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Alteration of phosphatidylinositol transfer protein during global brain ischemia–reperfusion in gerbils

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Abstract

Phosphatidylinositol transfer proteins (PI-TPs) are responsible for the transport of phosphatidylinositol and other phospholipids. Moreover, these proteins are involved in vesicle transport and in the function of cytoskeleton. Our previous data indicated that brain ischemia affected phosphoinositides metabolism and the level of lipid derived second messengers.

In this study, the effect of ischemia–reperfusion injury on the level of PI-TPs and of the role of NMDA receptor stimulation on the alteration of these proteins was investigated during reperfusion after 5 min of forebrain ischemia in gerbils. Some groups of animals were injected intraperitoneally with MK-801, an antagonist of NMDA receptor 30 min before ischemia. The levels of both PI-TP isoforms $\alpha + \beta$ and separately the α-isoform were determined in cytosol and membrane fraction from brain cortex and hippocampus using Western blot analysis.

In the cytosolic fractions, the concentration of both isoforms of PI-TP was 2 times higher when compared to the membrane fraction. In brain cortex, PI-TPα isoform consist about 32–44% but in hippocampus 72–82% of both isoforms (PI-TP$\alpha + \beta$) in cytosolic and membrane fraction respectively. Ischemia–reperfusion had no effect on PI-TPs in brain cortex. However, in hippocampus after 5 min ischemia and during whole reperfusion time up till 7 days the level of PI-TP$\alpha + \beta$ and PI-TPα was significantly higher by about 20–55%, respectively when compared to control. MK-801 eliminated ischemia–reperfusion evoked alteration of PI-TPs. To confirm the role of NMDA receptor in PI-TP alteration additional experiments were carried out on PC-12 cells in culture. The results indicated that activation of NMDA receptor enhances significantly the level of PI-TPα. The competitive antagonist of NMDA receptor inhibited this effect. These results indicated that activation of NMDA receptor is connected with PI-TPs alteration and plays an important role in modulation of PI-TPs during ischemia–reperfusion injury that may have important physiopathological consequence.

Keywords: Phosphatidylinositol; Transfer protein; Ischemia–reperfusion

1. Introduction

Phosphatidylinositol transfer protein (PI-TP) is a ubiquitous and abundant protein that can exchange PI and phosphatidylcholine (PC) between membranes in vitro (Wirtz, 1991, 1997; Geijtenbeek et al., 1994).

Recently, it was shown that the cellular functions of PI-TP are not only connected with phospholipid transfer per se but also with other important cell functions as vesicle traffic, dynamics of cytoskeleton protein (Thomas et al., 1993, Fensome et al., 1996, Hay and Martin, 1993). PI-TPs are also essential components of the polyphosphoinositides metabolic machinery and they are required for proper signalling of different agonists as for example epidermal growth factor (EGF) and f-MET-Leu-Phe (Thomas et al., 1993; Kauffmann-Zeh et al., 1995) as well as for exocytosis (Hay et al., 1995). The involvement of PI-TP in polyphosphoinositides synthesis may also explain its significance in intracellular vesicular traffic (Liscovitch and Cantley, 1995). It was suggested (by Kauffmann-Zeh et al., 1995) that PI-TP plays a role in delivery of PI to PI4-kinase, and that the proteins are required for the synthesis of PI 4,5-bisphosphate (PIP_2). Because of the high affinity of PI-TP for PIP and PIP_2, these lipids remain bound to PI-TPs that may also deliver these lipids for phospholipase C (PLC) (Thomas et al., 1993, Kauffmann-Zeh et al., 1995). Data of Snoek et al. (1999) indicated that PI-TPα is directly involved in activation of a PI-specific phospholipase A_2; in the liberation of arachidonic acid (AA) and the other products of PLA_2 i.e. lysoPI. These two compounds play also a very important role in ischemic reperfusion pathology (Bazan, 1970; Strosznajder et al., 1972; Noremberg and Strosznajder, 1986; Toborek et al., 1999).

