Volume 257, number 1, 110–112 FEB 07727 October 1989

Prolonged ischemia differently affects phospholipase C acting against phosphatidylinositol and phosphatidylinositol 4,5-bisphosphate in brain subsynaptosomal fraction

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Received 31 August 1989

The effect of 10 min ischemia on the activity of phospholipase C acting against [^{3}H]inositol-phosphatidylinositol (PI) and [^{3}H]inositol-phosphatidylinositol 4,5-bisphosphate (PIP_2) in the brain subsynaptosomal fractions was investigated. In the presence of endogenous $CaCl_2$, specific activity of phospholipase C acting on phosphatidylinositol was as follows: synaptic cytosol (SC) > synaptic vesicles (SV) > synaptic plasma membrane (SPM). Brain ischemia activated phospholipase C acting on PI by about 60% and 40% in SV and SPM, respectively. The enzyme of synaptic cytosol was not affected by ischemic insult. Phospholipase C acting against PIP_2 in the presence of endogenous calcium expressed the specific activity in the following order: SV > SPM > SC. After 10 min of brain ischemia, activity of phospholipase C acting on PIP_2 was significantly suppressed in all subsynaptosomal fractions by about 50–60%. These results indicate that prolonged ischemia produced activation exclusively of phospholipase C acting against phosphatidylinositol.

Ischemia; Subsynaptosomal fraction; Phospholipase C

1. INTRODUCTION

Intensive study on phosphoinositide metabolism clarified that some neurotransmitters and other agonists transduce signals through activation of phospholipase C action on phosphatidylinositol 4,5-bisphosphate (PIP_2). Hydrolysis generated two second messenger molecules: IP_3, which may liberate Ca^{2+} from extra mitochondrial stores and diacylglycerol (DG), activator of protein kinase C [1–3]. However, it was found that in some cells, such as mouse acinar cells, cholecystokinin and acetylcholine stimulated phosphodiesterase activity acting on phosphatidylinositol (PI) but not on PIP_2 or phosphatidylinositol-4-phosphate (PIP) [4]. In some other cells where agonist receptor interaction induced degradation of PIP_2, there was evidence that PI was also degraded by phospholipase C to maintain the appreciable DG level in the cells [5]. Recently, numerous studies demonstrated that phosphoinositide hydrolysis is a GTP-dependent reaction [6,7]. In our previous studies, we have observed that brain ischemia induced activation of phospholipase C acting on PI exclusively in synaptosomes [8]. Moreover, the level of radioactivity of polyphosphoinositides (PIP and PIP_2) and concomitantly polyphosphoinositols (IP_2 and IP_3) was significantly decreased in the brain after 10 min of ischemia [9]. The aim of these studies was to investigate the effect of 10 min ischemia on phospholipase(s) C acting against PI and PIP_2 in subsynaptosomal fractions. A preliminary report of the study has already appeared [10].

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2. MATERIALS AND METHODS

2.1. *Materials*

PI, PIP_2, NaDoCh were from Sigma GmbH. AG-1-X8 resin was purchased from Bio-Rad. HPTLC Silica Gel 60 plates were obtained from Merck and all other chemicals from Sigma. Inositol 2-[^{3}H]phosphatidylinositol 4,5-bisphosphate (spec. act. 4.0 Ci/mmol) and inositol 2-[^{3}H]phosphatidylinositol (spec. act. 6.5 Ci/mmol) were purchased from Radiochemical Du Pont.

Gerbils (*Meriones unquiculatus*) 50–70 g body weight were used for experiments. Animals were fed on a normal diet with water ad libitum.

2.2. *Experimental model of ischemia*

Ligation of both common carotid arteries was carried out in adult gerbils (*Meriones unquiculatus*) 60–70 g body weight with the aid of surgical threads under light diethyl ether anesthesia. Duration of ischemia was 10 min. The animals were decapitated and the brain hemispheres were quickly removed and placed into ice-cold isolation medium. The time for brain dissection was about 30 s.

2.3. *Isolation of subsynaptosomal fraction: synaptic plasma membrane, synaptic vesicles and synaptic cytosol*

The fraction enriched with synaptic endings was isolated by the method of Booth and Clark [11]. Dissected brain hemispheres were

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00145793/89/$3.50

homogenized in ice-cold isolation medium (0.32 M sucrose, 10 mM Tris-HCl, pH 7.4 and 0.1 mM EDTA). The homogenate 10%, w/v, was centrifuged for 3 min at 1100 × g. The resulting supernatant was centrifuged for 10 min at 17000 × g to yield a crude mitochondrial fraction (P_2).

