Alterations of Second Messenger and Neurotransmitter Receptor Systems in the Exo-Focal Postischemic Brain Areas of the Rat: In vitro Autoradiographic Study

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Introduction

Transient ischemia induces neuronal damage in specific brain areas in the rat[1-2] and in the Mongolian gerbil[3-4], both animals being reproducible models for ischemic neuronal damage. Postischemic delayed neuronal damage has been reported in the ipsilateral thalamus and substantia nigra which lie outside ischemic areas of rat brain after middle cerebral artery (MCA) occlusion[5-6]. In those reports, the histological appearance of these remote areas was characterized by 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 recent studies has revealed 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 the mechanism[7-10].

Protein kinase C(PKC) is a calcium-dependent and phospholipid-stimulated, phosphorylating enzyme present in high concentrations in the brain[11-14]. Its activity appears to be linked closely to the phosphoinositide (PI) cycle, since diacylglycerol, which is generated by the PI cycle, may be an endogenous activator of PKC[15]. In recent years, PKC is known to be a second messenger and is thought to play an important role in several cellular process[16] and synaptic transmission in the brain[17]. Phorbol esters are potent tumour promoters that bind to specific receptor sites with high affinity[18-19] and it is well known that phorbol ester receptor is identical to PKC[20-22]. The anatomical distribution of PKC activity in the brain has been mapped by monitoring the binding of the potent phorbol ester receptor ligand [^{3}H]phorbol 12, 13-dibutyrate[23-24].

Acetylcholine is a major neurotransmitter in the central nervous system and is thought to contribute to memory and cognitive processes[25]. The anatomical distribution of muscarinic acetylcholine binding sites in the brain has been mapped by an autoradiographic method using the radiolabeled antagonist [^{3}H]quinuclidinyl benzilate[26]. Autoradiographic

studies suggest that acetylcholine is very rich in the cerebral cortex, the striatum, the hippocampus, and the thalamus.

Cellular responses to neurotransmitter receptor activation depend on the integrity of the coupling of the recognition sites to their associated signal transduction mechanisms. Activation of a receptor results in its coupling to a guanine nucleotide binding protein and this in turn activates associated second messenger systems. In the present study, we examined chronological changes of PKC and acetylcholine receptor binding activities 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 from the viewpoint of the relationship between second messenger and neurotransmitter receptor systems.

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[27]. Briefly, 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, Germany) which was mixed with a hardener (Elastomer Activator, Bayer Dental) to increase the thickness of the distal 5 mm to 0.25-0.30 mm. After introduction of 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 neurological deficits characterized by left hemiparesis with upper extremity dominance and right Horner's syndrome. After 90 min of MCA occlusion, the 6 rats were decapitated with no recirculation, and in the 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. The rats were killed by decapitation 3 h, 6 h, 1 day, 3 days, 1 week, and 4 weeks after recirculation. After decapitation, the brains were quickly removed and frozen in powdered solid CO_2, 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. Adjacent sections were stained with cresyl violet and luxol fast blue, as well as with hematoxylin and eosin, in order to confirm the ischemic areas.

PROTEIN KINASE C ACTIVITY

The chronological changes of protein kinase C activity in the brain after 90 min of ischemia were measured by the autoradiographic method of Worley et al[23] using [^{3}H]PDBu with minor modifications. Sections were incubated for 60 min at 25 °C in a buffer (50 mM TrisHCl, pH 7.7; 100 mM NaCl; 1 mM $CaCl_2$) containing 2.5 nM [^{3}H]PDBu (New England Nuclear, spec. act. 13.2 Ci/mmol). Following incubation, sections were washed twice for 2 min each time at 4 °C in the same buffer, briefly rinsed in distilled water at 4 °C and rapidly dried under a cold stream of air. Non-specific binding was evaluated in serial sections which were incubated under identical conditions in the presence of unlabeled 1 μM PDBu (Sigma, Chemical Company, St. Louis, USA). Autoradiograms were prepared from the sections by exposing them to [^{3}H]sensitive hyperfilm (Amersham, Sweden AB, Solna, Sweden) with a tritium standard microscale (Amersham International plc, Buckingham, UK) for nine days in standard X-ray cassettes.

