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PROTEIN KINASE C SUBSPECIES IN RAT BRAIN SYNAPSES AND PHOSPHORYLATION OF GROWTH-ASSOCIATED PROTEIN

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INDEXING WORDS

diacylglycerol; GAP-43; protein kinase C; unsaturated fatty acid

SYNOPSIS

Synaptosomes isolated from rat hippocampus contained the α- and β-subspecies of protein kinase C (PKC), but not the γ-subspecies, whereas postsynaptic pyramidal cells contained all these three subspecies. The PKC enzymes were activated synergistically by diacylglycerol and *cis*-unsaturated fatty acids including docosahexaenoic acid which is abundant in brain phospholipids. The phosphorylation of a PKC-specific substrate, growth-associated protein (GAP-43), which is associated predominantly with presynaptic membranes, was also stimulated by diacylglycerol and *cis*-unsaturated fatty acids, particularly at the micromolar range

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Abbreviations used are: PKC, protein kinase C; GAP-43, growth-associated protein-43; PC, phosphatidylcholine; EGTA, ethylene glycol bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid; PS, phosphatidylserine.

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of Ca^{2+} concentrations. The results presented suggest that *cis*-unsaturated fatty acids together with diacylglycerol may take part in the phosphorylation of GAP-43 in presynaptic membranes presumably by the α- or β-subspecies of PKC even after the Ca^{2+} concentrations return to the basal level.

INTRODUCTION

Protein kinase C (PKC) is a family of multiple subspecies with distinct distribution and subtly different enzymatic properties, and has been proposed to play roles in the regulation of neuronal functions such as neurotransmitter release, modulation of membrane excitability, and long term potentiation.[6,9,12,15,19] This enzyme was initially thought to be activated by diacylglycerol that is generated by inositol phospholipid hydrolysis initiated by either receptor stimulation or opening of Ca^{2+} channels.[15] It is becoming clear that agonist-induced hydrolysis of other membrane phospholipids, particularly phosphatidylcholine (PC) by phospholipase D and phospholipase A_2 may also take part in cell signaling. For example, the hydrolysis of PC by phospholipase D results in the formation of phosphatidic acid, which is then converted to diacylglycerol by the action of a phosphomonoesterase.[5,8] This diacylglycerol plays a role in prolonging the activation of PKC, that is needed for long-term cellular responses.[1,4] It is also plausible that the primary products of PC hydrolysis by phospholipase A_2, *cis*-unsaturated fatty acids and lysophosphatidylcholine, potentiate PKC activation and thereby contribute to signal transduction through the PKC pathway.[2,22,24]

Previous reports from several laboratories have suggested that *cis*-unsaturated fatty acids such as oleic and arachidonic acids may function in long-term potentiation of synaptic processes.[11,12] An extracellular excretory phospholipase A_2 (group II) is also found in neuronal cells.[16] The present studies were undertaken, therefore, to identify the PKC subspecies present in the pre- and post-portions of the hippocampal synapses, and to explore the kinetics of protein phosphorylation in the presence of various *cis*-unsaturated fatty acids. The PKC specific substrate, growth-associated protein (GAP-43) that is present only in the pre-synaptic membrane [6,9] has also been employed.

MATERIALS AND METHODS

Preparation of Synaptosomes

Sprague-Dawley rats (6-8 weeks) were used. The hippocampus region was dissected, and synaptosomes were prepared using the Percoll gradient method described by Dunkley *et al.*[7)]

Analysis of PKC Subspecies

Synaptosomes were lyzed in 20 mM Tris-Cl, pH 7.5 containing 10 mM EGTA, 2 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride and 45 μM leupeptin, followed by sonication for 2 x 15 sec, and standing on ice for 20 min. Then, an equal volume of the above solution containing, in addition, 2% (v/v) Triton X-100 was added, and the mixture was sonicated again for 2 x 15 sec and incubated for additional 20 min at 4°C with stirring to solubilize membrane-bound PKC as much as possible. Following centrifugation for 60 min at 100,000 x g, the soluble fraction was subjected to PKC subspecies analysis upon hydroxyapatite chromatography as described.[18,20)] The enzyme subspecies in the hippocampus region were analyzed under the same conditions except that the tissue was homogenized in 10 volumes of the buffer with a Teflon-glass homogenizer.