This fraction was resuspended in 10 ml of 12% ficoll and placed on the bottom of a centrifuge tube, and then 5 ml of 7% ficoll and 5 ml of isolation medium were placed over and centrifuged for 30 min at 99000 × g. The material at the 7–12% ficoll interphase was collected, diluted 4 times with isolation medium, and centrifuged for 10 min at 17000 × g. The final synaptosomal pellet was lysed in 1 mM Tris-citrate buffer, pH 7.0 for 30 min, and then the membranes were sedimented by centrifugation at 8000 × g for 10 min and then the supernatant was collected and the pellet was lysed again. Combined supernatants were centrifuged at 48000 × g for 20 min. The final synaptosomal plasma membrane pellet was resuspended and used for the experiments. The supernatant was centrifuged at 100000 × g for 1 h to obtain the synaptic vesicles (pellet) and synaptic cytosol.

2.4. *Assays for phospholipase C activity, acting against [^{3}H]-phosphatidylinositol and [^{3}H]phosphatidylinositol 4,5-bis-phosphate (PIP_2)*

The enzyme activity was assayed by measuring the formation of radioactive inositol phosphate(s) from labelled substrate [12,13]. Exogenous substrate, 25 nmol PIP_2 (25000–28000 cpm) or 20 nmol PI (20000 cpm) was added per incubation vial, and organic solvent was evaporated under nitrogen. After addition of 10 mM Tris-HCl buffer, pH 6.6, for determination of phospholipase C acting on PIP_2 and 10 mM Tris-HCl buffer, pH 7.8 in the case of PI and 0.01% sodium deoxycholate, each tube was vigorously vortexed for 1 min. Moreover, the assay system contained 10 mM LiCl, 10 mM NaF, 0–2.5 mM $CaCl_2$ or 10 mM EGTA, and 20–30 μg protein in a final volume of 200 μl. Mixtures were incubated for 15 min at 37°C and the reaction was stopped with 1 ml chloroform/methanol/concentrated HCl 100:100:0.6 and 0.3 ml of 1 N HCl containing 5 mM EGTA. A 0.5 ml portion of the aqueous phase was mixed with 10 ml of Bray scintillation fluid for determination of radioactivity. The rest of the aqueous phase was used for separation of [^{3}H]inositol phosphates. Under the above conditions, the hydrolyzed products increased linearly with incubation time and the amount of protein.

2.5. *Separation of water soluble inositol metabolites*

The water-soluble inositol metabolites were applied to a small column (0.5 × 7.0 cm) containing 0.4 g Dowex AG 1 × 8 (200–400 mesh). Free inositol and inositol phosphates were eluted sequentially according to the procedure described by Berridge et al. [14]. Between 16 and 20 ml were collected for each fraction. Aliquots of each fraction (0.5 ml) were taken for radioactivity measurements. Total yield was between 80 and 90%.

3. RESULTS

Degradation of PI and PIP_2 was a time-, protein- and substrate-dependent reaction. Optimal conditions were chosen for the study: 15 min incubation time, 20–30 μg protein per incubation tube and 20–25 nmol of substrates in 200 μl of final volume.

Degradation of labelled PI by subsynaptosomal fractions was a calcium-dependent reaction. In the presence of 2 mM EGTA, this reaction did not occur. In the presence of endogenous calcium ions, specific activity calculated as nmol·mg protein^{-1}·min^{-1} was in the following order: synaptic cytosol, 9.5 > synaptic vesicles, 3.12 > synaptic plasma membrane, 2.54.

Contrary to PI, degradation of PIP_2 was a calcium-independent reaction. In the presence of 2 mM EGTA, there was quite a significant PIP_2 degradation (table 1).

Table 1

Degradation of phosphatidylinositol and phosphatidylinositol 4,5-bisphosphate by subsynaptosomal enzyme(s) in the presence of EGTA and endogenous calcium

Substrate	Addition	SPM	SV	SC
		(nmol·mg protein^{-1}·min^{-1})		
PI	EGTA (2 mM)	n.d.	n.d.	n.d.
	$CaCl_2$ (endogenous)	2.54 ± 0.39	3.12 ± 0.45	9.49 ± 1.59
PIP_2	EGTA (2 mM)	2.42 ± 0.39	3.86 ± 0.29	0.26 ± 0.31
	$CaCl_2$ (endogenous)	3.35 ± 0.64	4.33 ± 0.41	2.08 ± 0.18

The values are means ± SD from 3 experiments performed in triplicate. SPM, synaptic plasma membrane; SV, synaptic vesicles; SC, synaptic cytosol; n.d., not detectable

A higher specific activity (nmol·mg protein^{-1}·min^{-1}) was observed in synaptic vesicles (SV, 4.29) than in synaptic plasma membranes (SPM, 3.35) and in synaptic cytosol (SC, 2.08). In the presence of endogenous calcium ions, the enzyme was activated by about 50% and 20% in SPM and SV, respectively, and significantly about 9-fold in SC (table 1).