MUSCARINIC ACETYLCHOLINE RECEPTOR AUTORADIOGRAPHY

Muscarinic acetylcholine receptors were quantified using [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB, spec. act. 41.5 Ci/mmol, Amersham International plc, Buckingham, UK) according to the method of Onodera et al.[26] Sections were incubated with 1 nM [^{3}H]QNB in phosphate buffer (pH7.4) at room temperature for 90 min. The slides were then washed in the buffer at 4 °C for 5 min and dried under a cold stream of air. Non-specific binding was determined using 1 μM atropine (Sigma). Autoradiograms were prepared from the sections by exposing them to [^{3}H]sensitive hyperfilm with a tritium standard microscale for 2 weeks in standard X-ray cassettes.

STATISTICAL ANALYSIS

Areas of the brain were identified with reference to the atlas of Paxinos and Watson[28]. The optical density of the brain regions was measured with a computer-assisted image analyzer (Zeiss, IBAS image analyzer system, Germany) without the examiner knowing the experimental protocol. The relationship between optical density and radioactivity was obtained with reference to the [^{3}H]microscales co-exposed with the sections using a third-order polynomial function. The optical density of the brain regions measured in

the present study was in the range where optical density and radioactivity of the [^{3}H]micro-scales showed a near linear relationship. Values were expressed as means ± SD fmol/mg tissue using 6 animals. Data regarding the PKC and QNB binding activities in each structure of the brain were analyzed using Duncan's multiple range test with $p<0.05$ and $p<0.01$ considered to be statistically significant.

Results

PROTEIN KINASE C ACTIVITY

Autoradiographic localization of PKC using [^{3}H]PDBu in the control and postischemic brain sections is shown in Fig. 1. After 90-min ischemia followed by 3-hour recirculation, significant decreases of the [^{3}H]PDBu binding sites were first observed in the anterior neocortex (FrPaSS) and the lateral part of the caudate putamen (CPu-L) , both of which were supplied by the occluded MCA (Figs. 3 and 4). Thereafter, [^{3}H]PDBu binding sites of the ischemic side decreased to approximately 20 % in the FrPaSS and to 45 % in the CPu-L of each control value 4 weeks after the ischemic insult. On the contrary, there was no alteration on day 1, but 3 days after recirculation, marked reduction of [^{3}H]PDBu binding sites was observed in the ipsilateral thalamus and the substantia nigra, which were remote from the precedent ischemic areas (Figs. 5 and 6); thereafter, these binding sites of the ischemic side decreased progressively to approximately 40 % in the thalamus and to 55 % in the substantia nigra of each control value 4 weeks after the ischemia. There were no significant changes of [^{3}H]PDBu binding sites in the contralateral non-ischemic hemisphere .

MUSCARINIC ACETYLCHOLINE RECEPTOR

Autoradiographic localization of muscarinic acetylcholine binding sites using [^{3}H]QNB in the control and postischemic brain sections is shown in Fig. 2. After 90-min ischemia followed by 3-day recirculation, significant decreases of the [^{3}H]QNB binding sites were first observed in the FrPaSS and the CPu-L, both of which were supplied by the occluded MCA (Figs.3 and 4). Thereafter, [^{3}H]QNB binding sites of the ischemic side decreased to approximately 20 % in the FrPaSS and to 30 % in the CPu-L of each control value 4 weeks after the ischemic insult. Moreover, 3 days after the ischemia, a significant reduction of [^{3}H]QNB binding sites was observed in the ipsilateral thalamus, and also 7 days after the ischemia, in the substantia nigra, both areas were located remote from the precedent ischemic areas (Figs. 5 and 6). Thereafter, the binding sites decreased progressively in the thalamus and the substantia nigra on the ischemic side. There were no significant changes of [^{3}H]QNB binding sites in the contralateral non-ischemic hemisphere.

