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IMMUNOCYTOCHEMICAL DISTRIBUTION OF GAMMA ISOFORM OF PROTEIN KINASE C (PKC-γ) FOLLOWING INCOMPLETE ISCHAEMIA

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Abstract: Immunocytochemical distribution of PKC-γ was examined in rat brain in relation to molecular mechanisms of post-ischaemic neuronal modulation following incomplete ischaemia. Incomplete ischaemia was developed by either permanent occlusion of one common carotid artery (CA) or permanent occlusion of one CA with temporary occlusion of opposite CA. Unilateral CA (UCA) occlusion resulted in a pronounced increase in the intensity of staining and number of PKC-γ positive neurons in the neocortex ipsilateral to the insult after 3 h. The effect was maximum at 6-12 h and was undetectable after 7 days. CA1 neurons showed an increase immunoreactivity (IR) after 1 day, reached to a peak by 3 days, then reduced to basal levels after 7 days. Bilateral CA (BCA) occlusion showed almost similar changes in the neocortex, but on both sides and short durated. The altered patterns of PKC-γ IR in the neocortex and hippocampus following CA occlusion may reflect activation and/or down-regulation of PKC-γ in ischaemic neurons. PKC-γ may, therefore, potentially play an important role in the post-ischaemic modulation of synaptic efficacy in these neurons and in the neuronal damage following incomplete ischaemia.

Key words: ischaemia incomplete PKC-γ brain immunohistochemistry rat

INTRODUCTION

Protein kinase c plays an important role in the regulation of normal cellular homeostasis; and its role for the signal transduction of various extracellular signals into the cell to control many physiological processes has been suggested (1-3). So far, it is known that this Ca^{++} and phospholipid dependent enzyme is highly concentrated in the brain (4), and exists in several different isoforms, each with a characteristic distribution within the central nervous system (CNS) (5-7). Of which, gamma isoform (γ) is the most abundant isoform in CNS (6). Activated PKC-γ has been reported to be involved in long-term cellular regulation including cell growth and survival (8, 9), neurotransmitter release (3), membrane ionic pumps (10), non-ionic membrane transport (11), and synaptic plasticity (12). Since disturbances of each of these processes have been implicated in the pathophysiology of ischaemia, modulation of PKC activity could be an important determinant of neuronal survival following ischaemia.

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Cerebral ischaemia induces a wide spectrum of physiological and metabolic derangements resulting either non-recoverable functional losses or neuronal damage/death (13). A few minutes of forebrain ischaemia causes neuronal damage in specific brain areas in rat (14). The molecular mechanisms responsible for differential neuronal vulnerability to ischaemic trauma are not clear, but natural PKC inhibitors, such as gangliosides have been shown to improve recovery following brain ischaemia (15). Earlier studies have suggested the role of PKC in the development of neuronal damage after complete forebrain ischaemia (16-18), but reports on incomplete ischaemia, especially immunocytochemical studies are scarce.

In view of the central importance of PKC in neuronal function and cell survival, the present study was undertaken to determine the subcellular distribution of γ-isoform of PKC following incomplete ischaemia. The results are discussed in relation to a possible role of PKC-γ in the mechanisms of selective neuronal damage following incomplete ischaemia.

METHODS

SUBJECTS

Male adult Wistar rats (Shimizu Experimental Animals, Kyoto, Japan) weighing 300-400 g were used throughout the experiment. The rats had free access to food and water prior to the experiments, and housed under diurnal lighting conditions. The rats were fasted overnight prior to the surgery with free access to tap water. Surgery was carried out under ether anaesthesia. Animals were immobilized with gallamine triethiodine, intubated and placed on a Starling type of respirator for artificial ventilation with a gas mixture of CO_2 in oxygen. For EEG recording needle electrodes were inserted in the muscles lateral to the skull bone. BCA were exposed through a midline cervical incision, blood flow was occluded permanently by double ligatures and vessel transection between the ligatures. The temporary occlusion was carried out with aneurysm clips, and blood flow was restored by releasing the clips after 1 h of occlusion. In 44 rats, either left or right (in random order) CA was permanently occluded. In additional 36 rats, one CA was occluded permanently, and the opposite one was occluded temporarily (1 h). After surgery, all animals were allowed to awaken and were extubated 10-20 min post ischemia. Control animals were subjected to the same procedure but CA was not occluded. Body temperature was kept close to 37°C by thermostated warming pad during and following the operations until they began to move again. All rats were then allowed to recover for various time until immunocytochemistry.