PKC Subspecies and Assay

The α-, β-, and γ-subspecies of PKC employed were purified from the rat brain soluble fraction as described.[18)] All preparations were practically pure. For routine assay enzyme activity was assayed with calf thymus H1 histone as phosphate acceptor. The reaction mixture (0.25 ml) contained 20 mM Tris-Cl (pH 7.5), 10 mM $MgCl_2$, 10 μM [γ-^{32}P]ATP (100-200 cpm/pmole), 10^{-4} M $CaCl_2$, 200 μg/ml H1 histone, 8 μg/ml phosphatidylserine (PS), 0.8 μg/ml diacylglycerol, and a free fatty acid as indicated in each experiment. PS and diacylglycerol were mixed first in chloroform, and dried under nitrogen stream. The residue was then sonicated in a buffer solution to prepare lipid vesicles as described.[18)] Free fatty acids were normally dissolved in ethanol was directly added to the reaction mixture. Neither the

order of addition of these lipids nor the prior mixing of FFA with PS/diacylglycerol affected significantly of enzyme. After incubation for 3 min at 30°C, the acid-precipitable radioactivity was determined as described.[18] One unit of PKC was defined as that amount of enzyme which incorporated one nmol of phosphate from ATP into histone under the assay conditions.

Immunoblot Analysis

Antibodies that recognize each subspecies specifically were prepared, and immunoblot analysis of the PKC subspecies were made as described.[18]

Other Materials and Chemicals

PKC employed as standard was purified from the rat brain soluble fraction as described. GAP-43 was purified from bovine cerebral cortex by calmodulin affinity column chromatography according to.[14] Calf thymus H1 histone was prepared as described.[10] [γ-^{32}P]ATP was a product of New England Nuclear. Arachidonic acid was purchased from Kurita Water Industries. PS (bovine brain), 1,2-diolein, and other fatty acids were obtained from Serdary Research Laboratories.

RESULTS

PKC Subspecies in Hippocampal synaptosomes

Biochemical analysis with a hydroxyapatite column revealed that the rat hippocampus contained a large quantity of the γ-subspecies in addition to the α- and β-subspecies (Fig. 1A). The synaptosomes prepared from this brain region, however, contained a little or no γ-subspecies (Fig. 1B). These findings are consistent with those obtained by electron-microscopic analysis with specific antibodies.[3] The α- and β-subspecies were expressed both in synaptosomes (nerve endings) and in postsynaptic pyramidal cells. These subspecies were all identified by immunoblot analysis with specific antibodies.

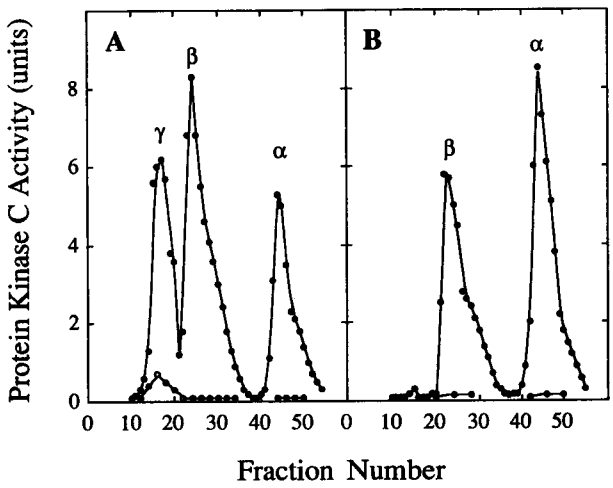

Fig. 1. Chromatographic profiles of PKC subspecies in rat brain tissues and synaptosomes. The detailed experimental conditions to isolate and identify the PKC subspecies are described under MATERIALS AND METHODS. α, β, and γ: the α-, β- and γ-subspecies of PKC, respectively. A: PKC subspecies from the adult rat hippocampus tissue; B: PKC subspecies in the synaptosomes from the adult rat hippocampus. ●—● assayed with 10^{-4} M $CaCl_2$, 8 μg/ml PS, and 0.8 μg/ml diacylglycerol: and O—O assayed with EGTA (0.5 mM) instead of $CaCl_2$, PS, and diacylglycerol.

Activation by Diacylglycerol

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

Although there was some kinetic variation, the diacylglycerol-dependent activation of the PKC subspecies was greatly enhanced by the addition of arachidonic acid (Fig. 3). In this experiment H1 histone was employed as a phosphate acceptor. 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+}. 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 50 μM range. However, the kinetic properties varied slightly with the phosphate acceptor protein used (see below). In the absence of PS, arachidonic acid plus diacylglycerol activated

significantly all three enzyme subspecies. However, this activation did not depend on the Ca^{2+} concentration. Arachidonic acid did not activate the enzyme simply by mimicking the action of PS. The effect of arachidonic acid was blocked by the addition of bovine serum albumin to the reaction mixture.

