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Introduction

It is well known that the brain contains large amounts of the substrates and enzymes of inositol lipid turnover and its associated second messenger systems and that it is enriched with neurotransmitters and their receptors which are closely linked to stimulated inositol lipid turnover. These facts are said to indicate that the phosphoinositides indeed play an important role in brain function. The literature contains several general reviews on stimulated inositol lipid turnover and its function in the nervous system[1-6].

Postischemic delayed neuronal damage was reported in the ipsilateral thalamus and the substantia nigra which lay outside ischemic areas of rat brain after middle cerebral artery (MCA) occlusion [7,8]. In these reports, the histological appearance of these remote areas was characterized by selective degeneration of most neurons with no necrotic changes of neuroglia and blood vessels. The mechanism of such a delayed phenomenon in the exo-focal remote areas is unclear, but it has been speculated that it might be caused by a transsynaptic process neuroanatomically associated with ischemic foci and that intracellular and transsynaptic signal transduction systems might play important roles in this mechanism[7,9,10].

The inositol phospholipid system plays a key role in signal transduction by the cleavage of phosphatidylinositol biphosphate into D-myo-inositol 1, 4, 5-trisphosphate (IP_3) and 1, 2-diacyl-sn-glycerol (DG)[11]. These two products are known to have separate second messenger properties: DG stimulates protein kinase C, a Ca^{2+}-activated protein kinase[12], whereas IP_3 mobilizes Ca^{2+} from intracellular stores[13]. It is thought that autoradiographic analysis of [^{3}H]IP_3 can provide an excellent biochemical marker of neuronal damage and plasticity of the postischemic rat brain[14]. In the present study, we examined chronological changes of ^{45}Ca accumulation and IP_3 binding sites of the rat brain after 90 min of MCA occlusion and after such occlusion followed by different periods of recirculation in order to clarify the mechanisms of exo-focal postischemic neuronal damage.

Materials and Methods

Ischemia model

Adult male Wistar rats of the SPF strain weighing 280-300 g were allowed free access to food and water before and after all procedures. Six rats were used in each experiment. A detailed description of the surgical procedure has been previously reported[15]. In brief, after induction of anesthesia with a gas mixture of 70% N_2O and 2% halothane (the balance being O_2), the right middle cerebral artery (MCA) was occluded with a silicone rubber cylinder attached to a nylon surgical thread introduced from the bifurcation of the internal carotid artery immediately after ligation of the ipsilateral common and external carotid arteries. The cylinder was made of 4-0 nylon surgical thread (Nitcho Kogyo Co., Ltd., Tokyo, Japan), 16 mm long. This cylinder was coated with silicone (Xantopren, Bayer Dental, Leverkusen) mixed with a hardener (Elastomer Activator, Bayer Dental, Leverkusen) to increase the thickness of the distal 5 mm to 0.25-0.30 mm. After introducing the embolus, the internal carotid artery was ligated just distal to the point of insertion. The embolus extended from the bifurcation of the internal carotid artery to the proximal portion of the anterior cerebral artery (ACA). The origin of the right MCA and that of the right posterior communicating artery were occluded by the silicone rubber cylinder. In 6 sham-operated control rats, the right internal and external carotid arteries were ligated. Surgery was performed within 15 min with no bleeding. Body temperature was kept at normal limits with a heating pad. Following surgery, anesthesia was discontinued and all rats exhibited neurologic deficits characterized by left hemiparesis with upper extremity dominancy and right Horner's syndrome. After 90 min of MCA occlusion, the 6 rats were decapitated with no recirculation and in other rats, recirculation was achieved by pulling the thread out of the internal carotid artery under the same anesthetic conditions as during surgery. Once again, the rats were allowed free access to food and water. Although the ipsilateral common and external carotid arteries had been ligated, the ischemic area could be reperfused via the cerebral arterial circle (circle of Willis) through the contralateral carotid and basilar arteries, and by collateral circulation of the cortical branches of the cerebral arteries.

