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Selective activation of the γ-subspecies of protein kinase C from bovine cerebellum by arachidonic acid and its lipoxygenase metabolites

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The γ-subspecies of protein kinase C (PKC) apparently is expressed only in central nervous tissues, and at a high level in the cerebellum and hippocampus. γ-PKC from bovine cerebellum, but not the α- or βI/βII-subspecies, is activated by micromolar concentrations of arachidonic acid (AA), in the absence of both phospholipid and diacylglycerol. A significant component of this activation is also calcium independent. Other unsaturated fatty acids are much less active in this respect. Among the AA metabolites tested, lipoxin A (5(*S*),6(*R*),15(*S*)-11-*cis*-isomer) was a potent, selective activator of the γ-subspecies, and also, to a lesser extent, 12(*S*)-hydroxy-5,8,10,14-eicosatetraenoic acid could support activation. These results raise the possibility that AA and some of its lipoxygenase metabolites may function as messenger molecules in neurones to activate the γ-subspecies of PKC.

Protein kinase C; Arachidonic acid; Lipoxygenase metabolite

1. INTRODUCTION

Molecular cloning analysis has revealed the existence of multiple PKC subspecies in mammalian brain tissue, that can be provisionally divided into two groups (α/βI/βII/γ and δ/ε/ζ) on the basis of their structural characteristics (see [1] for a review and references). The PKC enzyme molecules encoded by the α-, βI-, βII- and γ-sequences have been purified from rat whole brain tissue and resolved into three distinct fractions by hydroxyapatite chromatography, the identities of which have been established as type I(γ), type II(βI/βII) and type III(α) [2–5]. The γ-subspecies of PKC appears to be expressed only in central nervous tissue areas [6], and is present at a high level in the cerebellum and hippocampus [7,8].

Immunohistochemical analysis with subspecies-specific monoclonal antibodies has shown this enzyme to be confined to cerebellar Purkinje cells [5,9,10], where it is present in the dendrites, cell soma, axons and synaptic terminals [9,11]. In contrast, in the same tissue, βI-PKC is present in the granule cell soma, and βII-PKC is present in the molecular layer, seemingly associated with presynaptic terminals terminating on Purkinje cell dendrites [10]. α-PKC may be present in both Purkinje and granule cells [5]. Such a defined cell-specific expression of PKC subspecies prompted us

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Abbreviations: PKC, protein kinase C; PtdSer, phosphatidylserine; DO, diolein; FPLC, fast-protein liquid chromatography; AA, arachidonic acid; LxA, 5(*S*),6(*R*),15(*S*)-trihydroxy-7,9,13-*trans*,11-*cis*-eicosatetraenoic acid, lipoxin A; 12-HETE, 12(*S*)-hydroxy-5,8,10,14-eicosatetraenoic acid; 5-HETE, 5(*S*)-hydroxy-6,8,11,14-eicosatetraenoic acid; TxB_2, thromboxane B_2; PGE_2, prostaglandin E_2; LTC_4, leukotriene C_4; NMDA, *N*-methyl-D-aspartate; PLA_2, phospholipase A_2; LTP, long-term potentiation

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to investigate their potential roles in neuronal transmission in the cerebellum. As an initial step, we have purified and resolved the individual subspecies from bovine cerebellum in order to characterize their enzymological properties, and report here their sensitivity to activation by AA and its metabolites.

2. MATERIALS AND METHODS

2.1. *Materials and chemicals*

PtdSer, DO, AA and oleic acid were purchased from Serdary Research Laboratories. AA was also obtained from Nu-Check-Prep. Other fatty acids were generous gifts from Drs S. Satoh and Y. Nakano (Nippon Oil & Fats Company). AA metabolites were a generous gift from Dr S. Yamamoto (Tokushima University, Japan), and in addition, a batch of LxA was most kindly supplied by Dr J. Rokach (Merck Frosst Canada Inc.). [γ-^{32}P]ATP was purchased from Amersham.