Discussion

We examined the time courses of the binding activities of the second messenger system and the neurotransmitter receptor sites in the postischemic rat brain. The present

study indicated that two different alterations of PKC and muscarinic acetylcholine binding activities associated with the mechanisms of neuronal damage took place in the postischemic rat brain. First, in the ischemic foci, the ipsilateral FrPaSS and the CPu-L, more rapid changes of PKC than those of muscarinic acetylcholine binding sites were observed after the ischemic insult and these changes of PKC developed concurrently with abnormal calcium accumulation in these ischemic foci in our previous studies using the same ischemia model[5,9]. This finding indicates that second messenger system is more vulnerable to the ischemic insult and involved more rapidly than neurotransmitter receptor sites in the ischemic foci. In this model of ischemia, 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[27]. The reductions of PKC and muscarinic acetylcholine binding activities in the FrPaSS and the CPu-L are explained by the direct damage to intracellular components including cell membrane following damage of second messenger systems and disruption of calcium homeostasis by ischemia-induced energy failure.

Second, in the exo-focal remote brain areas, the ipsilateral thalamus and the substantia nigra, the activity of PKC was not significantly changed 1 day after the ischemia compared with the control values and a significant reduction of PKC activity was first detected 3 days after the ischemic insult. These delayed changes of second messenger system observed in the thalamus and the substantia nigra were concurrent with the abnormal calcium accumulation detected there in our previous study[5,9]. A significant decrease of muscarinic acetylcholine binding sites was observed in the thalamus 3 days after the ischemia. Moreover, in the ipsilateral substantia nigra, these receptor sites decreased significantly 1 week after the ischemia. The alteration of binding sites in the substantia nigra was minimal because of their initial low binding activity. Moreover, both phenomena, i.e., the reduction of second messenger and neurotransmitter receptor systems, in two remote areas on the ischemic side preceded the histologic findings of delayed neuronal damages. In contrast with the FrPaSS and the CPu-L, the ipsilateral thalamus and the substantia nigra were remote from these ischemic areas, and both areas had not been directly affected by the original ischemic insult[27]. Delayed neuronal damages in the exo-focal remote areas might be caused by a transsynaptic process associated with the ischemic foci[5]. Iizuka et al. reported that delayed neuronal degeneration of the ipsilateral thalamus was observed after somatosensory cortical infarct of rats using the Fink-Heimer silver staining method[29]. We suggest that reductions of both PKC and muscarinic acetylcholine binding activities in the thalamus on the ischemic side may be explained by membrane damage with retrograde neuronal degeneration due to thalamocortical fiber damage in ischemic cortical regions.

We realize, however, that the mechanisms of delayed neuronal damage in the exo-focal brain areas may be variable and are complicated in remote areas, i.e., the thalamus and the substantia nigra on the ischemic side. Yamada et al. reported that basic fibroblast growth

factor prevented neuronal degeneration of the thalamus after MCA occlusion in rats[30]. This indicates that trophic substances may play an important role in the mechanism of neuronal damage of the thalamus which might be caused by retrograde degeneration of the thalamo-cortical pathway after ischemic insult. On the other hand, in the substantia nigra, we speculate that delayed neuronal degeneration may be induced by disinhibitory overexcitation caused by diminished inhibitory regulation from the caudate putamen, which was affected by the precedent ischemia [5,10]. Further detailed investigation is required in order to clarify the mechanisms of delayed neuronal degeneration caused by neuronal network disturbances after ischemia.

It is well known that thalamic damage results in some behavioral neurological disturbances, such as amnestic syndromes, dementia, or aphasic syndrome[31-32]. There have been few studies on clinical symptoms in multi-focal brain damages during the chronic stage after stroke, and such delayed neuronal degeneration in the exo-focal remote areas after ischemia have only recently been revealed by animal experiments. Based on the present study, we conclude that postischemic alterations of second messenger and neurotransmitter receptor systems were involved not only in the ischemic foci due to ischemia-induced energy failure, but also in the exo-focal remote areas prior to the histologic changes which neuronal damage might be caused by transsynaptic delayed degeneration. Furthermore, we suggest that multi-focal postischemic alterations of both second messenger and neurotransmitter receptor systems may exacerbate the clinical symptoms of patients during the chronic stage of stroke.