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Recently, an evidence indicated that phosphoinositide synthesis is linked to a variety of membrane traffic events connected with the activity of PI 3′-kinase and with the formation of different class of lipids as PI(3) phosphate PI(3)P, PI(3,4)P_2 and PI(3,4,5)P_3. These lipids are not substrates for PLC but they are very active intracellular messenger molecules (Ptasznik et al., 1997; Toker et al., 1994).

Polyphosphoinositides containing phosphate in the D3 position of inositol, influence vesicular traffic by affecting the proteins that regulate vesicle formation and perhaps also docking and fusion. Hay et al. (1995) have suggested that PI-TP and PI4P5-kinase-dependent synthesis of PIP_2 promotes assembly of the actin of cytoskeleton. The cytoskeletal PIP_2-binding proteins may play a role in docking of secretary granules at the plasma membrane.

Transient global brain ischemia, results in the delayed degeneration of specific vulnerable neurons within the brain, with the most vulnerable regions being the CA_1 subfield of hippocampus (Kirino, 1982; Pulsinelli et al., 1982). Accumulating evidence indicates that the neuronal damage that follows either global or focal ischemia may involve an over stimulation of glutamate receptors, in particular of NMDA type (Choi, 1988; Benveniste et al., 1988; Osuga and Hakim, 1994; Obrenovitch and Urenjak, 1997; Obrenovitch, 1999). Activation of NMDA receptor during ischemia–reperfusion is associated with an increase of intracellular calcium ions and nitric oxide synthesis (NO) and with production of other free radicals and nitrosonium ions. All of these compound $NO^{\bullet}$, $O_2^{\bullet -}$ and $ONOO^-$ subsequently are involved in damage of mitochondria and DNA (Choi, 1988; Chalimoniuk and Strosznajder, 1998). Recently it was found that many proteins are functionally linked to the NMDA receptor; some of them are physically associated and some of them are indirectly interacting with scolfold proteins (postsynaptic density-95), (Sheng and Lee, 2000).

Our previous data indicated that brain ischemia affected phosphoinositide metabolism and the lipid derived second messengers (Strosznajder et al., 1972, 1987, 1999; Noremberg and Strosznajder, 1986). The aim of these studies was to characterize the distribution of PI transfer protein isoforms between cytosol and membrane fraction of hippocampus and brain cortex. Moreover, to determine the effect of ischemia–reperfusion on PI-TPα + β isoforms and PI-TPα in hippocampus and brain cortex and to determine the role of NMDA receptor stimulation in this protein alteration.

2. Materials and methods

2.1. Materials

Male Mongolian gerbils 50–60 g were supplied from Animal Farm, Lomna, Poland. PC-12 cells were obtained from Centre for Biomembranes and Lipid Enzymology, University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands. The complete inhibitors of proteases were obtained from Boehringer Manheim GmbH, Germany. Tris, EDTA, EGTA, DTT, HEPES, 2-mercaptoethanol, *p*-nitro blue tetrazolium chloride (NBT), 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (BCIP) and goat anti-rabbit IgG-alkaline phosphatase conjugate (GAR-AP) and all other reagents were obtained from Sigma. Anti-PI-TP antibodies, recognizing only the α-isoform (9093) and the α + β-isoforms (9026) were prepared according to Snoek et al. (1992), Geijtenbeek et al. (1994). An antibody that recognizes specifically PI-TPβ was not available.

2.2. Ischemia–reperfusion injury

Male Mongolian gerbils were anaesthetized with halotane in 70% N_2O and 30% O_2 (2% halotane for induction and 0.5% for the maintenance of anesthesia). The body temperature was kept at 37 °C by using a controlled heating pad and lamps during the time of ischemia and reperfusion. Brain ischemia was induced by ligation of both common carotid arteries using Heifetz clips for 5 min. The corresponding groups of animals were allowed to survive for 15 min, 2 h and 7 days after ischemia. One group of animals was injected intraperitoneally with a noncompetitive antagonist of NMDA receptor, a Ca^{2+} canal blocker MK-801 in a dose of 0.8 mg/kg body weight 30 min before ischemia. The sham-operated animals served as a control. Then, animals were quickly decapitated and the brains were removed and frozen in liquid nitrogen. The research project was accepted by the Ethic Committee.