^{45}Ca autoradiography

Immediately after recirculation and then after 1 day, 3 days, and 2 weeks of recirculation following 90 min of MCA occlusion, 300 μCi $^{45}CaCl_2$ (Amersham) in aqueous solution (0.3 ml of 0.9% NaCl) was administered intravenously followed immediately by a 0.2-ml saline flush according to the method of Dienel[16]. Six hours after ^{45}Ca injection, the rats were decapitated. The brains were quickly removed and frozen in powdered dry ice. Serial coronal sections 20 μm in thickness were cut from the frozen brain in a -20°C cryostat and dried at 60°C on glass cover slips. Autoradiograms were prepared from these sections by exposing them to X-ray film (Kodak NMC-1) for 4 weeks in standard X-ray cassettes.

In vitro [^{3}H]IP_3 *autoradiography*

The localization and the chronological changes of IP_3 binding sites were measured using radiolabeled [^{3}H]IP_3 (New England Nuclear, spec. act. 17 Ci/mmol) by a slight modification of the method of Worley et al.[17] The rats were killed by decapitation after 90 min of ischemia and after such ischemia followed by 3 h, 6 h, 1 day, 3 days, 1 week, 2 weeks, and 4 weeks of recirculation. After decapitation,the brains were quickly removed and frozen in powdered dry ice and stored at -80°C until assay. Serial coronal sections 12 μm in thickness were cut on a cryostat and thaw-mounted onto gelatin-coated slides. Brain sections were incubated for 10 min at 4°C in a buffer (20 mM Tris-HCl, pH 7.7; 20 mM NaCl; 100 mM KCl; 1 mM EDTA; 1 mg/ml of bovine serum albumin) containing 9.8 nM [^{3}H]IP_3. Following incubation, sections were washed twice for 2 min each time at 4°C in the same buffer and dried under a cold stream of air. Non-specific binding was calculated in the presence of unlabeled 10 μM IP_3 (Sigma) Autoradiograms were prepared from the sections by exposing them to [^{3}H]sensitive hyperfilm (Amersham) with a tritium standard microscale (Amersham) for 4 weeks in standard X-ray cassettes.

Statistical analysis

Cerebral [^{3}H] tissue concentrations of the autoradiograms were determined by means of a computerized microdensitometric system. Data regarding the IP_3 binding sites in each structure of the brain were analyzed using a t-test with $p < 0.05$ and $p < 0.01$ considered to be statistically significant.

Results

Chronological changes of ^{45}Ca *accumulation*

In the sham-operated control group, no ^{45}Ca accumulation was detected during the 2-week observation period. The chronological changes of ^{45}Ca autoradiograms after 90 min of MCA occlusion are shown in Fig. 1. In the groups subjected to 90 min of ischemia, ^{45}Ca accumulation was observed in the frontoparietal cortex (FrPaSS) and the lateral segment of the caudate putamen (CPu-L), both supplied by the occluded MCA after 6 hours of recirculation, and its accumulation in both ischemic foci became more prominent in proportion to the duration after reperfusion. Moreover, abnormal ^{45}Ca accumulation was also detected in remote areas which were not initially affected by ischemic insult. After 3 days of recirculation, ^{45}Ca accumulation was first detected in the thalamus and the substantia nigra, but only on the ischemic side.

[^{3}H]*Inositol* 1,4,5-*trisphosphate binding*

The values of control and chronological alteration of [^{3}H]IP_3 binding sites in each structure of rat brain after 90 min of ischemia and after such ischemia followed by different periods of recirculation are shown in Table 1. There was no alteration of [^{3}H]IP_3 binding in

each structure of rat brain during 90 min of ischemia and during such ischemia followed by 6 h of recirculation. One day after recirculation, significant decreases of [^{3}H]IP_3 binding sites were observed in the ischemic foci, both the FrPaSS ($p < 0.05$) and the CPu-L ($p < 0.01$), compared with each control value, respectively. Thereafter, [^{3}H]IP_3 binding sites gradually decreased to approximately 60% in the FrPaSS and to 40% in the CPu-L of each control value until 3 days after reperfusion.