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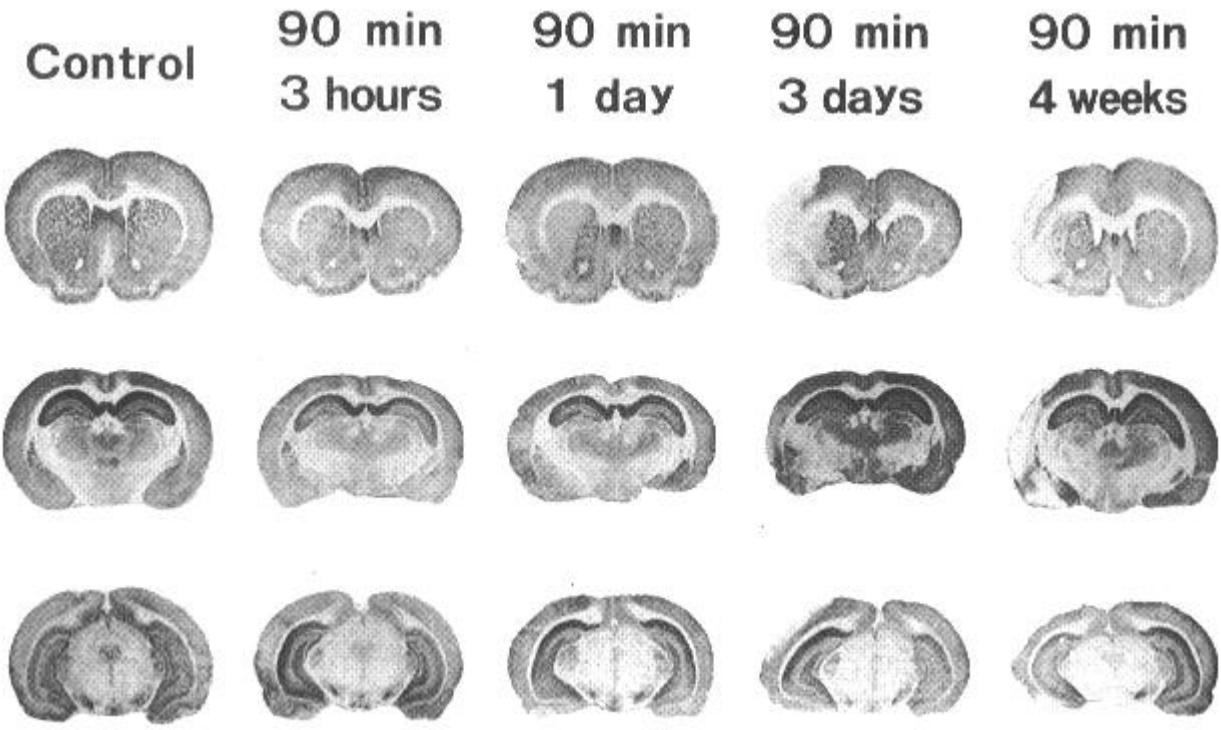

Fig. 1. [^{3}H]PDBu autoradiograms of the brains of sham-operated control rats and those obtained after 90 min of MCA occlusion followed by 3-hour, 1-day, 3-day, and 4-week recirculation. Representative autoradiograms show coronal sections at the level of the caudate putamen (top), the thalamus (middle), and substantia nigra (bottom).

