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SHORT COMMUNICATIONS

Phospholipase C and phospholipase D are independently activated in rat hippocampal slices

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Abstract—In order to investigate a possible G-protein-mediated activation of phospholipase D (PLD) and its relationship to the activation of phosphoinositide-specific phospholipase C (PI-PLC), we measured the effects of aluminium fluoride and carbachol on choline release, the PLD-specific transphosphatidylation reaction (generation of phosphatidylpropanol) and the formation of inositol phosphates in rat hippocampal slices. Aluminium fluoride markedly enhanced the formation of choline and phosphatidylpropanol but failed to increase the formation of inositol phosphates. In contrast, the muscarinic agonist carbachol strongly stimulated PI-PLC but failed to activate PLD. We conclude that PLD in hippocampal slices is activated by a G-protein independently of phosphoinositide hydrolysis.

Signal transduction through the receptor-mediated hydrolysis of phospholipids plays an important role in the CNS. In particular, the hydrolysis of PtdIns* by a specific phospholipase C (PI-PLC), leading to the generation of second messengers such as inositol phosphates and diacylglycerol, is well established [1]. In recent years, the hydrolysis of PtdCho by PLD, leading to the generation of phosphatidic acid and free choline, has also been shown to be controlled by neurotransmitter receptors [2, 3]. Since phosphatidic acid is rapidly dephosphorylated to diacylglycerol, both pathways may contribute to the activation of protein kinase C in the hippocampus and, therefore, may be involved in the process of memory formation [4]. Several neurotransmitters such as glutamate [5, 6], noradrenaline [7, 8] and acetylcholine [9–12], have been described to initiate the hydrolysis of both PtdIns and PtdCho in the CNS. PLD activation may be secondary to a G-protein coupled PI-PLC activation [13] because PtdIns hydrolysis leads to protein kinase C activation which, in turn, is expected to stimulate PLD [2, 3]. Stimulation of PLD by phorbol esters has been demonstrated in hippocampal slices [8, 14]. Alternatively, PI-PLC and PLD may be activated by a common or different G-proteins. In the present work, we tested these hypotheses by measuring in parallel the influences of aluminium fluoride and carbachol, a muscarinic agonist, on PI-PLC and PLD activation in rat hippocampal slices. The results give further evidence for the involvement of a G-protein in PLD activation and demonstrate that in rat hippocampus, PLD activation is a separate signal transduction pathway which is activated independently of PtdIns hydrolysis.

Materials and Methods

Preparation of hippocampal slices. Adult (12 weeks) male Wistar rats (Savo, Kisslegg, Germany) were killed by decapitation, and the brains were rapidly removed and placed on ice. Slices (400 μm) were obtained from the dissected hippocampi and superfused with KHB at 35°. The solution which was gassed with 95% O_2 and 5% CO_2 had the following composition (mM): Na^+ 143.0; K^+ 7.1; Ca^{2+} 1.3; Mg^{2+} 1.2; Cl^- 125.3; HPO_4^{2-} 1.2; SO_4^{2-} 1.2; HCO_3^- 25.0; glucose 11.7.

Measurement of choline efflux. Hippocampal slices were incubated with 0.1 mM diisopropylfluorophosphate (DFP) for 30 min and then washed for 40 min (basal efflux). Subsequently, drugs were added, and choline efflux was continuously measured for 90 min. Choline was determined by a chemoluminiscence assay described elsewhere [15]. The assay was linear from 1 to 5 pmol of choline.

Determination of PLD activity. PLD was determined in the slices by making use of the transphosphatidylation reaction which is catalyzed specifically by PLD [16]. The phospholipids were labelled by incubation of the tissue with 40 μCi [^{3}H]glycerol (NEN, Dreieich, Germany) in 5 mL KHB for 2 hr which was followed by superfusion with KHB. Part of the label (52%) in the lipid phase could be recovered in the major phospholipids. The distribution of label in the phospholipids was as follows: phosphatidylcholine, 46%; phosphatidylethanolamine, 25%; phosphatidylinositol, 23%; phosphatidylserine, 4%; phosphatidylglycerol and other phospholipids, <1%. After a 30 min wash, drugs were added to the superfusion medium in the presence of propanol (2%) for 60 min. For aluminium fluoride formation, 10 mM sodium fluoride and 10 μM aluminium chloride were added to the superfusion medium. At the end of the superfusion period, the slices were homogenized in 20 vol. of chloroform/methanol (2:1), and two phases were obtained by adding 4 vol. of 0.1 M KCl solution. Aliquots of the lipid phase were dried and radioactivity determined by liquid scintillation counting. The major phospholipids were separated by 2-dimensional TLC with chloroform/methanol/25% ammonia (13:7:1) and chloroform/methanol/acetone/acetic acid/water (20:4:8:4:1) as solvent systems. An additional TLC run was used for separation of [^{3}H]PtdPro (solvent: upper phase of ethylacetate/isooctane/acetic acid/water, 13:2:3:10). PtdPro standards were synthesized enzymatically as described [17]. The spots were visualized with iodine vapor and identified using standards of the major phospholipids. The spots were scraped off and radioactivity was counted. The formation of [^{3}H]PtdPro and of [^{3}H]phosphatidic acid was expressed as per cent of radioactivity present in the lipid phase.

