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Introduction

We have recently demonstrated that brief and non-lethal cerebral ischemia can injure selectively vulnerable neurons when such ischemia is induced repeatedly at certain intervals.[1-5] Two-minute bilateral common carotid artery occlusion in the gerbil produces no histopathological neuronal damage in the brain, whereas three such occlusions at 1-hr intervals consistently lead to severe destruction of hippocampal CA1 pyramidal neurons. Of interest is that the neuronal damage is most severe when ischemia is repeated at 1-hr intervals and is relatively mild when repeated at shorter or longer intervals.[2,5] However, the underlying mechanisms remain to be elucidated, although postischemic circulatory and metabolic disturbances, such as postischemic hypoperfusion which is maximal at 1 hr after 2-min ischemia[6] and impairment of protein synthesis which is depressed for several hours after 2-min ischemia,[7] as well as excitotoxic mechanisms,[8] may explain the cumulative damage.

Recent experimental evidence indicates that marked alterations in signal transmission systems, intracellular signal transduction systems, and in calcium channels precede delayed neuronal death of hippocampal CA1 pyramidal cells. The purpose of this study was, therefore, to reveal the sequential alterations in the binding of neurotransmitters, muscarinic acetylcholine and adenosine A1, and of an L-type calcium channel blocker, PN200-110, and in the second-messenger systems, IP_3, PKC and adenylate cyclase, in the gerbil hippocampus following three 2-min ischemic insults in comparison with those following an equivalent single period of 6-min ischemia and following single 2-min ischemia.

Materials and Methods

Induction of ischemia

A total of 75 male Mongolian gerbils (Seiwa Experimental Animals, Fukuoka, Japan), aged 12-15 weeks and weighing 68-92 g, were used. Anesthesia was induced with 2% halothane in a mixture of 30% oxygen and 70% nitrous oxide. Bilateral common carotid arteries were occluded with aneurysm clips. Anesthesia was discontinued when the clips

were in place. Body temperature during surgery and ischemia was maintained at close to 37°C. Animals were subjected to a 2-min occlusion, a 6-min occlusion or three 2-min occlusions at 1-hr intervals. Animals subjected to 2 or 6 min ischemia were decapitated at 1 hr, 6 hr, 1 day, 4 days and 1 month after ischemia. Animals subjected to three 2-min ischemic insults were decapitated at 6 hr, 1 day, 4 days and 1 month. Five control animals were also included. The brains were quickly removed and frozen in powdered dry ice. Coronal frozen sections, 15 μm in thickness, were cut in a cryostat and thaw-mounted onto getatin-coated cover-slips. Adjacent sections were stained with Cresyl violet and used for histopathology.

Ligand binding assays

Details of the ligand binding assays are described elsewhere[9,10] Muscarinic acetylcholine and adenosine A1 receptors were quantified using [^{3}H]quinuclidinyl benzilate (QNB; 41.5Ci/mmol, New England Nuclear) and [^{3}H]cyclohexyladenosine (CHA; 34.4 Ci/mmol, New England Nuclear), respectively. Autoradiographic visualization of L-type calcium channels was done using [^{3}H]PN200-110 (71.5 Ci/mmol, New England Nuclear), a 1,4-dihydropyridine calcium channel blocker. Autoradiographic visualization of IP_3 binding sites, protein kinase C, and adenylate cyclase was performed using [^{3}H]IP_3 (17.0 Ci/mmol, New England Nuclear), [^{3}H]phorbol 12, 13-dibutyrate (PDBu) (13.2 Ci/mmol, New England Nuclear), and [^{3}H]forskolin (40 Ci/mmol, New Englang Nuclear), respectively.

The incubated sections were apposed to Hyperfilm-^{3}H (Amersham) for 1-4 weeks. The optical density of the regions of interest was measured using a computer-assisted image analyzer system (IBAS image analyzer system, Zeiss). The relation between optical density and radioactivity was determined using a third order polynomial function with reference to tritium standards ([^{3}H]microscale, Amersham) exposed along with the tissue sections.

Statistics

Each group contained five animals. Binding assays were performed in duplicate. Because many values of both hippocampi were different, left and right hemispheres were analysed separately. Values were expressed as means ±SD. Statistical significance was analysed using the Kruskal-Wallis nonparametric analysis of variance and the two-tailed Mann-Whitney U-test.