TISSUE PREPARATION

At various times following unilateral/bilateral CA occlusion (3h-7days), the rats were transcardially perfused with 200 ml of a fixative containing 4% paraformaldehyde, 0·2% picric acid and 0·5% glutaraldehyde in 0·1 M phosphate buffer (pH 7·4) under ether anaesthesia. After careful removal from the cranial cavity, the perfused brains were stored in 0·1 M phosphate buffer containing 30% sucrose for cryoprotection (4°C). Frontal sections (30 μm) were cut on a freezing cryostate and during sectioning the sections were immersed in 0·1 M phosphate buffer saline (PBS).

IMMUNOHISTOCHEMICAL PROCEDURE

After washing with PBS containing 0·3% Triton X-100 (PBS-T), the sections were incubated for 30 min in 10% normal sheep serum (NSS) in PBS–T to block the nonspecific binding sites of proteins. Then, the free floating sections were incubated one overnight at 4°C under gentle movement with the primary antibody solution in PBS containing mouse anti-PKC-γ IgG (36G9; 1 : 200). After rinsing in PBS-T, the sections were exposed to biotinylated sheep antimouse IgG (1 : 200 : Amersham) for 1 h at room temperature (RT). Then, the sections were incubated with streptavidin-HRP (1 : 200; Zymed; 1 h at RT) after rinsing in PBS-T. The sections were again washed in PBS and stained with diaminobenzidine solution containing glucose oxidase and nickel (19). Finally, the sections were mounted and coverslipped for light microscopic inspection. Immunocytochemical control studies for the specificity were made by omission of primary antibody in the incubation cycle; or by

preabsorption test with purified protein kinase c; or using normal mouse serum instead of primary antibody.

QUANTIFICATION OF PKC-γ POSITIVE NEURONS

Though all the brain structures were looked for ischaemia induced changes in PKC-γ expression, after it became clear that the main structures of changes were invariable to be found in neocortex and CA1 subfields, incomplete ischaemia induced changes in PKC-γ expression were quantified by counting the number of PKC-γ positive neurons in the neocortex and CA1 area. The number of PKC-γ positive neurons was counted within a 520 X 520 μm grid placed over each of those areas at X100 magnification. The sections for quantification were selected on the grounds of complete cortical and hippocampal profiles. Schematic drawings illustrating the sampled area (dark squares) and AP position of sections used for cell counts are shown in Fig. 1A (20). Statistical analysis was carried out by analysis of variance, followed by Student's t-test.

RESULTS

In sham-operated control animals, a consistent distribution pattern of PKC-γ positive neurons was apparent for all animals studied throughout the cerebral cortex, piriform cortex, hippocampal formation, amygdaloid complex and entorhinal cortex. Intense PKC-γ IR in the neuron was seen both in the membrane and cytoplasm of the perikarya, dendrites, axons and axon terminals, while weak immunoreaction was observed in the nuclei but almost never in the nucleolus. Glial/astrocytes were not immunoreactive. Immunocytochemical specificity studies yielded negative results; i.e., absence of any detectable labelling.

After 3 h of UCA occlusion, a strong and pronounced increase in the intensity of PKC-γ IR in the neocortex ipsilateral to the occlusion was observed; which became maximum 6-12 h after incomplete ischaemia (Fig. 2). This increase was present in the cell bodies with nuclei and dendritic processes of both pyramidal and nonpyramidal neurons in layers 2/3, 5

