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Protein kinase C subspecies in adult rat hippocampal synaptosomes

Activation by diacylglycerol and arachidonic acid

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Synaptosomes isolated from the adult rat hippocampus contain the α- and β-subspecies of protein kinase C (PKC), but not the γ-subspecies which is abundantly expressed in the pyramidal cells in this brain region. Although the γ-subspecies is known to respond significantly to free arachidonic acid, it is found that both the α- and β-subspecies are also activated dramatically by arachidonic acid in synergistic action with diacylglycerol. Oleic, linoleic, and linolenic acids are all active. It is possible that unsaturated fatty acids may take part in the activation of α- and β-subspecies of PKC which are present in the presynaptic nerve endings terminating at the hippocampal pyramidal cells.

Protein kinase C; Arachidonic acid

1. INTRODUCTION

The activation of protein kinase C (PKC) has been proposed to play key roles in maintenance of long-term potentiation (LTP) in the hippocampus (for reviews, see [1–4]). Growth-associated protein (GAP43, F1, B50, neuromodulin) has been known to be one of the major target proteins of PKC which is associated with presynaptic nerve endings [5,6]. Although the biological function of this protein is not identified, its phosphorylation by PKC is implicated in the control of diverse synaptic processes, including transmitter release [7], calmodulin sequestration [8], neuronal development and regeneration [9–12]. It has been suggested, on the other hand, that unsaturated free fatty acids (FFAs) such as arachidonic and oleic acids may play a role in maintenance of LTP, particularly at the presynaptic portion of the hippocampus [13]. To explain this potential role of FFAs, the possibility has been discussed that FFAs activate PKC directly [13–19]. The γ-subspecies, in fact, shows properties that respond to FFAs to exhibit enzymatic activity [16,20,21]. Electron-microscopic analysis with specific antibodies, however, has shown that the γ-subspecies is not present in the nerve endings which terminate at the adult rat hippocampal pyramidal cells, although these cells express a large quantity of this PKC subspecies [22,23].

The biochemical studies described herein confirm that synaptosomes isolated from the adult rat hippocampus contain the α- and β- but not γ-subspecies, and that these PKC subspecies present in the synaptosomes are activated dramatically by synergistic action of an unsaturated FFA and diacylglycerol (DAG).

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Abbreviations: PKC, protein kinase C; LTP, long-term potentiation; FFA, free fatty acid; DAG, diacylglycerol; PS, phosphatidylserine; EGTA, [ethylenebis (oxyethylenenitrilo)]tetraacetic acid

2. MATERIALS AND METHODS

2.1. *Synaptosomes*

Sprague-Dawley rats (6–8 weeks) were used. The cerebrum and hippocampus regions were dissected, and synaptosomes were prepared using the Percoll gradient method described by Dunkley et al. [24].

2.2. *Analysis of PKC subspecies*

Synaptosomes were lysed in 20 mM Tris-HCl, 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 × 15 s, and staining 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 × 15 s 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 × *g*, the soluble fraction was subjected to PKC subspecies analysis upon hydroxyapatite chromatography as described [25]. The enzyme subspecies in the cerebrum and hippocampus regions were analyzed under the same conditions except that these tissues were homogenized in 10 volumes of the buffer with a Teflon-glass homogenizer.

2.3. *PKC subspecies and its assay*

The α-, β- and γ-subspecies of PKC employed were purified from the rat brain soluble fraction as described [26], and all preparations were practically pure. The enzyme activity was assayed with calf thymus H1 histone as phosphate acceptor. The reaction mixture (0.25 ml) contained 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 μM [γ-^{32}P]ATP (2000 cpm/nmol), 10^{-4} M $CaCl_2$, 100 μg H1 histone, enzyme, PS, DAG and FFA as indicted in each experiment. PS and DAG were mixed first in chloroform, and dried under a nitrogen stream. The residue was then sonicated in a buffer solution to prepare lipid vesicles as described [21]. FFA 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/DAG affected significantly the reaction velocity. The reaction was started by the addition of enzyme. After incubation for 3 min at 30°C, the acid-precipitable radioactivity was determined as described [26]. 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.

2.4. *Immunoblot analysis*

Antibodies that recognize specifically each PKC subspecies were prepared, and immunoblot analysis was carried out as described [26].

2.5. *Chemicals*

All chemicals were of analytical grade. [γ-^{32}P]ATP was a product of New England Nuclear. Calf thymus H1 histone was prepared as described [27]. PS, DAGs and FFAs were obtained from Serdary Research Laboratories.