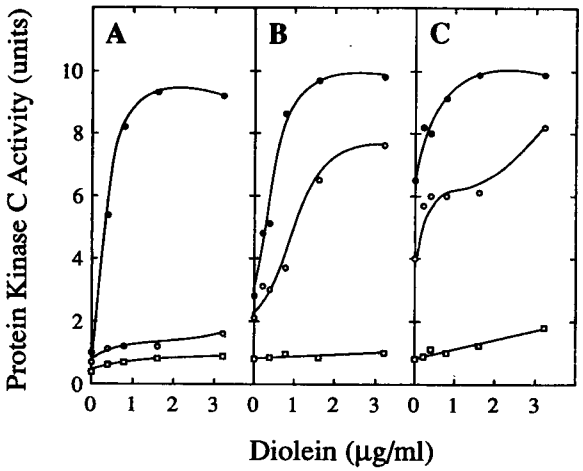

Fig. 2. Activation of PKC subspecies by arachidonic acid in the presence of various concentrations of diacylglycerol. The enzyme activity was assayed under the standard conditions with 50 μM arachidonic acid and diacylglycerol as indicated. A: the α-subspecies; B: the β-subspecies; and C: the γ-subspecies. ●—● assayed with Ca^{2+} and arachidonic acid; ○—○ assayed with arachidonic acid; and □—□ assayed with Ca^{2+}.

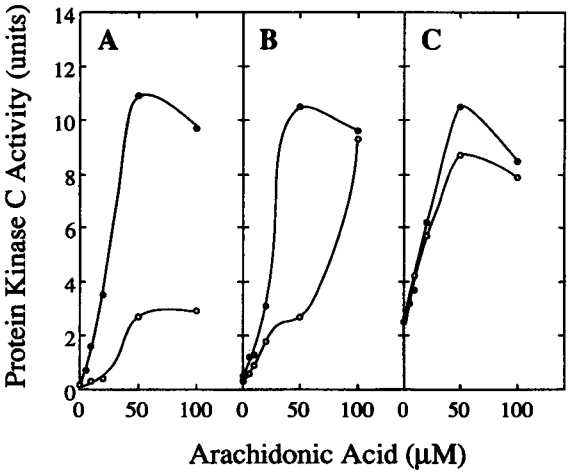

Fig. 3. Activation of PKC subspecies by diacylglycerol in the presence of various concentrations of arachidonic acid. The enzyme activity was assayed under the standard conditions with 0.8 μg/ml diacylglycerol and arachidonic acid as indicated. A: the α-subspecies; B: the β-subspecies; and C: the γ-subspecies. ●—● assayed with diacylglycerol; and ○—○ assayed without diacylglycerol.

Phosphorylation of GAP-43

GAP-43 was phosphorylated by PKC. The α-, β- or γ-subspecies all phosphorylated this protein in the presence of PS, diolein, and Ca^{2+}, and an apparent *Km* value for this substrate protein was about 25 μg/ml (Fig. 4). Approximately 0.5 mole of phosphate was incorporated in 1 mole of GAP-43 after prolonged incubation.

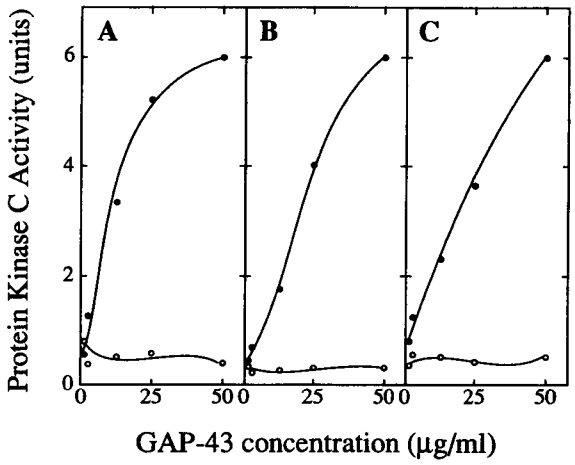

Fig. 4. Phosphorylation of GAP-43 by PKC subspecies. GAP-43 at various concentrations was phosphorylated by PKC subspecies as described under MATERIALS AND METHODS. The results are normalized to the value of the maximal phosphorylation of GAP-43 obtained at the final concentration of 50 μg/ml. A: the α-subspecies; B: the β-subspecies; and C: the γ-subspecies. ●—● assayed with 8 μg/ml PS, 0.8 μg/ml diacylglycerol, and 10^{-6} M $CaCl_2$; o—o assayed with 1 mM EGTA instead of $CaCl_2$, PS, and diacylglycerol.