2.3. Preparation of subcellular fraction

The brain cortex, hippocampus and PC-12 cells were homogenised in 10 mM Tris–HCl buffer pH 7.4 containing 0.25 M sucrose, 1 mM EDTA, protease inhibitors Boehringer (one tablet per 10 ml) in a Dounce homogenizer by 14 strokes. Then the homogenate was centrifuged at 10,000 × *g* for 10 min to obtain membrane fractions and supernatant, crude cytosolic fraction. The protein content of homogenate and each subcellular fraction were determined by the method of Bradford.

2.4. Treatment of PC-12 cells with NMDA

PC-12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum and 5% horse serum and 50 μl/ml penicillin/streptomycin. Cells were passaged every 5 days. PC-12 cells were plated on P-35 dish and before treatment the cells were washed with serum-free DMEM and subsequently incubated with fresh serum-free medium. Cells were exposed to agonist of glutamatergic receptor, *N*-methyl-D-aspartate (100 μM) and also to antagonist of NMDA receptor, APV5 (10 μM) and inhibitor of NO synthase *N*-nitro L-arginine (NNLA) (100 μM) for 15 min. For electrophoresis and Western blotting cells were washed 2 times with PBS buffer,

scraped into 10 mM Tris–HCl pH 7.4 containing 0.25 M sucrose, 1 mM EDTA and protease inhibitors Then cells were homogenised and the subcellular fractions was obtained as described above.

2.5. Gel electrophoresis and Western blotting

After precipitation with cold acetone the proteins were resuspended in 25 mM Tris–HCl buffer pH 6.8 and the suspension was mixed with an equal volume of sample buffer (62.5 mM Tris–HCl, 2% SDS, 100 mM DTT, 0.2 mM 2-mercaptoethanol, 20% glycerol and 0.2% bromophenol blue, pH 6.8). The samples were heated for 5 min at 95 °C. The proteins were analyzed by electrophoresis on 15% polyacrylamide gel (Laemmli, 1970). Then proteins were electrophoretically transferred from the SDS polyacrylamide gel at 1 mA/cm^2 for 75 min at room temperature to nitrocellulose membranes. The membrane was blocked in 2% milk powder (non fat dry-milk) in Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBS-T) for 1 h at 37 °C. Then, the blot was incubated with anti-PI-TPα + β antibody (raised against synthetic peptides) and anti-PI-TPα antibody (raised against recombinant PI-TPα, both diluted 1:100 in TBS-T containing 0.2% (w/v) non fat milk) overnight at room temperature. The PI-TP antibody complex was identified with goat-anti rabbit IgG conjugated with alkaline phosphatase (GAR-AP, 1:5000 diluted in TBS-T containing 0.2%(w/v) non fat milk) The GAR-AP was visualised with BCIP/NBT as colour development substrate for alkaline phosphatase. The optical densities of the PI-TP bands on the immunoblot were quantified using a BIO-RAD GS700 Densitometer equipped with an integrating program.

3. Results

3.1. The distribution of PI-TP isoforms in cytosol and membrane fraction of hippocampus and brain cortex

The levels of both PI-TP isoforms together α + β and the α-isoform separately were analysed using membrane fractions and cytosol from brain cortex and hippocampus (Table 1). It was observed that in the cytosolic fractions from brain cortex and hippocampus, both isoforms are present in the highest amount when compared to the membrane fraction. The results indicated that about 60–68% of PI-TPα + β is localised in cytosolic fraction in the investigated part of the brain. Cytosolic fraction from brain cortex contains 2 times more PI-TPβ when compared to the hippocampus. In brain cortex, the relative contribution of isoform α in the sum of both isoforms is 32 and 44% in cytosol and membrane respectively. In the hippocampus, PI-TPα contributes 82 and 72% of the total PI-TPα + β in the cytosol and in the membrane fraction respectively. These results show that in the hippocampus, the relative contribution of PI-TPα to the total amount of PI-TP is significantly higher when compared to the brain cortex (Table 1). However, in brain cortex, concentration of PI-TPα + β is significantly higher when compared to hippocampus (2.6–2.9 times higher in cytosol and membrane, respectively). These results indicate significant differences in distribution of PI-TPα and PI-TPβ isoform in brain cortex and hippocampus.