2.2. *Preparation of PKC subspecies from bovine cerebellum*

PKC subspecies were purified from freshly obtained bovine cerebellum soluble and particulate fractions, by DEAE-cellulose, threonine-Sepharose, TSK gel phenyl-5PW and G3000SW column chromatographies, essentially as described for rat whole brain [12]. All procedures were carried out at 4°C. The buffer system used was 20 mM Tris-HCl (pH 7.5) containing 0.5 mM EGTA, 0.5 mM EDTA and 10 mM 2-mercaptoethanol (buffer A), to which the appropriate concentrations of NaCl were added. The purified mixture of PKC subspecies was then resolved by loading the sample onto a packed hydroxyapatite column (1.07 cm × 10 cm, Type S, Koken Ltd, Tokyo), coupled to an FPLC system (Pharmacia), that was equilibrated with 20 mM potassium phosphate buffer (pH 7.5), containing 0.5 mM EGTA, 0.5 mM EDTA, 10 mM 2-mercaptoethanol and 10% (v/v) glycerol (buffer B). The type I(γ), type II(βI/βII) and type III(α) enzyme subspecies were then separately eluted by the application of a linear concentration gradient of potassium phosphate (20 mM to 215 mM) in 135 ml of buffer B, at a flow rate of 0.75 ml/min. Fractions comprising the three separate peaks of enzyme activity were pooled, dialyzed against buffer A containing 10% (v/v) glycerol, and stored at −80°C until use.

2.3. *Assay of PKC activity*

Enzyme activity was assayed at 30°C by measuring the incorporation of $^{32}P_i$ into H1 histone from [γ-^{32}P]ATP, as previously described [7]. To the standard reaction mixture were added 8 μg/ml PtdSer and 0.8 μg/ml DO or test compounds, calcium chloride or EGTA (as indicated) and 20 μl enzyme fraction (final assay volume 0.25 ml). PtdSer/DO, fatty acids or AA metabolites, were prepared by firstly, drying down aliquots of the stock solutions (stored either at −20°C or −80°C) under N_2, and then resuspension in 20 mM Tris-HCl (pH 7.5) by vortexing and sonication on ice (3 × 1 min). For LxA, 20 mM Tris-HCl containing 5% (v/v) methanol was used. This concentration of solvent had no effect on PKC activity. Each concentration of compound was prepared separately (as opposed to dilution after sonication), and added immediately prior to the start of the assay.

3. RESULTS

3.1. *Properties of PKC subspecies*

PKC activity from bovine cerebellum can be resolved into three distinct fractions by hydroxyapatite column chromatography (fig.1A). The relative elution position of these subspecies, their mode of activation, biochemical properties, and immunoblotting analysis with subspecies-specific monoclonal and polyclonal antibodies, have collectively established the identity of the three peaks as corresponding to type I(γ), type II(βI/βII) and type III(α) PKC subspecies, respectively. As previously described for rat and monkey tissue [7,8], cerebellum is a rich source for the γ-type en-