On the other hand, two different alterations of [^{3}H]IP_3 binding sites were observed in the exo-focal remote areas. In the ipsilateral substantia nigra, [^{3}H]IP_3 binding sites first decreased significantly compared with the control value in accordance with the findings of ^{45}Ca accumulation 3 days after the ischemia (Fig. 2). In the thalamus, however, there was no alteration until 1 week after the ischemia, and then [^{3}H]IP_3 binding sites increased significantly compared with the control value 2 weeks ($p < 0.05$) and 4 weeks ($p < 0.01$) after the ischemia (Fig. 2).

Discussion

In this ischemia model, the anterior neocortex (FrPaSS) and lateral segment of the caudate putamen (CPu-L), which were supplied by the occluded MCA, were the regions most frequently damaged as so called ischemic foci[15]. On the other hand, the ipsilateral thalamus and the substantia nigra were remote from these ischemic areas and both areas were not directly affected by the original ischemic insult[7].

In the present study three different alterations of the second messenger system in close relation to findings of ^{45}Ca accumulation were observed in each area of the postischemic rat brain. First, in the ischemic foci, the ipsilateral anterior neocortex (FrPaSS) and the lateral part of the caudate putamen (CPu-L), [^{3}H]IP_3 binding sites were significantly decreased compared with the control values 1 day after ischemic insult, while ^{45}Ca accumulation was first detected there 6 h after the ischemia. IP_3 binding sites are believed to be located on the endoplasmic reticulum in the nervous system[18-20]. The reduction of [^{3}H]IP_3 binding sites in the FrPaSS and CPu-L is explained by the direct damage to intracellular components including the endoplasmic reticulum by ischemia induced energy failure.

Second, in the ipsilateral substantia nigra which lay outside the ischemic areas, [^{3}H]IP_3 binding sites were not significantly changed compared with the control values 1 day after ischemic insult, and thereafter a significant reduction of these sites was first observed 3 days after the ischemia. This delayed change of second messenger system observed in the substantia nigra was quite similarly developed as abnormal calcium accumulation was detected. The alteration of [^{3}H]IP_3 binding sites in the substantia nigra was minimal because of their initial low binding activity[17]. Moreover, both phenomena, the reduction of [^{3}H]IP_3 binding sites and abnormal calcium accumulation, in the substantia nigra of ischemic side preceded the histologic findings of delayed neuronal damage[7]. Third, in the ipsilateral thalamus, there were no significant changes of [^{3}H]IP_3 binding sites until 1 week after the

ischemia, and [^{3}H]IP_3 binding sites increased significantly after 2 weeks ($p < 0.05$) and 4 weeks ($p < 0.01$) compared with the control values, while ^{45}Ca accumulation was observed in this area 3 days after the ischemia. How can the increase of [^{3}H]IP_3 binding sites in the ipsilateral thalamus at a chronic stage of ischemia be explained? In the next section, we discuss the mechanism of exo-focal delayed neuronal changes focusing on the results observed in the ipsilateral thalamus.

We have reported that a delayed reduction of forskolin binding sites was observed in the substantia nigra on the ischemic side using the same ischemic model, but that there were no significant changes of those binding sites in the ipsilateral thalamus after the ischemia[21]. These facts indicate that different mechanisms, including second messenger systems, are responsible for exo-focal delayed neuronal damage in the two individual areas on the ischemic side.

In our previous report using the same ischemia model, the damage to the thalamus was limited to the ventral posterior nucleus and the neuronal damage in this area might be explained as being due to retrograde degeneration resulting from thalamocortical fiber damage caused by the precedent ischemic insult in the postcentral gyrus of the cerebral cortex[7,21]. Moreover, it is known that ablation of the cerebral cortical fibers reduces glutamate uptake in the synaptic terminals of the cortico-striatal and cortico-thalamic fibers, and it is thus believed that these can act as glutaminergic fibers[23,24]. Therefore, it seems that the non-physiological release of a large quantity of neurotransmitters could occur at the axon terminals of the thalamus when neuronal cells in the cortex are damaged by an ischemic insult. Such a large amount of neurotransmitters could activate inositol lipid turnover and receptors of the postsynaptic membrane in the thalamus. Several neuropeptide receptors may be also coupled to inositol lipid turnover in the brain[2,25,26]. Moreover, receptors are found at both presynaptic and postsynaptic sites, but most of the limited investigations to date point to a postsynaptic localization for receptor-enhanced inositol lipid turnover[27-29]. The increase of [^{3}H]IP_3 binding sites in the thalamus at the chronic stage of ischemia may reflect the highly activated state of the signal transducing system at the postsynaptic sites. It can be hypothesized that the phenomenon in this area is due to abnormal function of some neurotransmitters in the transsynaptic process associated with the ischemic foci