3.2. Effect of ischemia–reperfusion on PI-TP in cytosolic and plasma membrane fractions of hippocampus and brain cortex

Subsequently, our data showed the higher levels of PI-TPα + β (15–20%) in hippocampus after short time of ischemia–reperfusion (15 min 2 h and 7 days) comparing to control (Fig. 2) (sham-operated animals). These changes were similar in cytosolic and membrane fractions (Fig. 1). After ischemia and reperfusion, the level of PI-TPα was significantly higher in hippocampus, about 15–20% in cytosol and by about 35–55% in membrane fraction when compared to the control (sham-operated animals, Fig. 2). We have not observed changes in the level or distribution of-PI-TPα + β and PI-TPα in brain cortex after ischemia–reperfusion (data not shown).

3.3. Role of NMDA receptor in PI-TPα alteration during ischemia–reperfusion injury in hippocampus

In further experiments, the involvement of NMDA receptor stimulation in the alteration of PI-TP concentration and localisation during ischemia–reperfusion injury was investigated. For this reason, an antagonist of the NMDA receptor, MK-801 was injected intraperitonaly 30 min before ischemia in a dose of 0.8 mg/kg body weight. A significantly lower level of PI-TPα was observed after ischemia and during reperfusion in hippocampus of animals pretreated with

Table 1
Distribution of PI transfer proteins PI-TPα + β and PI-TPα between cytosol and membrane fractions from gerbil hippocampus, and brain cortex

	Isoforms of PI-TP	Cytosol ng/mg protein (% of total)	Membranes fractions ng/mg protein (% of total)
Hippocampus	α, β	582 ± 25	270 ± 19
	α	475 ± 34 (81.6)	194 ± 11 (71.9)
Brain cortex	α, β	1540 ± 30	785 ± 25
	α	490 ± 15 (31.8)	347 ± 21 (44.2)

15 μg of cytosolic and membrane fraction protein was analysed using the Western blot. The data were evaluated as described in Section 2. The value are mean ± S.D. from 3–4 experiments The values in parenthesis represent the percentage of PI-TPα in total PI-TPα + β.