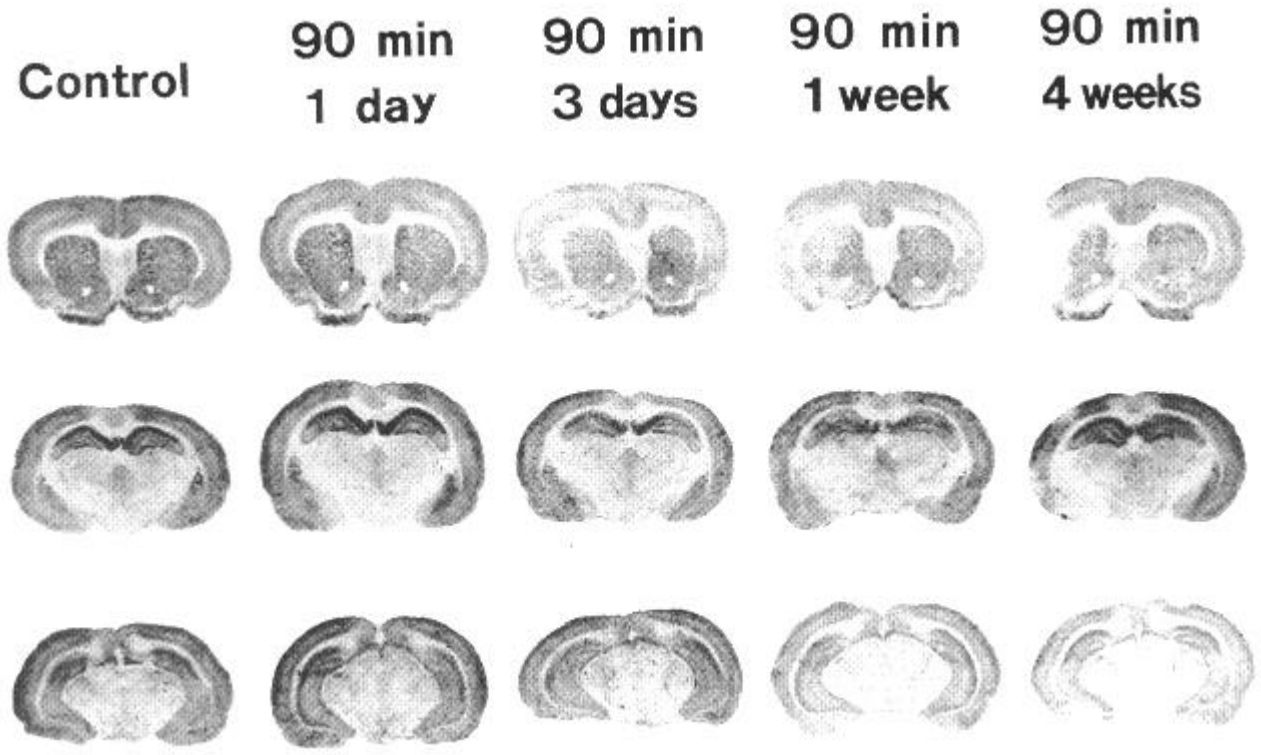

Fig. 2. [^{3}H]QNB autoradiograms of the brains of sham-operated control rats and those obtained after 90 min of MCA occlusion followed by 1-day, 3-day, 1-week, and 4-week recirculation. Representative autoradiograms show coronal sections at the level of the caudate putamen (top). the thalamus (middle), and the substantia nigra (bottom). Three days after the ischemia, a significant reduction of the binding sites was first detected not only in the cerebral cortex and the lateral segment of the caudate putamen, but also in the ipsilateral thalamus on the ischemic side and these findings continued up tp 4-week recirculation.