As an another hypothesis, it can be speculated that glia may play a role in stimulated phospholipid turnover in the thalamus, because mild gliosis was observed in this area at the chronic stage of ischemia[7]. The possible involvement of glia in receptor-mediated phosphoinositide turnover has been examined in primary cultures of cortical astrocytes.[30] In these cultures, the addition of muscarinic cholinergic and α_1-adrenergic ligands stimulated the release of insitol phosphate. However, using primary cultures of both neurons and glia, Gonzales et al.[31] reported that the phosphoinositide response was primarily observed in neurons. Nevertheless, the demonstration of functionally coupled muscarinic recepors on

both neuroblastoma[32] and astrocytoma[33] cells indicates that glial elements may also play an important role in the stimulation of inositol lipid turnover in the brain.

Recently, it has been postulated that the phosphoinositide second messengers could play an important role in neuronal plasticity in close relationship with axonally transported proteins associated with neural growth and regeneration[34,35]. The highly activated response of IP_3 binding sites of the thalamus in the chronic stage of ischemia may be new evidence of neuronal plasticity related to neurotransmission. However, as shown by the present study, the mechanism of exo-focal postischemic delayed neuronal damage is still far from being understood and further detailed investigation is required in order to clarify the mechanism.

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Table 1 [^{3}H]inositol 1,4,5-trisphosphate binding in each structure of the rat brain after 90 min of MCA occlusion followed by different periods of recirculation

Structure	Control	90-min ischemia	3 hours	6 hours	1 day
Ischemic side					
FrPaM	124 ± 5.2	116 ± 17.0	121 ± 5.8	122 ± 14.1	121 ± 7.0
FrPaSS	110 ± 12.3	106 ± 8.7*	110 ± 10.0	111 ± 7.6	91 ± 4.7*
CPu(L)	158 ± 12.3	160 ± 17.6	155 ± 13.5	166 ± 15.2	99 ± 11.1**
CPu(M)	144 ± 7.6	136 ± 17.0	149 ± 10.0	151 ± 15.2	145 ± 11.7
Hippocampus	255 ± 27.0	232 ± 28.2	262 ± 24.7	270 ± 22.3	260 ± 21.1
Thalamus(VPN)	77 ± 3.5	75 ± 4.7	79 ± 4.1	81 ± 4.1	76 ± 5.2
Amygdala	95 ± 10.5	84 ± 4.1	82 ± 10.5	85 ± 8.8	84 ± 9.4
Substantia nigra	81 ± 13.5	72 ± 9.4	72 ± 5.2	76 ± 7.6	78 ± 3.5
Pons	62 ± 2.3	61 ± 2.9	62 ± 1.7	62 ± 1.1	62 ± 1.7
Non-ischemic side					
FrPaM	125 ± 17.0	48.68 ± 14.7	128 ± 4.7	127 ± 15.8	128 ± 13.5
FrPaSS	118 ± 11.1	47.10 ± 10.5	120 ± 8.2	116 ± 10.0	121 ± 5.8
CPu(L)	160 ± 8.8	231.05 ± 13.5	170 ± 8.2	176 ± 17.0	172 ± 13.5
CPu(M)	144 ± 10.5	184.47 ± 5.8	153 ± 9.4	151 ± 6.4	158 ± 11.7
Hippocampus	244 ± 36.4	30.36 ± 23.5	254 ± 21.7	238 ± 20.0	242 ± 27.0
Thalamus(VPN)	72 ± 4.7	33.68 ± 4.7	78 ± 4.1	85 ± 12.3	87 ± 2.9
Amygdala	90 ± 15.2	42.36 ± 14.1	84 ± 11.7	84 ± 9.4	83 ± 9.4
Substantia nigra	80 ± 11.7	79.25 ± 9.4	77 ± 4.1	81 ± 4.1	85 ± 10.5
Pons	62 ± 4.1	22.39 ± 1.7	62 ± 1.7	64 ± 1.7	63 ± 1.7

Values are given in mean ± S.D. fmol/mg tissue using six animals.
FrPaM = frontoparietal cortex, motor area, supplied by anterior cerebral artery;
FrPaSS = frontoparietal cortex, somatosensory area, supplied by middle cerebral artery;
CPu(L) = lateral segment of caudate putamen; CPu(M) = medial segment of caudate putamen.
*$p<0.01$; **$p<0.001$, significant difference from control values using a t-test.