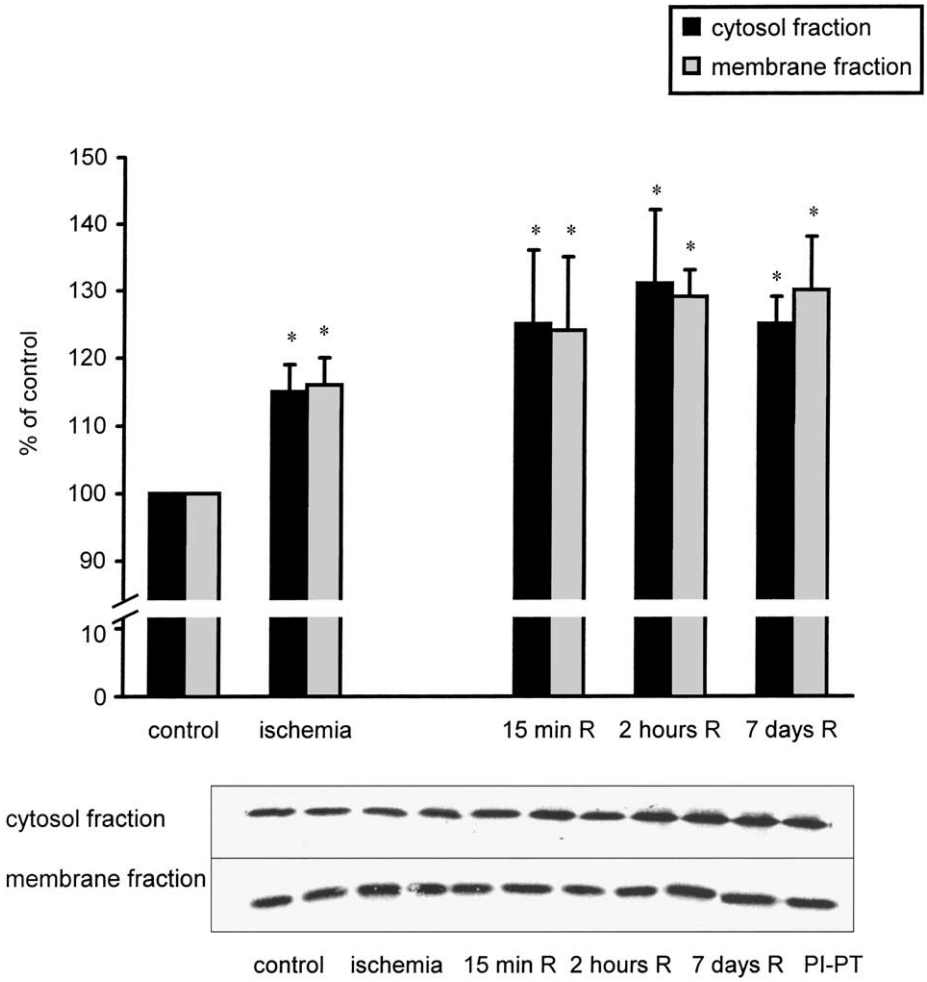

Fig. 1. Effect of ischemia–reperfusion on PI-TPα and β in the cytosolic and membrane fractions from hippocampus. 15 μg of cytosolic or membrane fraction protein was analysed as described in Section 2. The data are mean ± S.D. from 3–4 experiments. The statistical analysis was performed by one-way ANOVA, using Newman–Keules post-hoc test. ∗: Statistical probability of $P < 0.05$ was considered significant as compared with the control values (sham-operated animals).

MK-801. The results indicated that NMDA receptor antagonist MK-801 eliminated the alteration of PI-TPα evoked by ischemic-reperfusion (Fig. 3).

3.4. Effect of NMDA receptor stimulation on PI-TPα in PC-12 cells

To confirm the observation that the NMDA receptor is involved in the alteration of the level of PI-TP, several experiments were carried out using PC-12 cells in culture. PI-TPα protein level was determined after NMDA receptor stimulation. The time-dependent activation of the NMDA receptor in PC-12 cells enhances significantly the level of PI-TPα (Fig. 4). The NMDA receptor response was eliminated by APV, a competitive NMDA receptor antagonist (Fig. 5). *N*-nitro-L-arginine (NNLA), an inhibitor of NO synthase had no effect on the NMDA receptor-induced PI-TPα alteration. These results indicated that nitric oxide preferentially liberated during NMDA receptor activation is not involved in modification of PI-TPα protein.

4. Discussion

On the basis of our data, it is possible to suggest that brain ischemia significantly influences PI-TP protein probably by allosteric modification of the protein structure. It is known from our studies (Strosznajder et al., 1987), and also from the work of Yoshida et al. (1983) and Das (1994) that ischemia–reperfusion injury activates phospholipid breakdown and evoked liberation of AA and other lipid mediators (Bazan, 1970; Strosznajder et al., 1972, 1999). We have observed that in nerve endings (isolated as synaptosomal fraction) phosphoinositides are mainly degraded after ischemic insult (Noremberg and Strosznajder, 1986; Strosznajder et al., 1987). Degradation of phospho-