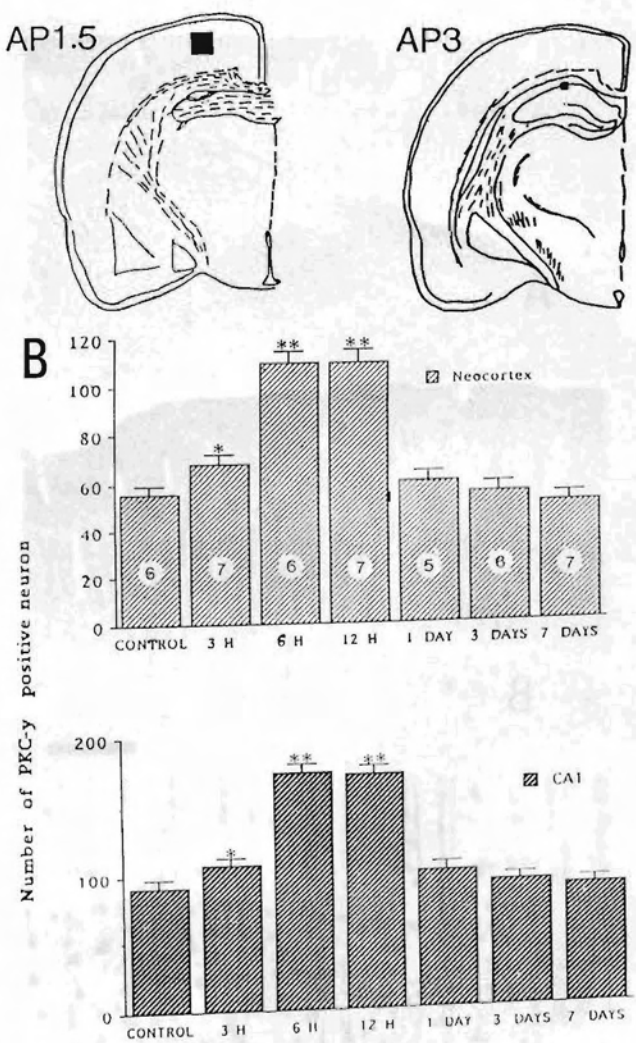

Fig. 1 A: Schematic drawing of sections of the rat brain demonstrating the standardized levels chosen for neuronal quantification. AP numbers indicate the distance from frontal zero plane (bregma). Boxed regions indicate the 520 X 520 μm sampled areas.

B: Time course of changes in PKC-γ expression in the neocortex and CA1 subfield of rat brain after various periods of UCA occlusion. Values are expressed as the mean (±SE) Number of PKC-γ positive neurons/ 520 X 520 μm area. *P < 0·05, **P < 0·01 significantly different from control. Values in parenthesis are the number of rats used.

and 6. Thereafter, intensity was decreased and PKC-γ positive staining in the neocortex returned almost to control level by 3 days. At 7 days, there was no apparent

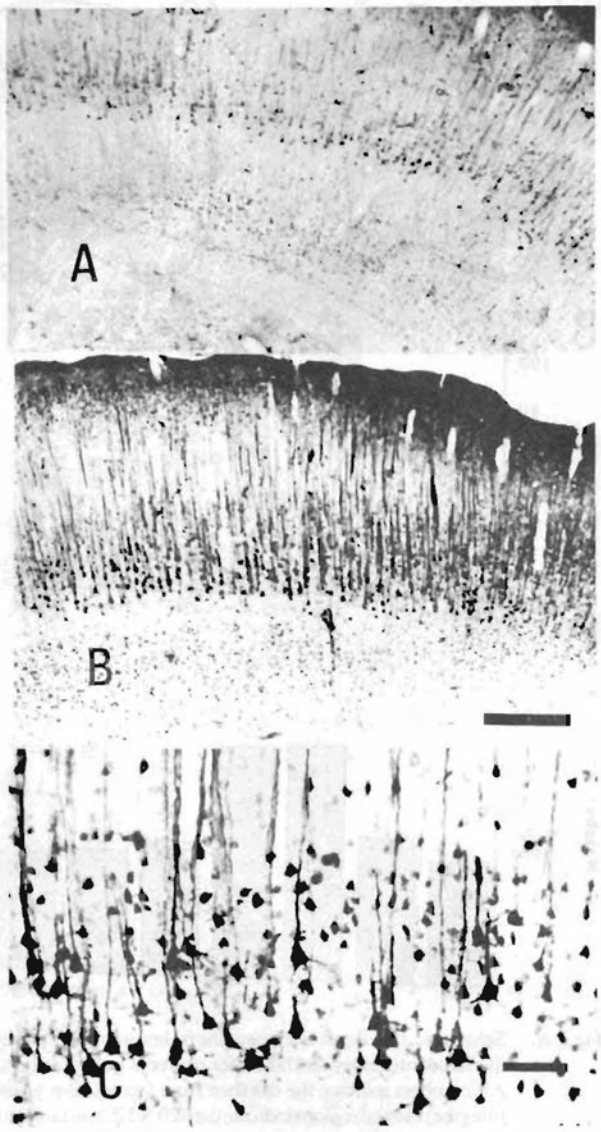

Fig. 2 : The effects of incomplete ischaemia on the IR for PKC-γ (B) compared to sham-operated control section (A). A clear increase in PKC-γ IR is found after 6 h of UCA occlusion. Incomplete ischaemia induced a redistribution of PKC-γ IR (C), most notably in the basal dendrites of the layer 5 pyramidal neurons with dendritic arborizations. Bar : A, B = 130 μm ; C = 30 μm.