Enhancement of GAP-43 Phosphorylation by Arachidonic Acid

Arachidonic acid enhanced significantly the phosphorylation of GAP-43 by the α- and β-subspecies of PKC (Fig. 5). This enhancement was dependent on the simultaneous presence of diacylglycerol and PS as quantitatively given in Table I. The enhancement of this phosphorylation was not observed at higher concentrations of arachidonic acid. The phosphorylation of GAP-43 by the γ-subspecies was rather inhibited by arachidonic acid.

Ca^{2+} Concentration on Arachidonic Acid Effect

The enhancement of protein phosphorylation by arachidonic acid was more predominant at lower concentrations of Ca^{2+} with H1 histone as well as GAP-43 as phosphate acceptor (Fig. 6).

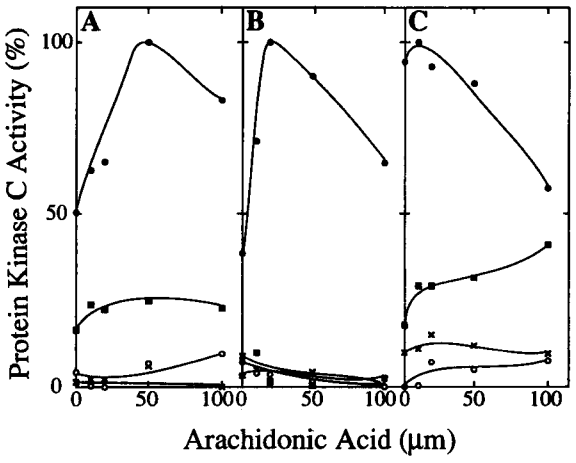

Fig. 5. Enhancement of GAP-43 phosphorylation by arachidonic acid. GAP-43 (25 μg/ml) was phosphorylated by PKC subspecies in the presence of various concentrations of arachidonic acid as described under MATERIALS AND METHODS. The results are normalized to the value of the maximal phosphorylation of GAP-43 by each PKC subspecies. A: the α-subspecies ; B: the β-subspecies; and C: the γ-subspecies. ●—● assayed with 8 μg/ml PS, 0.8 μg/ml diacylglycerol, and 10^{-6} M $CaCl_2$, ○—○ assayed with 0.8 μg/ml diacylglycerol and 10^{-6} M $CaCl_2$, ×—× assayed with 10^{-6} M $CaCl_2$.

Table I. Enhancement of phosphorylation of GAP-43 by arachidonic acid in the presence of PS and diacylglycerol.

System	Reaction rate (cpm)
without Arachidonic Acid	
none	210
PS	510
Diacylglycerol	200
PS+Diacylglycerol	3,540
with Arachidonic Acid	
none	210
PS	510
Diacylglycerol	240
PS+Diacylglycerol	5,180

GAP-43 was phosphorylated by the β-subspecies of protein kinase C in the presence or absence of PS (8 μg/ml), diacylglycerol (0.8 μg/ml), and arachidonic acid (20 μM). Calcium concentration was 10^{-6} M. The reactions were carried out in the same condition for H1 histone as substrate, that is described in the text.

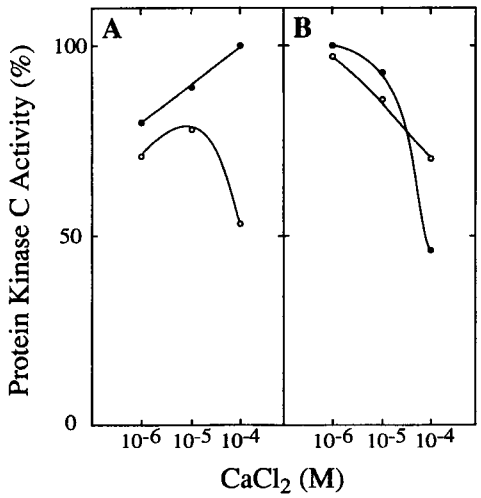

Fig. 6 Effects of Ca^{2+} on PKC activation by arachidonic acid. GAP-43 (25 μg/ml) or H1 histone (200 μg/ml) was phosphorylated by the β-subspecies of PKC in the presence of various concentrations of Ca^{2+} as described under MATERIALS AND METHODS. The results are normalized to the value of the maximal phosphorylation of each substrate protein. A: GAP-43; B: H1 histone. ●−● assayed with 8 μg/ml PS, 0.8 μg/ml diacylglycerol, and arachidonic acid (20 μM and 50 μM for GAP-43 and H1 histone, respectively); ○−○ assayed with 8 μg/ml PS and 0.8 μg/ml diacylglycerol.