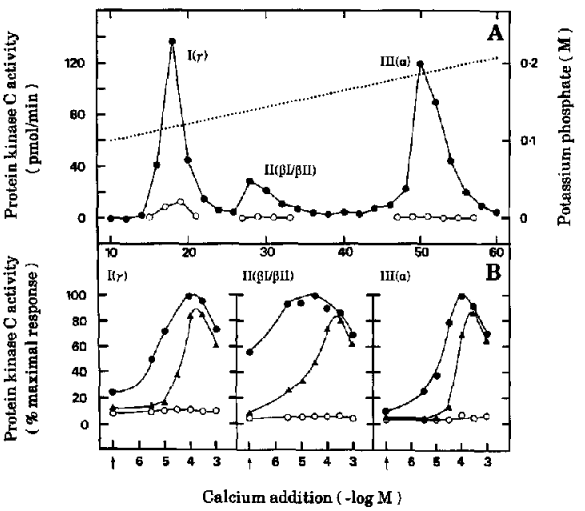

Fig.1. Resolution of PKC subspecies purified from bovine cerebellum, and their calcium, phospholipid and diacylglycerol sensitivity. (A) A mixture of PKC subspecies purified from bovine cerebellum was resolved into three distinct fractions upon hydroxyapatite column chromatography, as described in section 2. Based on a number of criteria (see text), the identity of each peak was established as shown. Protein kinase activity was assayed for each column fraction (20 μl sample) in the presence of 8 μg/ml PtdSer, 0.8 μg/ml DO and 0.3 mM calcium chloride (●) or in the presence of 0.5 mM EGTA (○), as described in section 2. (B) For each enzyme subspecies, protein kinase activity was assayed in the presence of 0.8 μg/ml DO (○), 8 μg/ml PtdSer (▲) or both lipids (●), at various concentrations of calcium chloride, as described in section 2. Where indicated with arrows, 0.5 mM EGTA was added instead of calcium chloride. Data are expressed as a percentage of the maximal activity obtained for each subspecies in the presence of 8 μg/ml PtdSer, 0.8 μg/ml DO and 0.3 mM calcium chloride (= 100%), and are the mean value of three or more separate determinations.

zyme. In bovine tissue the relative activity of the three peaks is a little different from that of the latter tissues, with an enrichment of both γ- and α-PKC, and a less prominent β-PKC ($\alpha/\beta/\gamma$, 42:17:41%, respectively; mean of four separate enzyme preparations). Immunoblotting analysis with polyclonal antibodies raised against C-terminal (V5 region) peptide sequences of the βI- and βII-subspecies [7,10], gave an estimate of their ratio in the type II fraction from bovine cerebellum as 23:77%, respectively, which is identical to that of the rat cerebellum enzymes [7].

In the presence of PtdSer, all three enzyme subspecies showed an increase in protein kinase activity that was dependent on the calcium concentration in the assay (fig.1B). In the presence of PtdSer and DO, a shift to higher affinity in the calcium dose-response curves of approximately one order of magnitude for γ- and α-subspecies, and two to three orders of magnitude for the β-subspecies was observed. In the presence of both cofactors, the β-subspecies showed considerable activity in the nominal absence of calcium. None of the enzymes were responsive to DO alone, at any calcium concentration. These properties are the same as those of the corresponding rat brain enzymes [12].

3.2. *Activation by free fatty acids*

Micromolar concentrations of AA, in the absence of PtdSer and DO, selectively activates the γ-subspecies of PKC (fig.2). In the presence of calcium (0.3 mM), activation of γ-PKC by AA (12.5 μM) exceeded 50% of the activity obtained in the presence of PtdSer, DO and calcium, whereas the β- and α-subspecies were activated to less than 10% of the maximal activity, at this concentration. These subspecies are known only to be responsive to high concentrations of AA (>100 μM; [12]). A significant component of the activation of γ-PKC by AA was independent of calcium, as similarly described for the γ-subspecies from rat hypothalamus [13]. Calcium-independent activation of PKC does not appear to be a general property of fatty acids, but is limited to AA and, possibly, other *cis*-unsaturated molecules, such as linoleic and oleic acid, which were found, however, to be relatively less active (fig.3). Saturated fatty acids, and the *trans*-isomer of oleic acid, elaidic acid, were practically inactive.

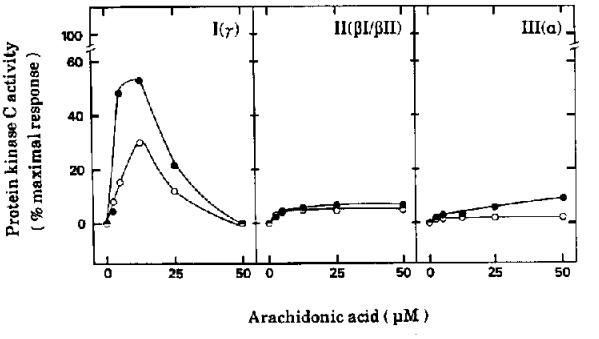

Fig.2. Arachidonic acid activation of PKC subspecies isolated from bovine cerebellum. Samples (20 μl) of type I(γ), type II(βI/βII) and type III(α) PKC subspecies were assayed following the addition of increasing concentrations of AA (0–50 μM) either in the presence of 0.3 mM calcium chloride (●), or with 0.5 mM EGTA (○), as described in section 2. Data are expressed as a percentage of the maximal activity obtained for each subspecies in the presence of 8 μg/ml PtdSer, 0.8 μg/ml DO and 0.3 mM calcium chloride (= 100%), and are the mean value of two or more separate determinations.