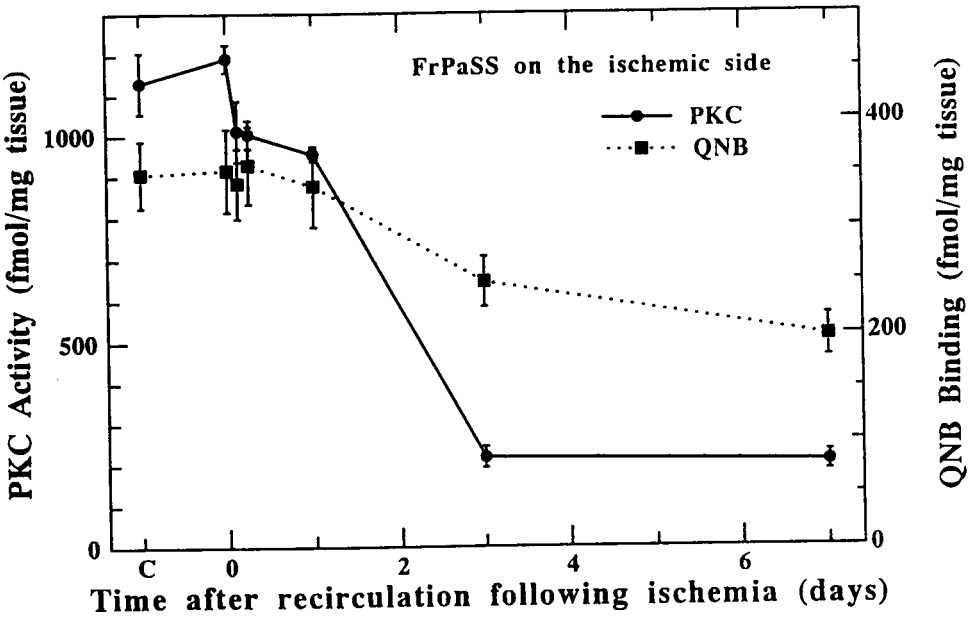

Fig. 3. Time course of PKC and muscarinic acetylcholine (QNB) binding activities in the frontoparietal cortex, somatosensory area (FrPaSS), on the ischemic side after 90 min of MCA occlusion with no recirculation and such occlusion followed by various periods of recirculation. Values are given in mean ± S.D. fmol/mg tissue using six animals. * $P<0.05$. ** $P<0.01$ vs sham-operated control (C) values using Duncan's multiple range test.

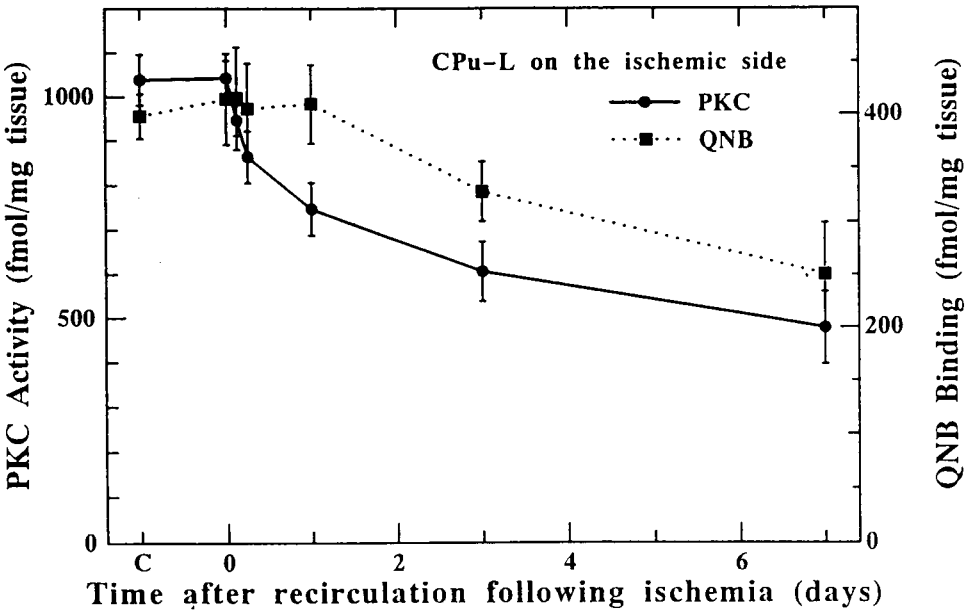

Fig. 4. Time course of PKC and muscarinic acetylcholine (QNB) binding activities in the lateral segment of caudate putamen (CPu-L) on the ischemic side after 90 min of MCA occlusion with no recirculation and such occlusion followed by various periods of recirculation. Values are given in mean ± S.D. fmol/mg tissue using six animals. * P<0.05, ** P<0.01 vs sham-operated control (C) values using Duncan's multiple range test.