Results

Histopathological observations revealed that CA1 pyramidal cells exhibited delayed neuronal death following 6-min ischemia and three 2-min ischemic insults. The CA1 neurons appeared intact at 1 and 6 hr and 1 day, but almost all pyramidal cells had been destroyed 4 days and 1 month after ischemia. CA3 pyramidal cells and dentate granule cells showed no visible damage at any time. Two-minute ischemia caused no damage in the hippocampus.

The results of ligand binding assays are summarized in Figs. 1 through 6. Two-minute ischemia produced no persistent alterations in binding sites. We observed a transient and mild increase in binding activities, especially in [^{3}H]CHA binding, at 1 hr of recirculation. Following 6-min ischemia and three 2-min ischemic insults, [^{3}H]QNB and [^{3}H]PN200-110 binding decreased by more than 50% in the CA1 subfield by 1 month, but [^{3}H]CHA binding decreased transiently by 20-30% at 4 days when delayed neuronal death of hippocampal CA1 pyramidal cells took place. Reductions in binding, especially in [^{3}H]QNB binding, following three 2-min ischemic insults were greater and appeared earlier than those after 6-min ischemia. Furthermore, alterations extended to the CA3 subfield and the dentate gyrus following repeated insults.

[^{3}H]IP_3 binding was extremely sensitive to ischemic insult, and more than 80% of the binding sites were lost after destructon of CA1 pyramidal cells following 6-min ischemia and three 2-min ischemic insults. Furthermore, a 30% reduction was observed after 2-min ischemia which leads to no neuronal loss. [^{3}H]PDBu binding in the CA1 subfield decreased by 1 day after three 2-min ischemic insults and by 4 days after 6-min ischemia, and 40-50% reductions were observed at 1 month. In contrast, [^{3}H]forskolin binding was relatively preserved. [^{3}H]PDBu and [^{3}H]forskolin binding transiently increased early in the reperfusion period. We also observed a difference in the pattern and severity of alterations between repeated ischemic insults and single ischemia.

Discussions

[^{3}H]QNB and [^{3}H]PN200-110 binding in the CA1 subfield decreased to 45% and 40% of control, respectively, 1 month after 6-min ischemia and three 2-min ischemic insults. However, at 4 days when CA1 pyramidal cells had been destroyed, a significant decrease in [^{3}H]QNB binding was observed only in the stratum oriens (by 13%), and a reduction in [^{3}H]PN200-110 binding was not observed. Therefore, recognition sites labeled by [^{3}H]QNB and [^{3}H]PN200-110 may be resistant to degradation processes. Furthermore, because almost all CA1 pyramidal cells are destroyed following 6-min ischemia and following three 2-min ischemic insults, [^{3}H]QNB and [^{3}H]PN200-110 binding sites in the CA1 subfield for up to 40-45% of control may be localized on interneurons and presynaptic sites which are resistant to ischemic insult.

In contrast, [^{3}H]CHA binding in the CA1 subfield decreased to 70-80% of control at 4 days after 6-min ischemia and after three 2-min ischemic insults when CA1 pyramidal cells were destroyed, but the binding had returned to control level 1 month after 6-min ischemia and even increased after three 2-min ischemic insults. Therefore, [^{3}H]CHA binding sites may be located on cells other than CA1 pyramidal neurons such as presynaptic sites, interneurons, blood vessels and glial cells.

[^{3}H]QNB, [^{3}H]CHA and [^{3}H]PN200-110 binding increased in the CA3 subfield and the dentate gyrus at 1 month after three 2-min ischemic insults. Such an increase was not

observed after 6-min ischemia except for an increase in [^{3}H]PN200-110 binding in the dentate gyrus. The physiological meaning of the increase at 1 month is difficult to explain in the present study. Elimination of CA1 pyramidal cells may play a role to induce synaptic modification of the neurotransmitter system resulting in an increase in receptor density on surviving neurons or glial cells. Increase in [^{3}H]CHA binding in the CA1 subfield which was observed 4 days after 2-min ischemia and 1 month after three 2-min ischemic insults may also be explained by the synaptic modification of this inhibitory neurotransmitter system.