Subsynaptosomal fractions (SPM and SV) isolated from the brains submitted to 10 min ischemia, hydrolyzed PI about 40–60% more actively compared to the membrane fractions from normoxic brain. The cytosolic enzyme was not affected by ischemic insult (table 2).

The activity of phospholipase C acting against PIP_2 in brain subsynaptosomal fractions was in the order of SV > SPM > SC.

Hydrolysis of PIP_2 by the enzyme(s) in subsynaptosomal fractions isolated from the brain submitted to 10 min ischemia was significantly decreased to 40% of the control value in SV and 50% of control in SPM and SC (table 3).

4. DISCUSSION

Our previous studies on the effect of ischemia on phosphoinositides [8,9,15] indicated the higher suscep-

Table 2

Activity of phospholipase C acting against inositol 2-[^{3}H]phosphatidylinositol in subsynaptosomal fractions isolated from brain submitted to ischemia

Subsynaptosomal fraction	Control	Ischemia	% of control
	(nmol·mg protein^{-1}·min^{-1})		
Synaptic plasma membrane	2.54 ± 0.39	3.57 ± 0.63*	140.6
Synaptic vesicles	3.12 ± 0.45	5.02 ± 0.26*	160.9
Synaptic cytosol	9.49 ± 1.59	9.04 ± 0.21	

The results are means ± SD from 3–5 experiments performed in triplicate. * $P < 0.05$, Student's t-test

Table 3

Activity of phospholipase C acting against inositol 2-[^{3}H]phosphatidylinositol 4,5-bisphosphate in subsynaptosomal fractions isolated from brains submitted to ischemia

Subsynaptosomal fraction	Control	Ischemia	% of control
	(nmol·mg protein^{-1}·min^{-1})		
Synaptic plasma membrane	3.35 ± 0.64	1.76 ± 0.66*	52.5
Synaptic vesicles	4.29 ± 0.44	1.83 ± 0.40*	42.7
Synaptic cytosol	2.08 ± 0.18	1.03 ± 0.15*	49.5

The results are means ± SD from 3 experiments performed in triplicate. * $P < 0.05$, Student's t-test

tibility of synaptosomal phosphoinositides to ischemic insult. After 10 min of ischemic insult, there was a significantly lower level of poly-PI and concomitantly a lower level of IP_3 and IP_2, but the level of IP_1 was increased. The studies on phospholipase C activity acting against PI in brain synaptosomes demonstrated the activation of this enzyme by ischemic insult by about 60%.

These studies confirmed that phospholipase C acting against PI is stimulated by ischemic insult and this result demonstrated additionally that more significant activation was observed in SV and then in SPM, with no changes in SC.

It was previously described that PI-phospholipase C is present in both cytosolic and membrane fractions in brain and that the enzyme activity is dependent on high Ca^{2+} ion concentration. In in vitro assay of phospholipase C, the presence of sodium deoxycholate and calcium ions is necessary for the determination of phospholipase C activity [6,8,16,17].

It is known that a number of lipid compounds and proteins are involved in regulation of phospholipase C activity [18–20]. It seems that the elevation of calcium ion concentration occurring during ischemia may be an important factor responsible for the activation of phospholipase C acting on PI.

The massive release of neurotransmitters known to occur during ischemia and phosphorylation reactions mediated by cAMP or DG which may occur during the first second of ischemia may be involved in suppression of phospholipase C acting on PIP_2 in subsynaptosomal fractions isolated from the brain submitted to 10 min ischemia.

It was previously observed [21,22] that long-term activation of cell growth by diglyceride oleoyl-acetylglycerol (synthetic analog of diacylglycerol) produced inhibition of phospholipase C, probably by phosphorylation of enzyme molecules by DG-activated protein kinase C. Recent studies demonstrated that phospholipase C is a good substrate for protein kinase C [23].

It seems that also in the case when subsynaptosomal fractions are isolated from brain submitted to 10 min ischemia, phosphorylation of phospholipase C acting on PIP_2 may be involved in suppression of enzyme activity.

Acknowledgements: Thanks are due to Miss D. Kacprzak for her expert technical assistance and to Mrs M. Izak for typing the manuscript. This work was supported by the Polish Academy of Sciences, Grant 06-02.II.6.2.

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