3. RESULTS

3.1. *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 [22,23]. The α- and β-subspecies were expressed both in synaptosomes (nerve endings) and in post-synaptic pyramidal cells [22]. The whole cerebrum and synaptosomes isolated therefrom contained the three PKC subspecies (Figs. 1C and 1D). These subspecies were all identified by immunoblot analysis with specific antibodies.

3.2. *Synergistic action of arachidonic acid and DAG*

Although arachidonic acid and Ca^{2+} could activate significantly the γ- but not α- and β-subspecies, both the α- and β-, particularly α-subspecies, were found to respond highly to arachidonic acid in the presence of DAG and Ca^{2+} (10^{-4} M) to exhibit an enzymatic activity nearly equivalent to that obtained in the presence of PS and DAG (Fig. 2 and Table I). It did not appear, however, that arachidonic acid exerted this effect simply be being substituted for PS. The maximum activity was obtained at approximately 50 μM arachidonic acid (Fig. 3).

3.3. *Specificity of fatty acid*

The synergistic action of fatty acid and DAG was observed not only with arachidonic acid but also with other unsaturated FFAs such as oleic, linoleic and linolenic acids (Table I). Lonolenic acid was less active for the β- and γ-subspecies at 10^{-4} M Ca^{2+}. In the absence of DAG, unsaturated FFAs activated the γ-

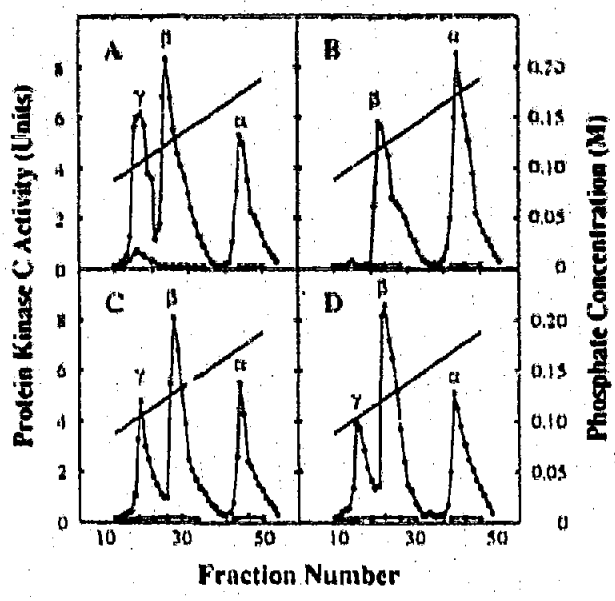

Fig. 1. Chromatographic profiles of PKC subspecies in rat brain tissues and synaptosomes. The detailed experimental conditions to isolate and identify the PKC subspecies are described in section 2. α, β and γ: the α-, β- and γ-subspecies of PKC, respectively. (A) PKC subspecies from the adult rat hippocampus tissue; (B) PKC subspecies in the synaptosomes from the adult rat hippocampus; (C) PKC subspecies from the adult rat cerebrum; and (D) PKC subspecies in the synaptosomes from the adult rat cerbrum. (•—•) assayed with 10^{-4} M Ca^{2+}, 8 μg/ml PS and 0.8 μg/ml DAG; and (○—○) assayed with EGTA (0.5 mM) instead of Ca^{2+}, PS and DAG.

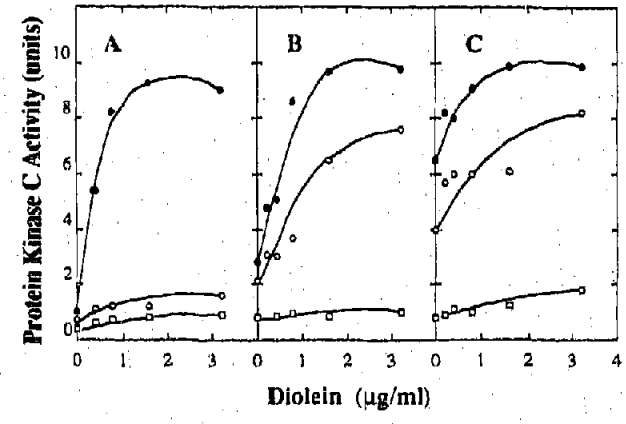

Fig. 2. Activation of PKC subspecies by arachidonic acid in the presence of various concentrations of DAG. The enzyme activity was assayed under the standard conditions with 50 μM arachidonic acid and diolein as indicted. (A) the α-subspecies; (B) the β-subspecies; and (C) the γ-subspecies. (•—•) assayed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

Table I

Stimulation of PKC activity by unsaturated fatty acids.

Each PKC subspecies was assayed under the standard conditions in the presence of 0.8 μg/ml diolein and 50 μM FFA as indicated. Numbers show the acid-precipitable radioactivity (cpm), and those in parentheses show percentages of that obtained in the presence of 10^{-4} M $CaCl_2$, 8 μg/ml PS and 0.8 μg/ml diolein as 100%.