PKC-γ positive neurons in any layers over that seen in control animals. PKC-γ IR was markedly increased in the dendrites and perikarya of CA1 neurons after 1 day and was peaked at 3 days (Fig. 3); and returned to control levels 7 days after CA occlusion. Granule cells in the dentate gyrus and other areas of hippocampal formation did not show any increase in the staining intensity following ischaemia, though the CA3 and CA4 neurons presented a moderate increase in staining intensity (Fig. 3). Other structures, such as piriform cortex, amygdaloid complex or entorhinal cortex failed to show any change in their staining patterns following incomplete ischaemia over that of their control parts. These qualitative results were affirmed by quantification of PKC-γ positive neurons in the neocortex and CA1 subfields (Fig. 1B).

After BCA occlusion (with one CA was occluded

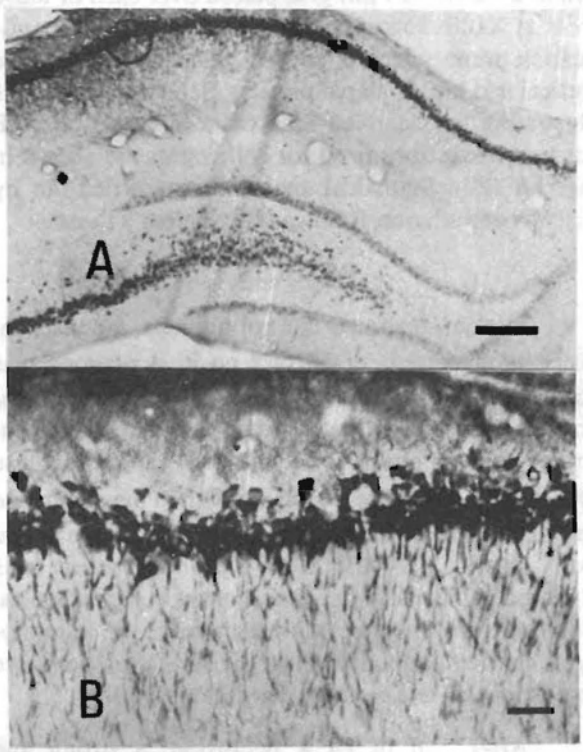

Fig. 3: Representative photomicrographs of hippocampus 1 day after UCA occlusion in rats. A. CA1 pyramidal cells show intense IR for PKC-γ with a mild staining in CA3 and CA4 neurons. But, dendate gyrus show almost background staining, Bar = 130 μm. B. Higher magnification of CA1 are area (○) of A, Bar = 30 μm.

temporarily for 1 h), PKC-γ like IR was seen in a similar distribution, as in unilateral occlusion; but on the both sides of the neocortex. At 6 h, PKC-γ positive neurons were more numerous and darkely stained than that of unilateral occlusion, but returned to control level within 1 day. Moreover, there was no increased hippocampal staining in any subfields over that seen in the control sections at any time interval studied.

DISCUSSION

In this study we attempted to define the pathophysiologic role of PKC-γ in ischaemic neurons. In sham-operated animals, the distribution patterns observed for PKC-γ resembled previously reported PKC-γ distribution patterns (21, 22). The distribution patterns, as well as the negative control studies indicate the specificity of the PKC-γ immunostaining in our experiment. The present results showed that IR for PKC-γ in the cell bodies and dendrites of the neocortical and CA1 neurons were significantly enhanced 6-12 h and 1 day after the ischaemic insult, respectively (Fig. 1B). There are at least two possible explanations for the observed increase IR in ischaemic neurons. Increased PKC-γ IR may reflect either a real increase in the quantity of PKC as a result of *de novo* synthesis (23), or a synaptic modulation, by which IR is enhanced, is shifted from the cell body to the cell membrane or dendrites. Earlier study support the concept of redistribution of PKC-γ to the membrane in ischaemic neurons (24). The present increased IR may, therefore, reflect activation and redistribution of PKC-γ to cellular membranes; activation and membrane binding of PKC-γ may lead to enhanced dendritic and perikaryal staining.