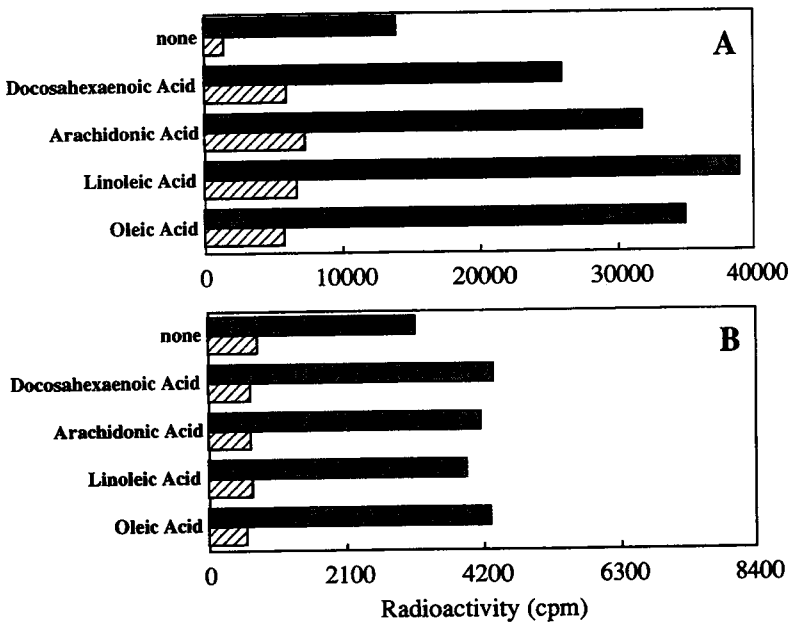

Fig. 7. Effects of various fatty acids on PKC activation. GAP-43 (25 μg/ml) or H1 histone (200 μg/ml) was phosphorylated by the β-subspecies of PKC in the presence of various fatty acids (20 μM and 50 μM for GAP-43 and H1 histone, respectively) as described under MATERIALS AND METHODS. The results are normalized to the value of the phosphorylation of each substrate protein in the presence of PS, diacylglycerol, and $CaCl_2$. A: GAP-43; B: H1 histone. Shaded bars assayed with 8 μg/ml PS, 0.8 μg/ml diacylglycerol, and 10^{-6} M $CaCl_2$; hatched bars assayed with 10^{-6} M $CaCl_2$.

Effects of Various Fatty Acids

cis-Unsaturated fatty acids other than arachidonic acid, such as docosahexaenoic, linoleic, and oleic acids all enhanced the phosphorylation of H1 histone as well as, to lesser extents, GAP-43. The results with the β-subspecies were given (Fig. 7). Palmitic and stearic acids were inactive.

DISCUSSION

Routtenberg and his coworkers[12] have proposed that oleic acid may be involved in maintenance of long-term potentiation by activating PKC. Bliss and his colaborators[13] have postulated a possible role of arachidonic acid as a retrograde messenger from postsynaptic to presynaptic portion. Although biochemical mechanism of developing long-term potentiation and the precise role of PKC in synaptic transmission have not been clarified, it is noted that, in the hippocampus, the γ-subspecies is not detected at presynaptic portion. Instead, the α- and β-subspecies are present in both pre- and postsynaptic portions, and the phosphorylation of proteins, particularly GAP-43, by these PKC subspecies is enhanced by *cis*-unsaturated fatty acids.

GAP-43 is expressed specifically in presynaptic membranes and known as a major substrate of PKC.[6,21] This protein is also rich in the growth cone, and binds calmodulin in the absence of free Ca^{2+}.[14] The phosphorylation of GAP-43 at Ser-41 by PKC reduces its affinity for calmodulin, suggesting that calmodulin becomes available when PKC is activated. The phosphorylation of GAP-43 by PKC has also been implicated in additional neuronal functions such as long-term potentiation, the regulation of neurotransmitter release, and neuronal development and regeneration.[6,23] The results presented above show that several *cis*-unsaturated fatty acids such as arachidonic acid activated GAP-43 protein phosphorylation by the α- or β-subspecies, synergistically with diacylglycerol in the presence of physiologically low concentrations of Ca^{2+}. The results are consistent with the observation that GAP-43 is colocalized with the βII subspecies in synaptosomes.[21] GAP-43 is known to be phosphorylated not only by PKC but by other protein kinases such as casein kinase II.[17]

Further studies are necessary to explore the regulation as well as the roles of GAP-43 in synaptic processes, notably long-term potentiation through PKC activation.

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