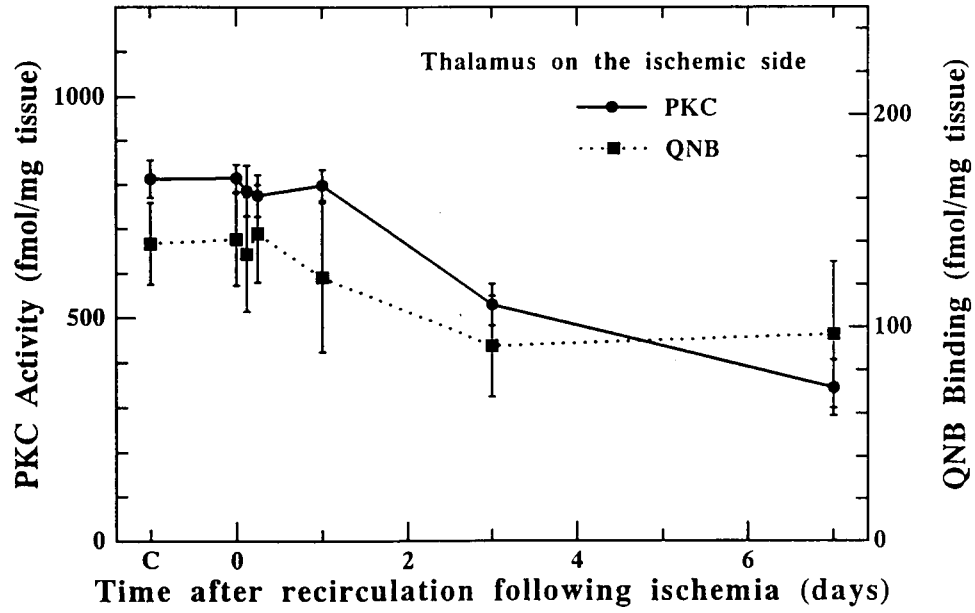

Fig. 5. Time course of PKC and muscarinic acetylcholine (QNB) binding activities in the thalamus on the ischemic side after 90 min of MCA occlusion with no recirculation and such occlusion followed by various periods of recirculation. Values are given in mean ± S.D. fmol/mg tissue using six animals. ** P<0.01 vs sham-operated control (C) values using Duncan's multiple range test.

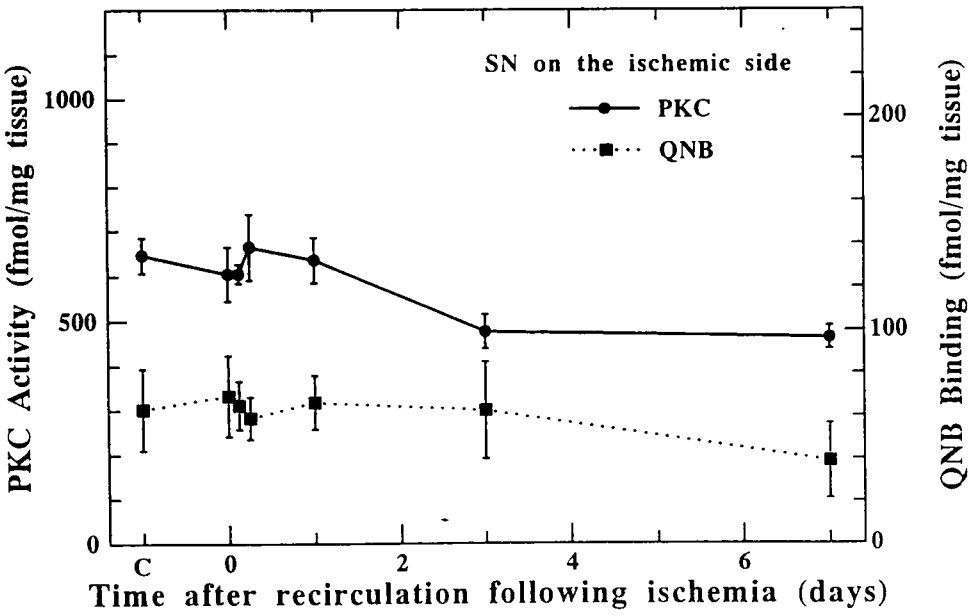

Fig. 6. Time course of PKC and muscarinic acetylcholine (QNB) binding activities in the substantia nigra (SN) on the ischemic side after 90 min of MCA occlusion with no recirculation and such occlusion followed by various periods of recirculation. Values are given in mean ± S.D. fmol/mg tissue using six animals. * $P<0.05$, ** $P<0.01$ vs sham-operated control (C) values using Duncan's multiple range test.