The mechanism by which forebrain ischaemia leads to increase in PKC activity is not clear. An increased intracellular CA^{++} concentration and release of free fatty acids and diacylglycerol from membrane phospholipids are among the initial events of the ischaemic cascade (25, 26). In addition, it has been suggested that massive release of excitatory amino acids, such as glutamate and asparate may be one of the causal factors in the damage to certain neurons observed after ischaemia (27). Increased intracellular Ca^{++} levels (3) and/or glutamate release (27) are believed to facilitate translocation of PKC from the cytosol to the plasma membrane to provide the lipid requirments for the enzyme activation. Therefore, the observed increase IR in specific neurons following incomplete ischaemia may be due to the excessive receptor activation caused by release of neurotransmitters including neurotoxic excitatory amino acids, the increased intracellular calcium concentrations and the formation of PKC activators and of translocation of PKC-γ during early phase of collateral circulation/reperfusion.

We observed a time dependent increase IR (Fig. 1B). In the neocortex, IR began to fall 1 day after ischaemia, but in CA1 neurons IR was increased after 1 day and decreased by 3-7 days. These findings indicate an early (in neocortex) and delayed (in CA1) increase in PKC-γ activation in these neurons. Our recent histological study reveals that there is a delayed recoverable neuronal degeneration (after 5 days) in CA1 subfields following UCA occlusion; and after BCA occlusion, there is an recoverable early (after 1 day) neuronal damage in the neocortex with a delayed (after 5 days) damage of CA1 neurons (28). These findings indicated that post-ischaemic PKC-γ activation were present in the histologically intact neurons preceding ischaemic damage with a down-regulation of PKC-γ activity in the ischaemic-lesioned neocortical/CA1 neurons. Time dependent activation and down-regulation of PKC-γ in ischaemic neurons lead us to the speculation that activation of PKC may bridge up the gap from a transient signal to a long-lasting change. This concept is supported by the fact that a marked alteration of second messenger and neurotransmitter systems occurs in morphologically normal neurons (29) following ischaemia.

The altered patterns of PKC-γ IR in the neocortex and CA1 subfields may reflect activation and/or down-regulation of PKC-γ isozyme, indicating that this isozyme play an important role in the post-ischaemic modulation of synaptic efficacy in the neocortex and hippocampal formation and in the post-ischaemic neuronal damage and recovery.