Among three ligands used, [^{3}H]QNB binding was most susceptible to ischemic insult. The binding decreased as early as 6 hr after three 2-min ischemic insults. Thus, the decrease in [^{3}H]QNB binding preceded delayed neuronal death of CA1 pyramidal cells following repeated insults as reported previously in transient ischemia. Furthermore, [^{3}H]QNB binding transiently decreased after 2-min ischemia which causes no morphological neuronal damage.

One of the unique features of neuronal damage following repeated ischemic insults is that the damage is most severe when ischemia is repeated at 1-hr intervals.[2,5] This phenomenon may be explained by the alterations in cerebral blood flow and metabolism which is maximally disturbed 1 hr after ischemia.[6,7] In this study, we found a transient increase in [^{3}H]QNB, [^{3}H]PN200-110, and especially [^{3}H]CHA binding in the hippocampus 1 hr after 2 or 6 min ischemia. The increase may reflect the possible alterations in receptor sensitivity and calcium conduction. Therefore, these changes in receptor binding might explain the mechanism of the cumulative damage following repeated ischemic insults which is most severe at 1-hr intervals.

Patterns of alterations in the binding activities after three 2-min ischemic insults were different from that after 6-min ischemia. Reductions in [^{3}H]QNB binding after three 2-min insults were greater and earlier that those after 6-min ischemia. Furthermore, changes were extended to the CA3 subfield and the dentate gyrus after repeated ischemic insults. These observations may further demonstrate the cumulative effect of neuronal damage after three 2-min ischemic insults which is greater than that after 6-min ischemia.

The present study also demonstrated that [^{3}H]IP$_3$ binding is remarkably susceptible to ischemic insult. [^{3}H]IP$_3$ binding in the CA1 subfield decreased as early as 1 hr after 6-min ischemia. As much as 80% of the binding was lost by 4 days after 6-min ischemia and after three 2-min ischemic insults. Therefore, [^{3}H]IP$_3$ binding sites are suggested to be predominantly localized on the CA1 pyramidal cells because almost all CA1 pyramidal cells are selectively destroyed by these ischemic insults. Furthermore, we demonstrated that 2-min ischemia causes a 30% reduction in [^{3}H]IP$_3$ binding in the CA1 subfield, although this length of ischemia does not lead to histopathological neuronal damage. Thus, [^{3}H]IP$_3$ binding is a sensitive marker for intracellular derangements after ischemia. The reduction in [^{3}H]IP$_3$ binding may be down-regulation of the intracellular receptors following excess stimulation with IP$_3$ and calcium during and after ischemia. On the other hand, IP$_3$ binding sites are believed to be localized on the endoplasmic reticulum, and IP$_3$ modulates calcium channels.

Therefore, the reductions in $[^3H]IP_3$ binding might reflect dysfunctions of intracellular calcium buffering system.

$[^3H]$PDBu binding was moderately reduced after ischemic insult. Two-minute ischemia caused no alterations in the binding except for a reduction in the stratum lacunosum-moleculare of the CA1 subfield at 1 month. Both 6-min ischemia and three 2-min ischemic insults caused only a 20-30% reduction in $[^3H]$PDBu binding in the strata oriens and radiatum of the CA1 subfield even after almost all CA1 pyramidal cells had been eliminated. However, $[^3H]$PDBu binding in the strata pyramidale and lacunosum-moleculare of the CA1 subfield decreased by 40-50% after 6-min ischemia or three 2-min ischemic insults when CA1 pyramidal cells had been destroyed. The reduction in $[^3H]$PDBu binding in the CA1 subfield following three 2-min ischemic insults was observed by 1 day when reductions were not found in animals subjected to 6-min ischemia. The earlier reductions following repeated insults may confirm the phenomenon that neuronal damage following repeated ischemic insults is greater than that after equivalent single period of ischemia.

$[^3H]$forskolin binding was most resistant to ischemic insult. Only a 25% reduction in $[^3H]$forskolin binding was observed in the CA1 subfield 4 days or 1 month after 6-min ischemia or three 2-min ischemic insults. Therefore, many $[^3H]$forskolin binding sites may be localized on cells other than postsynaptic CA1 pyramidal cells such as presynaptic sites, interneurons, glia and blood vessels. Furthermore, $[^3H]$forskolin binding is low in the CA1 region.