Table 1 [^{3}H]inositol 1,4,5-trisphosphate binding in each sgtructure of the rat brain after 90 min of MCA occlusion followed by different periods of recirculation (continued)

Structure	3 days	1 week	2 weeks	4 weeks
Ischemic side				
FrPaM	120 ± 15.8	128 ± 11.7	129 ± 11.1	123 ± 17.6
FrPaSS	64 ± 2.3**	63 ± 1.7**	64 ± 1.1**	63 ± 4.1**
CPu(L)	64 ± 2.3**	64 ± 2.9**	64 ± 1.1**	67 ± 5.8**
CPu(M)	139 ± 8.8	135 ± 13.5	131 ± 18.2	125 ± 8.2
Hippocampus	272 ± 18.2	268 ± 13.5	271 ± 14.7	267 ± 35.2
Thalamus(VPN)	78 ± 5.8	76 ± 1.1	97 ± 13.5	239 ± 51.1**
Amygdala	61 ± 1.1**	62 ± 1.7**	62 ± 2.3**	65 ± 12.3**
Substantia nigra	63 ± 1.7*	62 ± 1.1*	62 ± 2.3*	63 ± 1.1*
Pons	64 ± 3.5	63 ± 4.2	61 ± 3.1	60 ± 1.7
Non-ischemic side				
FrPaM	122 ± 15.2	124 ± 14.7	128 ± 6.4	120 ± 17.6
FrPaSS	120 ± 5.8	120 ± 11.7	124 ± 8.2	129 ± 12.9
CPu(L)	170 + 13.5	175 ± 27.36	175 ± 11.1	167 ± 10.5
CPu(M)	156 ± 10.5	153 ± 17.10	153 ± 4.7	134 ± 12.3
Hippocampus	276 ± 18.8	285 ± 3.42	285 ± 14.7	284 ± 35.8
Thalamus(VPN)	87 ± 8.2	78 ± 1.31	78 ± 8.8	75 ± 5.8
Amygdala	84 ± 11.7	83 ± 5.52	83 ± 9.4	87 ± 12.3
Substantia nigra	78 ± 6.4	72 ± 11.05	72 ± 5.9	80 ± 12.9
Pons	64 ± 2.3	61 ± 1.57	61 ± 2.3	62 ± 2.3

Values are given in mean ± S.D. fmol/mg tissue using six animals.
FrPaM = frontoparietal cortex, motor area, supplied by anterior cerebral artery;
FrPaSS = frontoparietal cortex, somatosensory area, supplied by middle cerebral artery;
CPu(L) = lateral segment of caudate putamen; CPu(M) = medial segment of caudate putamen.
*$p<0.01$; **$p<0.001$, significant difference from control values using a t-test.