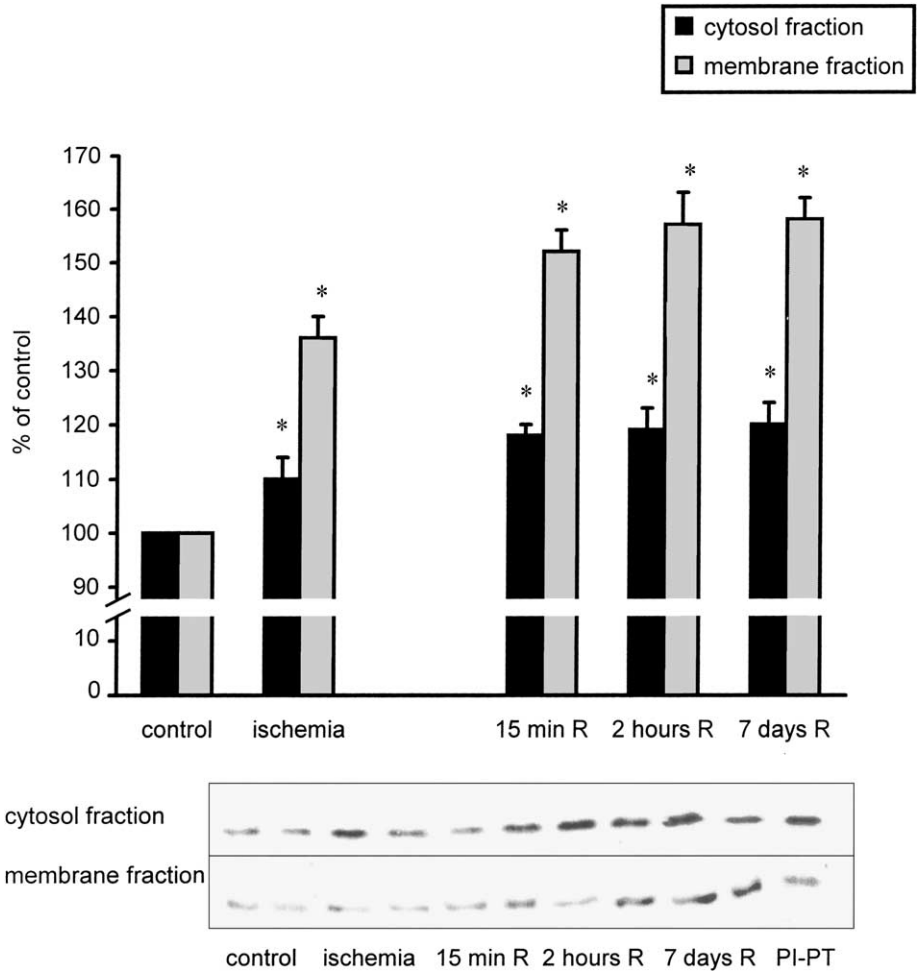

Fig. 2. Effect of ischemia–reperfusion on PI-TPα in the cytosolic and membrane fractions from hippocampus. 15 μg of cytosolic or membrane fraction protein was analysed as described in Section 2. The data are mean ± S.D. from 3–4 experiments. The statistical analysis was performed by one-way ANOVA, using Newman–Keules post-hoc test. ∗: Statistical probability of $P < 0.05$ was considered significant as compared with the control values (sham-operated animals).

inositides and phosphatidyicholine may have a lot of consequences for the cell function. The products of phosphoinositides degradation are diacylglycerol (DAG), AA, lysophospholipids and water-soluble inositol (1,4,5)trisphosphates (IP_3). All of these mediators are very potent second messengers (Berridge, 1987; Berridge and Irvine, 1989; Nishizuka, 1988). AA additionally is the retrograde messenger. However, if they are released in excessive amount they may be involved in neurodegeneration after ischemic insult. Alteration of PI-TP evoked by ischemia may affect phosphoinositides content and metabolism observed during reperfusion (Yoshida et al., 1983; Strosznajder et al., 1987; Strosznajder et al., 1999). The exact mechanism of ischemia–reperfusion injury remains till now unknown. Among three major hypothesis concerning, the mediators of reperfusion injury one is connected with phospholipid degradation (Das, 1994) and the other with excitotoxicity of glutamatergic receptor and disturbances of calcium homeostasis. These results indicate that ischemia–reperfusion injury affects the concentration of PI-TPs, proteins that are suggested to be involved in phospholipid biosynthesis, phospholipid transport and also in the dynamics of cytoskeleton and in function of Golgi system. Moreover, data of Snoek et al. (1999) presented a role of PI-TP in PI degradation, AA release and lysophospholipids formation. It is possible that PI-TPs are involved in modulation of the concentration of the lipid messengers and in activation of AA liberation by PLA_2 during ischemia–reperfusion.