Of interest is that $[^3H]$forskolin and $[^3H]$PDBu binding transiently increased 1 hr after 2-min or 6-min ischemia. We observed a 20-30% increase in $[^3H]$forskolin binding in the CA1 subfield, dentate hilus and dentate gyrus following 6-min ischemia and in the dentate hilus and dentate gyrus following 2 min ischemia. We also noted an 11-13% increase in $[^3H]$PDBu binding in the strata oriens and radiatum of the CA1 subfield and the dentate gyrus following 6-min ischemia. The increase may reflect translocation of PKC from cytosol to membrane because glutamate which is massively released during ischemia can translocate PKC. Hydrolysis of phosphoinositides into diacylglycerol and IP_3 during and after ischemia may also enhance PKC activity. Redistribution and activation of PKC early in the reperfusion period may be a prerequisite for CA1 pyramidal cell death because pretreatment with PKC inhibitors prevents the cell death.

We observed an increase in $[^3H]$PDBu binding in the stratum moleculare of the dentate gyrus at 4 days and 1 month of reperfusion after 6-min ischemia. $[^3H]$forskolin binding in the dentate hilus also increased 1 month after 6-min ischemia. The observations may suggest postischemic alterations in neurotransmission in the dentate gyrus which may be induced by lesioning of the synaptic circuit in the hippocampus. Following three 2-min insults, $[^3H]$PDBu binding increased at 4 days but not at 1 month, and $[^3H]$forskolin binding also did not increase. The reason is not clear but the phenomena may reflect greater damage to these areas following repeated ischemic insults.

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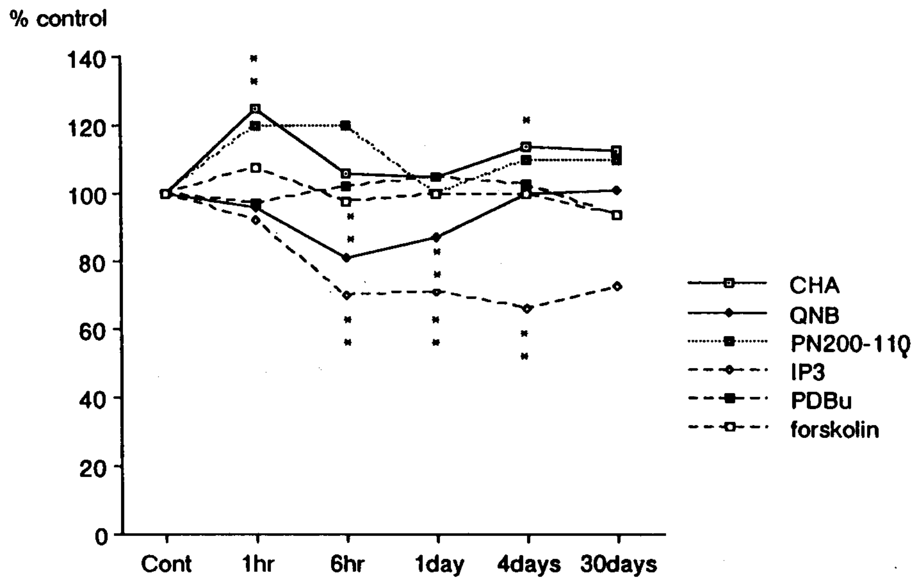

Fig. 1. Binding of neurotransmitter and second messenger systems in CA1 subfield following 2-min ischemia