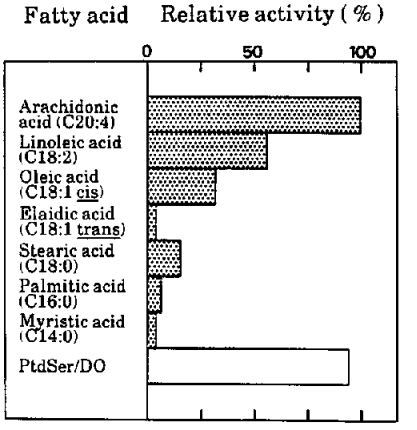

Fig.3. Calcium-independent activation of γ-PKC from bovine cerebellum by free fatty acids. Samples of γ-PKC (20 μl) were assayed with the fatty acids indicated, at a concentration of 12 μM, in the absence of PtdSer and DO, with 0.5 mM EGTA, as described in section 2. The response to 8 μg/ml PtdSer and 0.8 μg/ml DO is also shown. Data are expressed as percentage activity relative to that obtained with AA (= 100%), and are the mean value of two or more separate determinations.

3.3. *Activation by arachidonic acid metabolites*

Fig.4 shows the ability of some AA metabolites, produced either by the cyclooxygenase (PGE_2, TxB_2) or lipoxygenase (5-HETE, 12-HETE, LTC_4, LxA) pathways to support the activation of γ-type PKC in the absence of phospholipid,

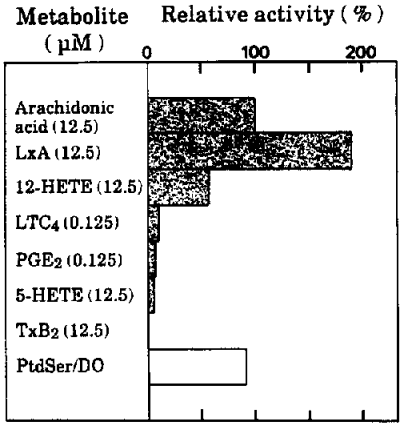

Fig.4. Calcium-independent activation of γ-PKC from bovine cerebellum by various arachidonic acid metabolites. Samples of γ-PKC (20 μl) were assayed with the AA metabolites indicated, in the absence of PtdSer and DO, with 0.5 mM EGTA, as described in section 2. Each compound was tested at 0.125, 1.25 and 12.5 μM, and the data for the most effective dose are given. The response to 8 μg/ml PtdSer and 0.8 μg/ml DO is also shown. Data are expressed as percentage activity relative to that obtained with AA (= 100%), and are the mean value of two or more separate determinations.

diacylglycerol and calcium. At equivalent concentrations, LxA (5(*S*),6(*R*),15(*S*)-11-*cis*-isomer) was found to be more effective than AA itself (189% activity relative to AA), and 12-HETE could also partially mimic AA (56% activity). LTC_4, PGE_2, 5-HETE and TxB_2 were essentially inactive at the range of concentrations tested (0.125–12.5 μM). The selectivity of activation by LxA and 12-HETE for the different PKC subspecies is shown in table 1. As reported for AA (fig.2), both LxA and 12-HETE (12.5 μM) showed poor activation of the α- and β-subspecies. Activation of the γ-subspecies by LxA was enhanced by the presence of calcium (0.3 mM), such that, at the higher concentration, LxA could activate the γ-PKC to approx. 90% of the maximal activity obtained in the presence of PtdSer, DO and calcium.

Table 1

Activation of bovine cerebellum PKC subspecies by arachidonic acid metabolites

		% maximal response					
		I(γ)		II(βI/βII)		III(α)	
Calcium addition:		−	+	−	+	−	+
AA	(2.5)	6	4	2	3	1	2
	(12.5)	18	45	4	7	1	4
LxA	(2.5)	15	63	4	20	0	1
	(12.5)	34	91	4	23	0	1
12-HETE	(1.25)	9	15	3	0	2	3
	(12.5)	10	23	1	0	2	4

