$^{2+}$ in the absence of PtdSer and diacylglycerol (×) or in the presence of PtdSer (●), diacylglycerol (○), or both (●). Each PKC subspecies (=0.15 unit) was assayed under the standard conditions with H1 histone as a model substrate as described except that various concentrations of arachidonic acid were added as indicated. (A) The α subspecies. (B) The β subspecies. (C) The γ subspecies.
fatty acid was most remarkable for the α subspecies (Fig. 5). It was also noted that docosahexaenoic acid was able to synergize with diacylglycerol to enhance the PKC activation and by itself was able to activate the γ subspecies significantly in the absence of diacylglycerol and PtdSer. Polyanions such as heparin were inhibitory.

**Phosphate Acceptor Proteins.** It has been reported (21, 31) that the requirements of Ca**2+**, PtdSer, and diacylglycerol for PKC activation and the stimulatory effects of unsaturated fatty acids on PKC vary significantly with the phosphate acceptor protein. The synergistic action of diacylglycerol and unsaturated fatty acids described above was similarly observed with bovine myelin basic protein as a model substrate, although the kinetics of this action was slightly different from that observed with H1 histone. With myelin basic protein, the maximum activation was obtained with 20 μM arachidonic acid. The experiment given in Fig. 6 shows that the phosphorylation of GAP-43, a physiological substrate of PKC—notably that of the β subspecies (32), which is present in a presynaptic region of the nervous system, was stimulated by the simultaneous addition of diacylglycerol and arachidonic acid. A part of the results is shown quantitatively in Table 1. In the absence of PtdSer, diacylglycerol with arachidonic acid per se was practically inactive with GAP-43 as a phosphate acceptor. Similar results were obtained with docosahexaenoic acid instead of arachidonic acid.

**DISCUSSION**

The activation of PKC by synergistic action of oleic acid and diacylglycerol has been described by Verkest et al. (22) with enzyme preparations purified from the rat brain and bovine spleen. This observation was made in the absence of PtdSer, and the extent of enzyme activation was equivalent to that obtained with diacylglycerol, PtdSer, and Ca**2+**. Seifert et al. (23) reported similar activation of the rat brain PKC by unsaturated fatty acids and diacylglycerol, both in the presence and absence of Ca**2+** and PtdSer, although the extent of the synergistic action of fatty acids and diacylglycerol was not very prominent. El Touny et al. (24) have observed that oleic acid sometimes could synergize with diacylglycerol for the platelet PKC activation by PtdSer. However, all of these studies were made with a mixture of several PKC subspecies.
cell types. Kinetic analysis shows that these unsaturated fatty acids together with diacylglycerol dramatically increase an apparent affinity of PKC for Ca^{2+} and almost fully activate the enzyme at the basal level of Ca^{2+} concentrations.

The fatty acids do not appear to exert their effects simply by ionic interaction with the basic phosphate acceptor proteins, since polyanions such as heparin were inhibitory. GAP-43 is an acidic protein that serves as a specific substrate for PKC (28, 29). Neither the order of the addition of fatty acid and other lipids to the reaction mixture nor the premixing of fatty acid with diacylglycerol and PtdSer significantly affected the rate of enzymatic reactions. The biochemical mechanism of the action of unsaturated fatty acids remains unclear.

The physiological significance of the synergistic action of unsaturated fatty acids and diacylglycerol remains to be clarified. One obvious possibility is that, in stimulated cells, PKC once activated initially by the hydrolysis of inositol phospholipids may sustain its enzymatic activity even after the Ca^{2+} concentration returns to the basal level, if both diacylglycerol and unsaturated fatty acids are still available. It is attractive to surmise, then, that phospholipase C and phospholipase A_2, which are activated in a signal-dependent manner, are both involved in the activation of the PKC family. Such a potential role of unsaturated fatty acids has been suggested by Seifert et al. (33), who have observed that some unsaturated fatty acids such as linoleic acid show synergistic action with permeable diacylglycerol, dioctanoyl-glycerol, to activate human platelet PKC. Szamel et al. (34) have recently reported that linoleic and arachidonic acids potentiate interleukin 2 synthesis in human T cells, which is induced by ionomycin and permeable diacylglycerol, although they interpreted this observation in a different way. Sustained activation of PKC has recently been shown to be necessary for gene activation and cell proliferation (35, 36).

Docosahexaenoic acid is most abundant in the phospholipids in the central nervous system and retina, particularly during early postnatal development and synaptogenesis (37, 38). This fatty acid is apparently very effective in potentiating the activation of PKC, notably the γ subunits, which is expressed only in the central nervous system after birth (39, 40). In the hippocampus, for example, the γ subunits are present normally in the postsynaptic pyramidal cell body of the adult rat, but transiently appears in the presynaptic nerve endings at an early stage of development (41). We presently can only speculate that docosahexaenoic acid could play some role in the activation of several PKC subtypes during development of neuronal systems. The finding that the kinetics of this synergistic action of diacylglycerol and unsaturated fatty acids may vary with the phosphate acceptor protein and the fact that the PKC subtypes are differently located in particular cell types and at limited intracellular locations greatly complicate the search for elucidation of the physiological relevance of the synergistic action of diacylglycerol and unsaturated fatty acids described above. Nevertheless, it may be noted that the phosphorylation of GAP-43, a specific substrate of PKC present in the presynaptic nerve endings and growth cones, is significantly enhanced by synergistic action of diacylglycerol and arachidonic or docosahexaenoic acid. This protein has been proposed to be phosphorylated preferentially by the β subtypes (32).

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### Table 1. Phosphorylation of GAP-43 by PKC β subpecies

<table>
<thead>
<tr>
<th>Additions</th>
<th>Radioactivity, cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>PtdSer</td>
<td>510</td>
</tr>
<tr>
<td>+ 20:4</td>
<td>510</td>
</tr>
<tr>
<td>+ diolein</td>
<td>3540</td>
</tr>
<tr>
<td>+ diolein and 20:4</td>
<td>5180</td>
</tr>
<tr>
<td>Diolein and 20:4</td>
<td>240</td>
</tr>
</tbody>
</table>

The phosphorylation reaction was carried out on a microscale under the conditions (in a total volume of 50 μl) similar to those described in the legend to Fig. 6. The acid-precipitable radioactivity was determined with 100 μg of H1 histone as a carrier protein during acid precipitation. The concentration of arachidonic acid (20:4) in this experiment was 20 μM.