It is widely known also from the previous studies that stimulation of the glutamatergic receptor, NMDA type, plays an important role in ischemic pathology (Choi, 1988; Obrenovitch and Urenjak, 1997; Chalimoniuk and Strosznajder, 1998; Obrenovitch, 1999). The last data of Snoek et al. (1999) indicated that overexpression of PI-transfer protein alpha in NIH3T3 cells activates a phospholipase A2 (PLA_2) acting on PI. Our previous investigation indicated significant increase of AA during short time of ischemia and reperfusion. Analysis of lipid products

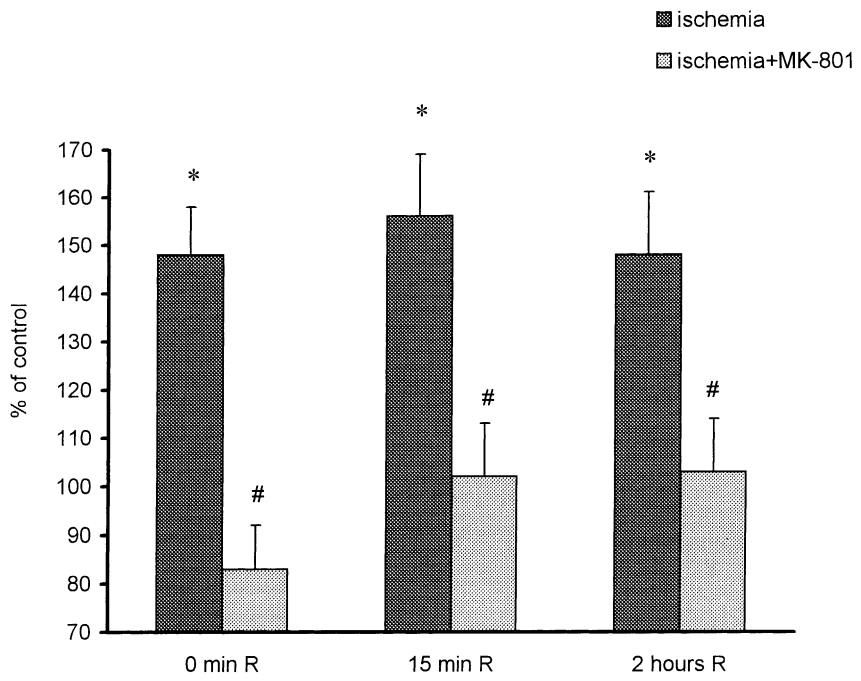

Fig. 3. NMDA receptor blocked prior ischemic insult by MK-801, eliminated ischemia–reperfusion evoked alteration of PI-PTα in hippocampus. 15 μg of cytosolic protein was analysed as described in Section 2. The data are mean ± S.D. from 3–4 experiments. The statistical analysis performed by one-way ANOVA, using Newman–Keules post-hoc test. ∗: Statistical probability of $P < 0.05$ was significant compared with the control values (sham-operated animals). #: Statistically significant as compared with ischemia values.