Samples of each PKC subspecies (20 μl) were incubated with AA, LxA or 12-HETE, at the concentrations indicated, in the absence of PtdSer and DO, and in the presence of either 0.5 mM EGTA or 0.3 mM calcium chloride, as described in section 2. Data are expressed as a percentage of the maximal activity obtained in the presence of 8 μg/ml PtdSer, 0.8 μg/ml DO and 0.3 mM calcium chloride (= 100%), and are the mean value of two or more separate determinations

4. DISCUSSION

The present studies demonstrate that, at micromolar concentrations, *cis*-unsaturated fatty acids, particularly AA, activate directly, in the absence of phospholipid and diacylglycerol, only the γ-subspecies of PKC from bovine cerebellum. Under the same conditions, two lipoxygenase metabolites of AA, LxA and 12-HETE, were also found to promote the selective activation of γ-PKC. In each case, a large component of this activation occurs independently of calcium.

Previous reports have described that unsaturated fatty acids, or AA and some of its metabolites activate PKC in human neutrophils [14], human placenta cytosol [15] and rat brain [16]. These studies differ from the present report in that a mixture of PKC subspecies was employed, γ-PKC being absent in neutrophils and placenta, and in general relatively high concentrations of fatty acid (>100 μM) were necessary for significant activation. As reported here, and in previous studies from this laboratory [12,13], this would lead to activation of the α- and β-subspecies, but have little effect on the γ-subspecies of PKC, if it were present. Methyl esters of LxA [15] were found to be much less active than the parent free acids, implying the importance of the carboxylic acid group.

Although it has been established that PKC subspecies, particularly the γ-type, are responsive to low concentrations of AA and LxA in in vitro experiments, evidence for a direct activation of PKC by these molecules under physiological situations is lacking at present. 5- and 12-HETE, and 12-HPETE (hydroperoxy derivative) have been

reported to participate in pre-synaptic inhibition of *Aplysia* sensory neurones [17], and recently, agonist binding to post-synaptic NMDA receptors was shown to stimulate the release of AA, 11-HETE and 12-HETE, from striatal neurones, probably by activation of PLA_2 [18]. In an analogous manner to the 'transcellular' metabolism of eicosanoids that takes place during platelet-neutrophil interaction [19], these derivatives could be retroactive 'transsynaptic' messenger molecules, acting to modify pre-synaptic function. Certainly, their ability to pass easily through the cytosolic and membrane compartments of the cell makes them attractive candidates for such a role. Based on some recent reports, a role for AA and its metabolites in synaptic responses in the hippocampus can also be inferred. Induction of LTP in the presence of millimolar calcium, following tetanic stimulation of afferent neurones and NMDA receptor activation, has been shown to be greatly reduced by the lipoxygenase inhibitor nordihydroguaiaretic acid [20], and inhibition of the time course of the persistent LTP response in rat hippocampus, as a result of a blockade of the PLA_2 enzyme by mepacrine, has been reported to be reversed by application of the *cis*-unsaturated fatty acid oleate ([21], but see [20]). Although there is both biochemical and electrophysiological evidence implicating the involvement of PKC at some stage(s) of the generation of LTP, its exact role is still obscure, as many of the proposed mechanisms are contentious. The involvement of AA or its lipoxygenase metabolites as activators of γ-PKC is an intriguing possibility, but provides yet a further source of conjecture. Interestingly, hippocampal pyramidal cells have been shown to stain heavily with antibodies against the γ-subspecies of PKC, whereas pre-synaptic components appear not to express this subspecies [9].

From our studies of the differential expression of PKC subspecies in central nervous tissues [7], we found that γ-PKC was isolated predominantly in the cytosolic fraction. It could be speculated that the function of AA and/or its lipoxygenase metabolites, such as LxA, is either to activate the soluble form of the γ-type enzyme, independent of the hydrolysis of polyphosphoinositides (for example, during its release following activation of the phospholipase A_1-lysophospholipase pathway [22]), or to reinforce activation of the membrane-bound form of the enzyme by diacylglycerol, following its release by the calcium-dependent PLA_2, thereby increasing the effectiveness of the signal and the potential range of substrates. In this way, the nature of the activator may play a part in determining the substrate specificity of the enzyme. The existence of such pathways, and their relative contribution to activation of the brain γ-subspecies of PKC is an important aspect of future research.

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