Measurement of inositol phosphate formation. Inositol phosphates were determined using a radiometric procedure [7]. Briefly, the tissue was incubated with 40 μCi [^{3}H]myo-

* Abbreviations: PLD, phospholipase D; PI-PLC, phosphoinositide-specific phospholipase C; PtdCho, phosphatidylcholine; PtdIns, phosphoinositides; PtdPro, phosphatidylpropanol; KHB, Krebs–Henseleit buffer.

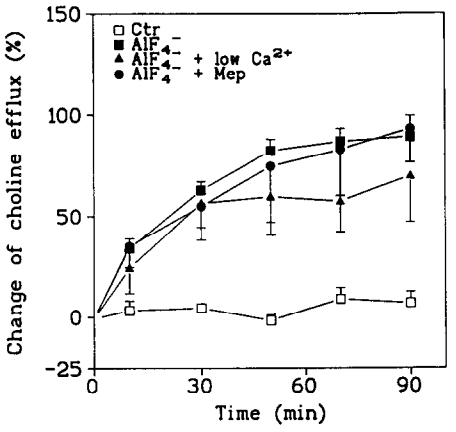

Fig. 1. Choline efflux from hippocampal slices. Hippocampal slices were placed in a superfusion chamber, and choline efflux was determined using a chemiluminescence assay (N = 4–6). At zero time, 10 μM aluminium chloride and 10 mM sodium fluoride were added. Experiments labelled "Mep" were done in the presence of 0.1 mM mepacrine, and in experiments labelled "Low Ca^{2+}" the calcium concentration was reduced to 0.23 mM.

inositol in 5 mL KHB for 2 hr. After a 10 min wash, the slices were superfused with KHB containing 10 mM lithium chloride for 20 min. Drugs were added to the superfusion medium, and the slices were superfused for a further 60 min in the presence of lithium. After homogenization, the aqueous phase was transferred to a column containing Bio Rad AG 1-X8 anion exchange resin, and the individual inositol phosphates were eluted by the stepwise addition of solutions containing increased concentrations of formiate and counted for radioactivity in a liquid scintillation counter.

Protein content was determined by the Lowry procedure. Data are presented as means ± SEM of N experiments. Statistical significance was evaluated by Student's *t*-test.

Results

Effects of aluminium fluoride and carbachol on choline release. Aluminium fluoride is an activator of trimeric G-proteins presumably acting by mimicking the structure of the third phosphate group of GTP [18]. In a first experiment, we tested the influence of aluminium fluoride (10 μM aluminium chloride plus 10 mM sodium fluoride) on the formation of choline from hippocampal slices. The results are shown in Fig. 1. Aluminium fluoride increased the effect of choline (basal efflux: 72.6 ± 6.4 pmol/min/mg protein) by about 90% compared to control incubations. After 60 min, the tissue contents of choline (basal level: 1.59 ± 0.36 nmol/mg protein, N = 6) and phosphocholine (basal level: 1.22 ± 0.13 nmol/mg protein, N = 6) were also increased by 74 and 86%, respectively. The increase in choline efflux was not reduced by the addition of 100 μM mepacrine or by the reduction of calcium (to 0.23 mM) in the superfusion fluid indicating that activation of phospholipase A_2 was not responsible for the effect of aluminium fluoride. In parallel experiments, carbachol (1 mM) did not increase choline efflux or tissue content (not illustrated).

Effects of aluminium fluoride and carbachol on PLD activity. PLD activity was determined by following the formation of PtdPro in the presence of propanol, a reaction which is specific for PLD [16]. Under basal conditions, rat hippocampal slices displayed a considerable PLD activity as indicated by the formation of a relatively high amount of PtdPro (0.64 ± 0.1% of lipid label after 60 min; N = 6).