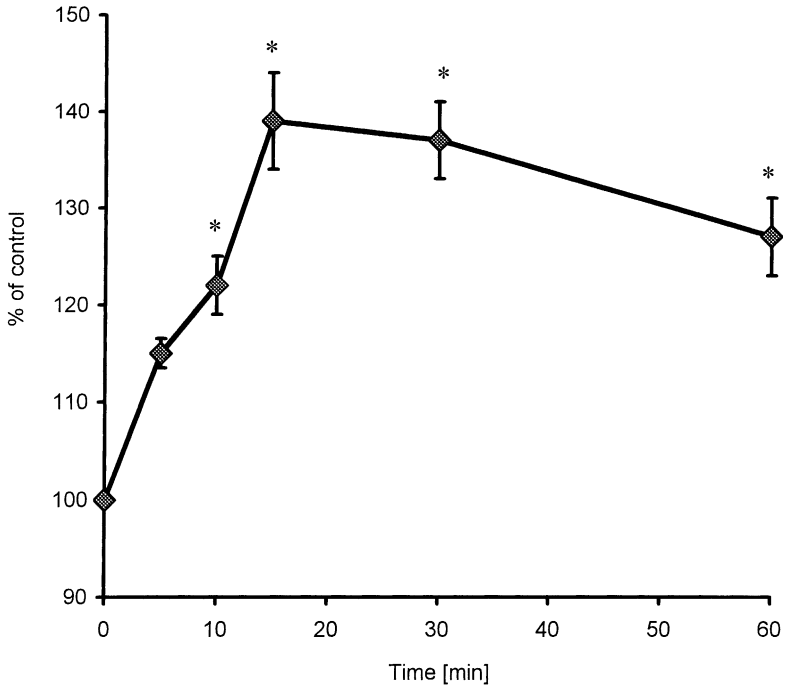

Fig. 4. Time-dependent effect of NMDA receptor stimulation on the level of PI-PTα in PC-12 cells. 15 μg of cytosolic protein was analysed as described in the Section 2. The data are mean ± S.D. from 3–4 experiments. The statistical analysis performed by one-way ANOVA, using Newman–Keules post-hoc test. ∗: Statistical probability of $P < 0.05$ was considered significant as compared with the control values.

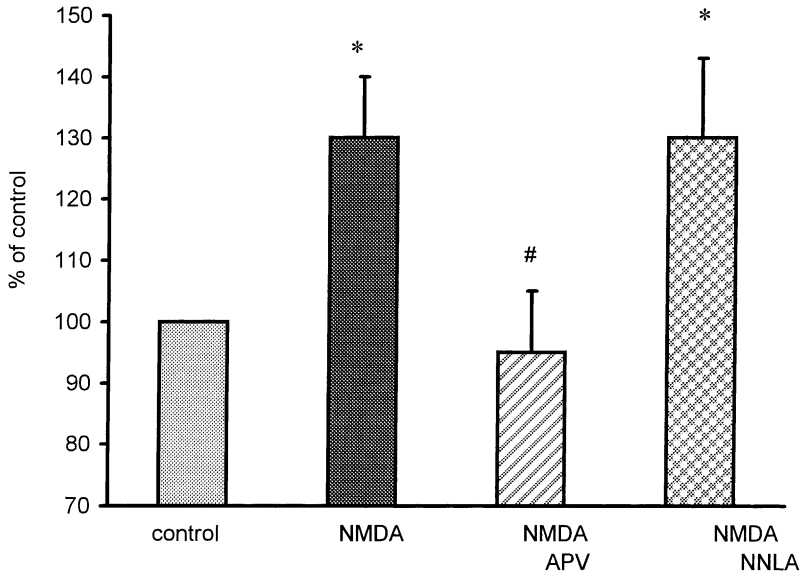

Fig. 5. Effect of NMDA receptor antagonist and inhibitor of nitric oxide synthase on NMDA receptor evoked PI-PTα alteration in PC-12 cells. The antagonist of NMDA receptor APV at 10 μM or NNLA at 100 μM was added 5 min before NMDA at 100 μM and cells were cultivated during 15 min. 15 μg of homogenate protein was analysed as described in the Section 2. The data are mean ± S.D. from 3–4 experiments. The statistical analysis performed by one-way ANOVA, using Newman–Keules post-hoc test. ∗: Statistical probability of $P < 0.05$ was considered significant as compared with the control values. #: Significant different as compared with the NMDA values.

accumulated during ischemia indicated that both phospholipase PLA_2 and also PLC are involved in phosphoinositides degradation. Snoek et al., 1999 have found that the addition of PI-TPα to a total lysate of myo-[(3)H]inositol labelled NIH3T3 cells stimulated the formation of lyso-PtdIns. Based on these observations, it was proposed that PI-TP is involved in the production of lyso-PtdIns by activation of phospholipase A2. The increased level of lyso-PtdIns is very dangerous for nerve cells and it may be responsible for the partial loss of cell contact inhibition. AA liberated in excessive amount may exert a lot of effects, it may be responsible for the uncoupling of oxidative phosphorylation in mitochondria and may be involved in the mechanism of neurodegeneration and in cell death (Strosznajder et al., 1972, Mattson, 1997, Toborek et al., 1999). A short time exposure to this fatty acid induces oxidative stress, increases of intracellular calcium levels, activation of PKC and nuclear factor kB (Wang et al., 1995; Okuda et al., 1994). Our results indicated that ischemia–reperfusion injury evoked alteration of PI-TPs only in hippocampus and that hippocampus is high enriched in PI-TPα isoform that is mainly affected by ischemia insult. Moreover our data presented evidences that activation of NMDA receptor is directly involved in PI-TPs protein alteration.

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