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REFERENCES

1. Nishizuka Y. The role of protein kinase c in cell surface signal transduction and tumor promotion. *Nature* 1984; 308 : 693-698.
2. Nishizuka Y. Turnover of inositol phospholipids and signal transduction. *Science* 1984; 255 : 1365-1370.
3. Nishizuka Y. Studies and perspective of protein kinase c. *Science* 1986; 233 : 305-312.
4. Kikkawa U, Takaki Y, Minakuchi R, Inohara S, Nishizuka Y. Calcium-activated, phospholipid-dependent protein kinase from rat brain: subcellular distribution, purification and properties. *J Biol Chem* 1982; 257 : 13341-13348.
5. Huang K-P, Nakabayashi H, Huang FL. Isozymic forms of rat brain Ca^{++}-activated and phospholipid-dependent protein kinase. *Proc Natl Acad Sci USA* 1986; 83 : 8535-8539.
6. Huang FL, Yoshida Y, Nakabayashi H, Young WS, Huang K-P. Immunocytochemical localization of protein kinase c isozymes in rat brain. *J Neurosci* 1988; 8 : 4734-4744.
7. Kikkawa U, Ono Y, Ogita K, et al. Identification of the structures of multiple subspecies of protein kinase c expressed in rat brain. *FEBS Let* 1987; 217 : 227-231.
8. Hsu L. Neurite-promoting effects of 12-0-tetradecanoyl-phorbol-13-acetate on chick embryo neurons. *Neurosci Lett* 1985; 62 : 283-289.
9. Montz HP, Davis GE, Skaper SD, Manthorp, Varon S. Tumor-promoting phorbolester mimics two distinct neurotrophic factors. *Brain Res* 1985; 355 : 150-154.
10. DeReimer SA, Strong JA, Albert KA, Greengard P, Kamarek LK. Enhancement of calcium current in aplysia neurons by phorbol ester and protein kinase c. *Nature* 1985; 313 : 313-316.
11. Witters LA, Vater CA, Leinhard GE. Phosphorylation of the glucose transporter *in vitro* and *in vivo* by protein kinase c. *Nature* 1985; 315 : 777-778.
12. Malenka RC, Madison DV, Nicoll RA. Potentiation of synaptic transmission in the hippocampus by phorbol esters. *Nature* 1986 ; 321 : 175-177.
13. Siesjo BK. Historical overview: Calcium, ischemia and death of brain cells. *Ann NY Acad Sci* 1989; 582 : 638-661.
14. Pulsinelli WA, Brierley JB, Plum F. Temporal profile of neuronal damage in a model of transient forebrain ischemia. *Ann Neurol* 1982; 11 : 491-498.
15. Karpiak S, Li YS, Mahadik SP. Gangliosides (GM_1 and AGF_2) reduce mortality due to ischemia: protection of membrane function. *Stroke* 1987; 18 : 184-187.
16. Curmrine RC, Dubyak G, LaManna JC. Decreased protein kinase c activity during cerebral ischemia and after reperfusion in the adult rat. *J Neurochem* 1990; 55 : 2001-2007.
17. Louis JC, Magal E, Brixi A, Steiberg R, Yavin E, Vincendon G. Reduction of protein kinase c activity in the adult rat brain following transient forebrain ischemia. *Brain Res* 1991; 541 : 171-174.
18. Onodera H, Araki T, Kogure K. Protein kinase c activity in the rat hippocampus after forebrain ischemia: autoradiographic analysis by [^{3}H] phorbol 12, 13-dibutyrate. *Brain Res* 1989; 481 : 1-7.
19. Shu S, Ju G, Fan L. The glucose oxidase-DAB-nickel method in peroxidase histochemistry of the nervous system. *Neurosci Lett* 1988; 85 : 169-171.
20. Marsala, Fifkova E. Stereotaxic atlases for the cat, rabbit and rat. In: Abures J, Petran M, Zachar J, eds Electrophysiological methods in biological research *Prague : Academia* 1967; 653-731.
21. Saito N, Kikkawa U, Nishizuka Y, Tanaka C. Distribution of protein kinase c-like immunoreactive neurons in rat brain. *J Neurosci* 1988; 8 : 369-382.
22. Tsujino T, Kose A, Saito N, Tanaka C. Light and electron microscopic localization of βI-, βII- and γ-subspecies of protein kinase c in rat cerebral neocortex. *J Neurosci* 1990; 10 : 870-884.
23. Dragunow M, Currie RW, Faull RLM, Robertson HA, Jansen K. Immediate early genes, kindling and long-term potentiation. *Neurosci Biobehave Rev* 1989; 13 : 301-313.
24. Weiloch T, Cardell M, Bingren H, Zivin J, Saitoh T. Changes in the activity of protein kinase c and the differential subcellular redistribution of its isozymes in the rat striatum during and following transient forebrain ischemia. *J Neurochem* 1991; 56 : 1227-1235.
25. Abe K, Kogure K, Yamamoto H, Imzawa M, Miyamoto K. Mechanisms of arachidonic acid liberation during ischemia in gerbil cerebral cortex. *J Neurochem* 1987; 48 : 503-509.
26. Bazan NG. Effects of ischemia and electroconvulsive shock on free fatty acid pool in the brain. *Biochem Biophys Acta* 1970; 218 : 1-10.
27. Benveniste H, Drejer J, Schusboe A, Diemer NH. Elevation of the extracellular concentrations of glutamate and asparate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. *Neurochem* 1984; 43 : 1369-1374.
28. Aftabuddin M, Islam N, Moriwaki A, Hori Y. Carotid artery occlusion causes reversible brain damage in rat. *Ind Heart J* (in press).
29. Hara H, Onodera H, Kato H, Araki T, Kogure K. Autoradiographic analysis of second messenger and neurotransmitter system receptors in the gerbil hippocampus following transient forebrain ischemia. *Brain Res* 1991; 545 : 87-96.