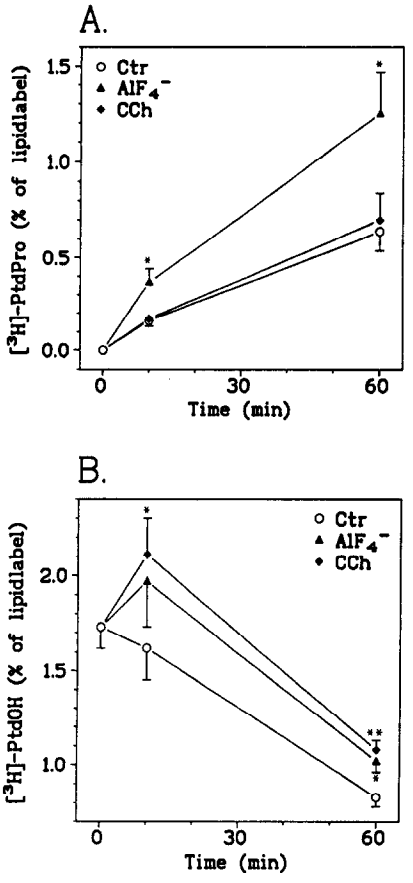

Fig. 2. Activation of PLD determined by the formation of phosphatidylpropanol (A) and phosphatidic acid (B). Hippocampal slices were prelabelled with [^{3}H]glycerol and superfused with KHB containing aluminium fluoride (AlF^{4-}; 10 μM aluminium, 10 mM fluoride) or carbachol (CCh; 1 mM) in the presence of 2% propanol. At the time points indicated, the tissue was extracted, and the radioactivity associated with PtdPro and phosphatidic acid (PtdOH) was determined after separation of the phospholipids by 2-dimensional TLC (N = 4–6). Statistical significance: *, $P < 0.05$; **, $P < 0.01$ vs control.

Concomitant with the formation of PtdPro, the label associated with phosphatidic acid was reduced by half indicating that PtdPro formation occurred at the expense of phosphatidic acid. Aluminium fluoride strongly increased PtdPro by about 2-fold (Fig. 2A) and also enhanced the formation of ^{3}H-labelled phosphatidic acid (Fig. 2B). The muscarinic agonist carbachol (1 mM) did not change the formation of PtdPro within 60 min. However, carbachol significantly increased [^{3}H]phosphatidic acid (Fig. 2B). A lower concentration of carbachol (0.1 mM) or the muscarinic antagonist, atropine (1 μM), did not affect the formation of PtdPro (data not shown). More importantly, the presence of 1 mM carbachol did not affect the PtdPro formation elicited by aluminium fluoride (data not shown).

Effects of aluminium fluoride and carbachol on PI-PLC activity. The activation of PI-PLC was determined by measuring the formation of [^{3}H]inositol phosphates (Fig. 3). Carbachol strongly increased the formation of inositol monophosphates within 10 min (by 5-fold) and of total inositol phosphates (by 4-fold) in hippocampal slices. Very similar increases were obtained if the incubations were terminated after 60 min (data not shown). In contrast, in

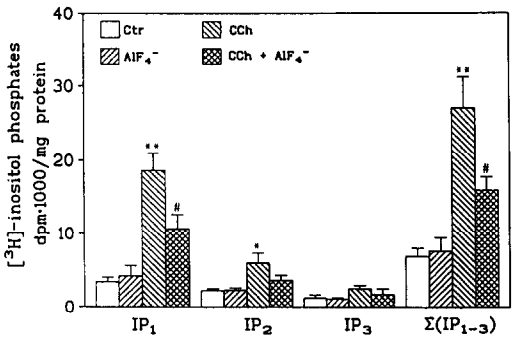

Fig. 3. Activation of PLC determined by the formation of inositol phosphates. Hippocampal slices were prelabelled with [^{3}H]inositol and superfused with KHB containing aluminium fluoride (AlF^{4-}; 10 μM aluminium, 10 mM fluoride) or carbachol (CCh; 1 mM) or both (CCh + AlF^{4-}). After 10 min, the tissue was extracted, and the radioactivity associated with inositol monophosphates (IP_1), inositol diphosphates (IP_2) and inositol triphosphates (IP_3) was determined after separation of the inositol phosphates by anion exchange chromatography (N = 4). Last column: sum of IP_1, IP_2 and IP_3. Statistical significance: *, $P < 0.05$; **, $P < 0.01$ vs control. #, $P < 0.05$ vs carbachol alone.

parallel experiments, aluminium fluoride did not affect basal inositol phosphate formation. However, in the presence of aluminium fluoride, the carbachol-induced increase of inositol phosphates was strongly attenuated (Fig. 3). Aluminium fluoride significantly reduced the accumulation of inositol monophosphate and of total inositol phosphates by 42 and 46%, respectively.