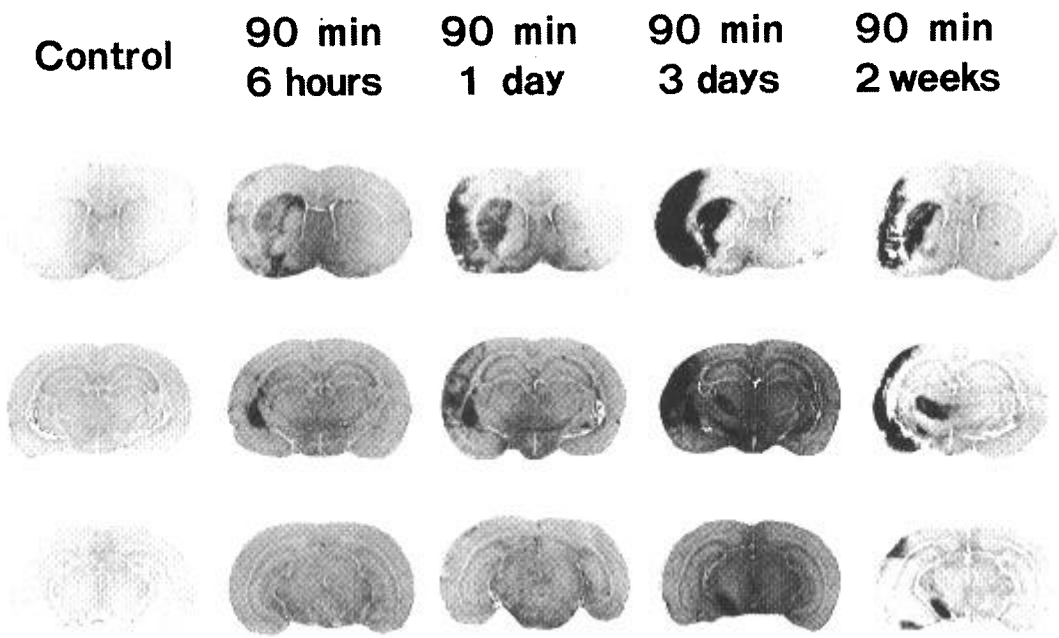

Fig. 1. ^{45}Ca autoradiograms of the brains of sham-operated control rats and those obtained after 90 min of MCA occlusion followed by 6-h, 1-day, 3-day, and 2-week recirculation. Representative autoradiograms show coronal sections at thelevel of the caudate putamen (top), the thalamus (middle),and the substantia nigra (bottom).

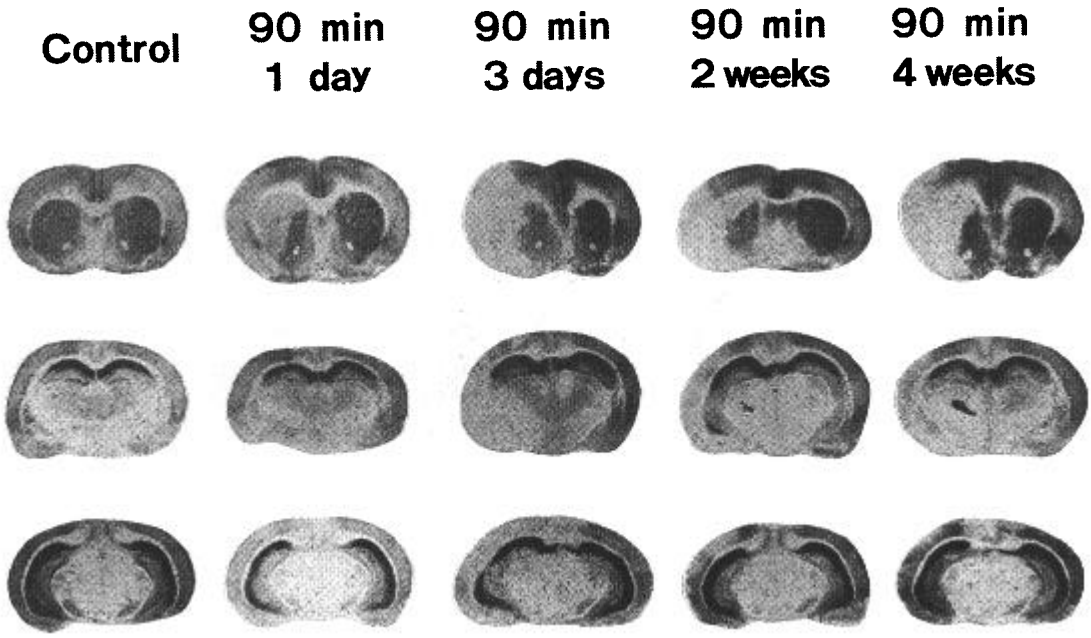

Fig. 2 [^{3}H]IP_3 autoradiograms of the brains of sham-operated control rats and those obtained after 90 min of MCA occlusion followed by 1-day, 3-day, 2-week, and 4-week recirculation. The levels of coronal sections are the same as described in Fig. 1.