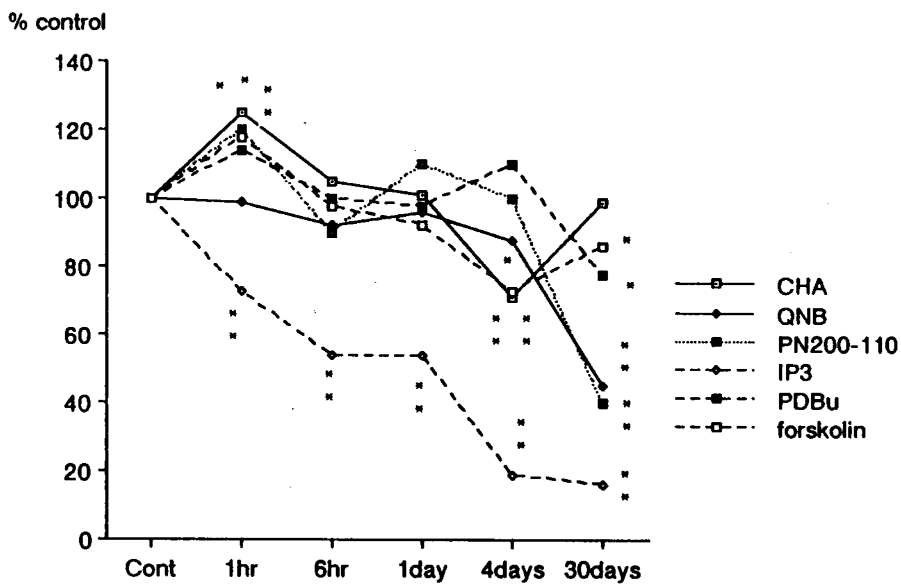

Fig. 2. Binding of neurotransmitter and second messenger systems in CA1 subfield following 6-min ischemia

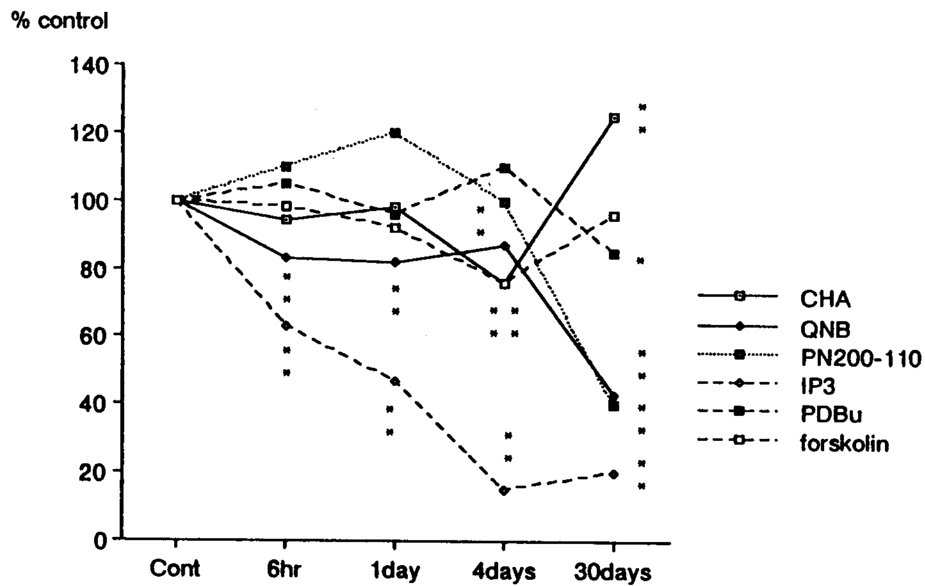

Fig. 3. Binding of neurotransmitter and second messenger systems in CA1 subfield following three 2-min ischemic insults

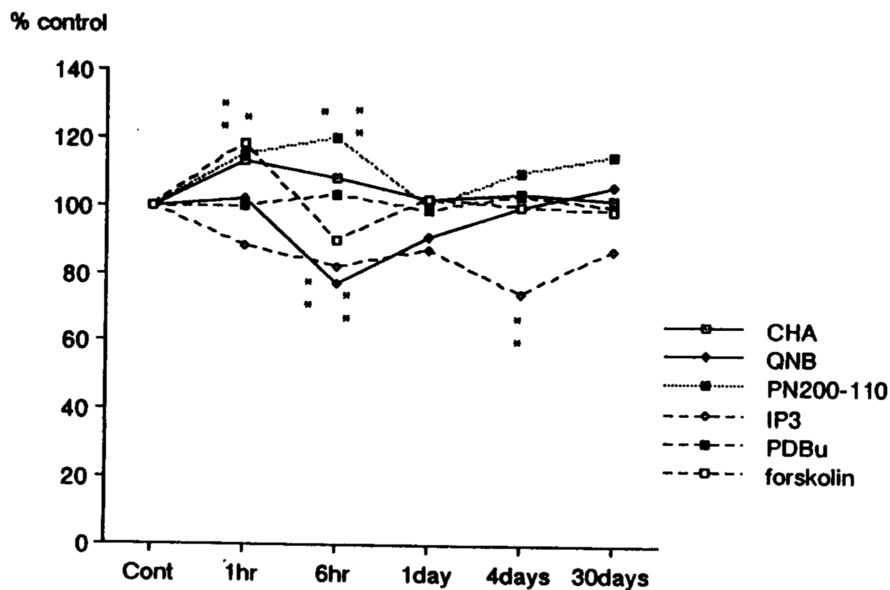

Fig. 4. Binding of neurotransmitter and second messenger systems in dentate gyrus following 2-min ischemia.