Discussion

Signal transduction pathways which involve the hydrolysis of phospholipids by phospholipases such as phospholipase A_2, PI-PLC and PLD display close interrelationships ("cross-talk") in many tissues and are often activated in parallel [19]. In the brain, both PLD and PI-PLC are activated by norepinephrine (via α 1-receptors) and by glutamate (via metabotropic receptors) suggesting the possibility that the two responses are mediated by a common G-protein. In the present work, we have investigated the effects of the G-protein activator, aluminium fluoride, in the hippocampal slice preparation. Aluminium fluoride led to a strong increase of choline release and also to an increase of PtdPro formation reflecting PLD activation. The enhanced release of choline was resistant to mepacrine and to reduction of extracellular calcium which excludes an involvement of phospholipase A_2. These findings demonstrate that rat hippocampus contains a PLD activity which is coupled to and regulated by a (presently unknown) G-protein.

The idea that the amount of choline released by aluminium fluoride (Fig. 1) is indeed formed by the action of PLD is substantiated by the following calculation. The increases of choline efflux and choline and phosphocholine tissue content by aluminium fluoride can be calculated from the results as 4.65 nmol/hr per mg protein. This corresponds to a hydrolysis of 2.9% of the PtdCho present (158 nmol/mg protein, cf. Ref. 20) and, consequently, to a theoretical reduction of label in PtdCho from 23.9 to 23.2% (of lipid phase). The difference of 0.7% is in good agreement with the combined increases of label in PtdPro and phosphatidic acid which can be calculated from Fig. 2 as 0.80%. Thus, PLD appears to be the only or predominant phospholipase acting on choline-containing phospholipids which is activated by aluminium fluoride.

In contrast to PLD activation, the formation of inositol phosphates by PI-PLC was not increased by aluminium fluoride in hippocampal slices. Moreover, aluminium fluoride inhibited the carbachol-induced increase of inositol phosphates. The lack of fluoride activation does not agree with results obtained in cortical slices which were, however, incubated in the absence of calcium [21], and with experiments in rat cerebral-cortical membranes [22] which were performed in the absence of cytosolic factors. As aluminium fluoride activates all types of trimeric G-proteins, this latter discrepancy may be explained by an indirect mechanism of action which possibly involves additional pathways requiring cytosolic factors e.g. of the adenylate cyclase pathway. Alternatively, the existence of G-proteins which inhibit PtdIns *hydrolysis* has been suggested in recent studies [23, 24], and the inhibition of agonist-induced PtdIns hydrolysis by aluminium fluoride may be due to the parallel activation of G-proteins which inhibit PI-PLC activation. Moreover, an inhibition of PtdIns *synthesis* may also be involved in fluoride action [25]. Whatever the mechanism of action, the obvious lack of PI-PLC activation by aluminium fluoride in hippocampal slice could be used in the present study focussing on a comparison of PI-PLC and PLD activities.

The activation of muscarinic receptors elicits a very prominent PtdIns hydrolysis in the brain [1] which is confirmed in the present work. Carbachol has also been shown to activate PLD in canine synaptosomal membranes [11] and in astrocytoma [10] and neuroblastoma cells [12]. However, in recent work using brain slices a muscarinic activation of PLD could not be demonstrated [8]. In our hands, basal PLD activity in the hippocampal slice, as reflected by the formation of PtdPro (Fig. 2A), was remarkably high but could not be inhibited by atropine and could not be stimulated by up to 1 mM carbachol. It remains to be determined whether the high basal PLD activity prevents the demonstration of a cholinergic effect. The increase of labelled phosphatidic acid (Fig. 2B) by carbachol can be explained by the observed activation of PI-PLC leading to a sequential formation of diacylglycerol and, through the action of diacylglycerol kinase, phosphatidic acid (cf. Ref. 2).

In conclusion, the present experiments are a clear-cut example of an independent activation of PLD and PI-PLC by different stimuli in the same experimental system. In parallel experiments, carbachol was able to activate PI-PLC but not PLD, while aluminium fluoride activated PLD but not PI-PLC. The effects of aluminium fluoride are strong evidence for a G-protein regulation of PLD. Thus, it appears plausible from the present experiments that PLD in hippocampus can be activated independently of PI-PLC, both phospholipases being regulated by separate G-proteins. Independent activation of PLD may offer a mechanism to increase diacylglycerol levels and to activate protein kinase C in the absence of calcium mobilization.

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