	α-Subspecies		β-Subspecies		γ-Subspecies	
	+ DAG	− DAG	+ DAG	− DAG	+ DAG	− DAG
PS	29 880 (100)		27 280 (100)		32 150 (100)	
None	540 (2)	890 (3)	900 (3)	1100 (4)	1 710 (5)	1 110 (3)
Oleic acid	26 370 (88)	3130 (10)	19 370 (71)	6730 (25)	26 460 (82)	20 170 (63)
Linoleic acid	23 120 (77)	2700 (9)	10 480 (38)	4330 (16)	25 230 (78)	13 800 (43)
Linolenic acid	17 920 (60)	2480 (8)	5 420 (20)	3550 (13)	7 430 (23)	7 520 (23)
Arachidonic acid	14 330 (48)	2670 (9)	14 270 (52)	4663 (17)	16 100 (50)	8 280 (26)

subspecies most efficiently. Saturated FFAs, palmitic and stearic acids, were practically inactive for all enzyme subspecies.

4. DISCUSSION

Routtenberg and co-workers [13] have proposed that oleic acid may be involved in the maintenance of LTP by activating PKC. Bliss and collaborators [28] have postulated a possible role for arachidonic acid as a retrograde messenger from the postsynaptic to the presynaptic portion to maintain LTP. Although the biochemical mechanism of developing LTP and the precise role of PKC in synaptic transmission have not yet been clarified, one of the puzzles resulting from immuno-cytochemical studies is that the γ-subspecies of PKC, which is most sensitive to unsaturated FFAs for its activation, is localized in the postsynaptic portion but not in the presynaptic nerve endings, at least in the adult rat hippocampus [22]. The biochemical analysis described above supports this uneven distribution of the γ-subspecies in the hippocampal pre- and postsynaptic portions, and further shows that the α- and β-subspecies, which are clearly present in the presynaptic portion, respond to unsaturated FFAs such as arachidonic and oleic acids in the presence of a small quantity of DAG.

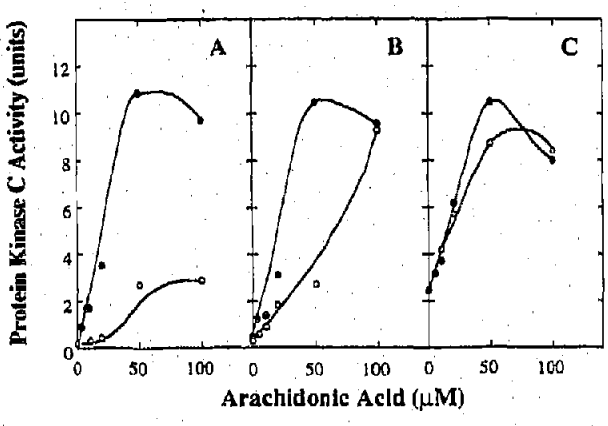

Fig. 3. Activation of PKC subspecies by DAG in the presence of various concentrations of arachidonic acid. The enzyme activity was assayed under the standard conditions with 0.8 μg/ml diolein and arachidonic acid as indicated. (A) the α-subspecies; (B) the β-subspecies; and (C) the γ-subspecies. (●—●)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

Verkest et al. [7] have described that the mixture of PKC subspecies obtained from the rat brain and from the bovine spleen is greatly activated by the simultaneous addition of oleic acid and DAG. Seifert et al. [18] and Touny et al. [19] have subsequently reported that some unsaturated FFAs and DAG synergistically activate platelet PKC, although platelets contain another structurally unknown, FFA-sensitive type of PKC [29]. Seifert et al. [30] and Szamel et al. [31] have described some evidence suggesting that unsaturated FFAs may have potentials to enhance the activation of human platelets and lymphocytes, respectively, presumably through the PKC signal pathway. It is possible that these obervations made by these previous authors are related to those described in the present report.

Such synergistic action of unsaturated FFAs and DAG on the PKC activation appears to imply a poten-

tial role of FFAs in the activation of PKC, particularly its α-subspecies universally present in all tissues and cell types. It has been recently shown that phospholipase A_2 is indeed activated in a signal-dependent manner to produce unsaturated FFAs [32]. More recently, Felder et al. [33] have demonstrated the release of arachidonic acid in the hippocampal neuron by activation of phospholipase A_2 with serotonin. Then, it is attractive to surmise that several phospholipases may be involved in the regulation of cell functions, and that activation of the PKC family is integrated into the degradation cascade of various membrane phospholipids which is elicited by extracellular signals.

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