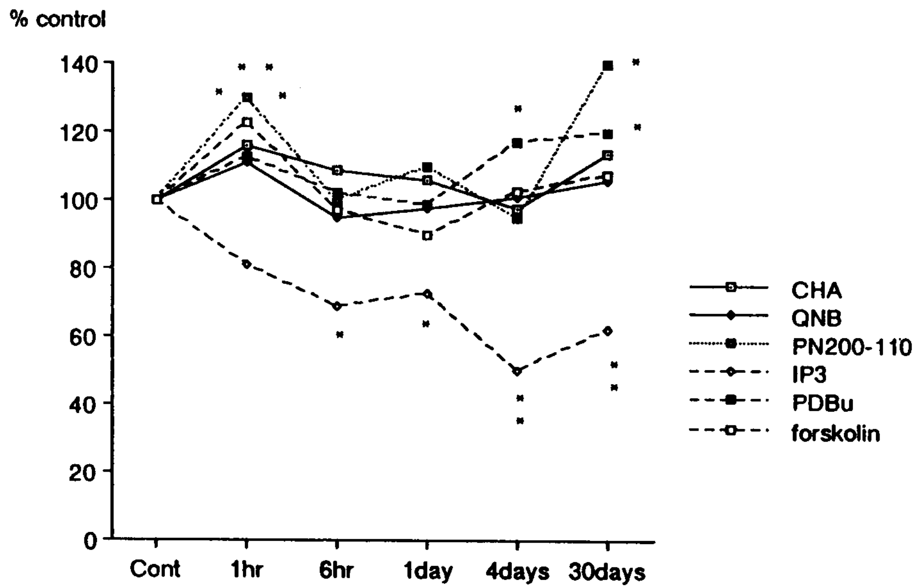

Fig. 5. Binding of neurotransmitter and second messenger systems in dentate gyrus following 6-min ischemia

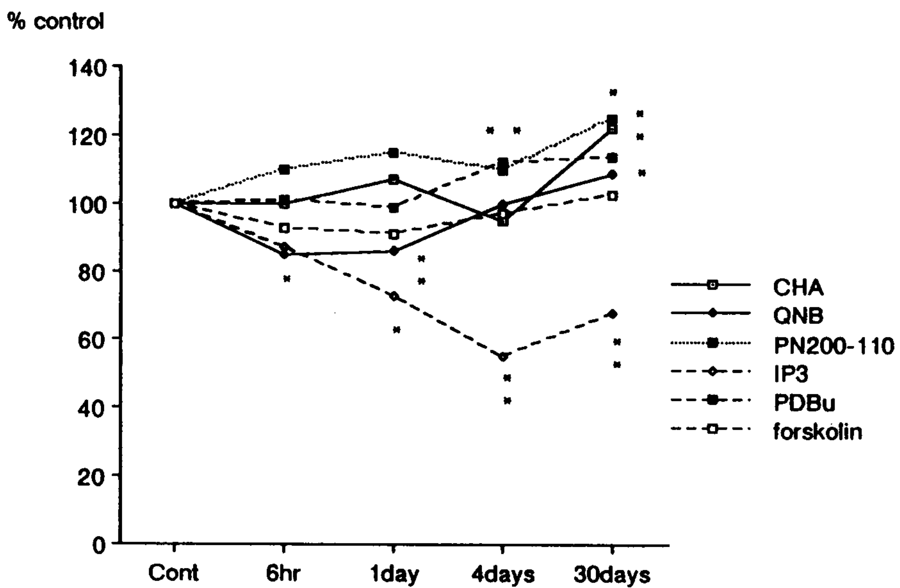

Fig. 6. Binding of neurotransmitter and second messenger systems in dentate gyrus following three 2-min ischemic insults