Autoradiographic Analysis of Protein Kinase C Activity in the Postischemic Rat Brain Using [3H] Phorbol 12,13-Dibutyrate

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Introduction

Transient cerebral ischemia leads to neuronal damage in selectively vulnerable areas. The extent of neuronal damage is dependent on the duration of ischemia [1]. Neuronal degenerative processes occur especially in the hippocampus of the rat [2] and the mongolian gerbil [3] induced by transient forebrain ischemia. Transient focal ischemia also induces neuronal degeneration in specific brain areas in the rat. Postischemic delayed neuronal damage has been reported in the ipsilateral thalamus and substantia nigra which lay outside ischemic areas of rat brain after middle cerebral artery (MCA) occlusion [4-5]. In these 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 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 [6-10].

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 processes [16] and synaptic transmission in the brain [17]. Phorbol esters are potent tumor promoters that bind to specific receptor sites with high affinity [18-19] and it is well known that the 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 ([^{3}H]PDBu) [23].

In the present study, we examined chronological changes of PKC activity of the rat brain using [^{3}H] PDBu autoradiography 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 [24]. 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, FRG) and mixed with a hardener (Elastomer Activator, Bayer Dental) 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 neurological 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 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, 2 weeks, and 4 weeks after 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. 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.

[^{3}H] PDBu autoradiography

The localization and the chronological changes of PDBu binding sites in the brain after 90 min of ischemia were measured by the autoradiographic method of Worley et al. [25] with minor modifications. Sections were incubated for 60 min at 25 °C in a buffer (50 mM Tris-HCl, 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). Autoradiograms were prepared from the sections by exposing them to [^{3}H] sensitive hyper-film (Amersham, UK) with a tritium standard microscale (Amersham) for nine days in standard X-ray cassettes.

Areas of the brain were identified with reference to the atlas of Paxinos and Watson [26]. The optical density of the brain regions was measured by a computer-assisted image analyzer (Zeiss, IBAS image analyzer system, FRG). The relationship between optical density and radioactivity was obtained with reference to the [^{3}H] microscale coexposed with the tissue sections using a third-order polynomial function. The optical density of the brain regions measured in the present study was in the range in which the optical density and radioactivity of the [^{3}H] microscale showed a near linear relationship.

Statistical analysis

Values were expressed as the means ± S.D.fmol/mg tissue using six animals. Data regarding the PDBu binding sites in each structure of the brain were analyzed using the Mann-Whitney U-test with $p < 0.05$ and $p < 0.01$ considered to be statistically significant.

Results

Autoradiographic localization of PKC using [^{3}H] PDBu in the control and postischemic brain sections is shown in Fig. 1. The values of control and chronological alteration of [^{3}H] PDBu binding sites in each structure of rat brain after 90 min of MCA occlusion and after such occlusion followed by different periods of recirculation are summarized in Table I. In sham-operated control animals, a high number of [^{3}H] PDBu binding sites was observed in the hippocampus, the neocortex, and the caudate putamen. The thalamus and the substantia nigra also exhibited relatively high numbers of [^{3}H] PDBu binding sites. These results are consistent with previous reports [23, 25].

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. Thereafter, [^{3}H] PDBu binding sites on 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 (Table I). On the contrary, there was no alteration on day 1, but 3 days after

recirculation, a marked reduction of the number of [^{3}H] PDBu binding sites was observed in the ipsilateral thalamus and the substantia nigra, which were remote from the precedent ischemic areas; thereafter, these binding sites on 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 ischemic insult (Table I). There were no significant changes in the number of [^{3}H] PDBu binding sites in the contralateral non-ischemic hemisphere.

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 regions most frequently damaged as so called ischemic foci [24]. On the other hand, 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 [4].

The present study indicated that two different alterations of the second messenger system took place in postischemic rat brain. First, in the ischemic foci, the ipsilateral FrPaSS and the CPu-L, the number of [^{3}H] PDBu binding sites rapidly decreased after 90 min of ischemia followed by 3 hours of recirculation. The reduction of PKC activities in the FrPaSS and the CPu-L is explained by the direct damage to intracellular components including the cell membrane by ischemia-induced energy failure. Second, in the exo-focal postischemic brain areas, the ipsilateral thalamus and the substantia nigra, the number of [^{3}H] PDBu binding sites was not significantly changed compared with changes in the control value 1 day after the ischemic insult; a significant reduction of these binding sites was not observed until 3 days after the ischemic insult.

This delayed change of the second messenger system observed in the thalamus and the substantia nigra was concurrent with the abnormal calcium accumulation detected there in our previous study [4]. Moreover, both phenomena, i. e., the reduction of [^{3}H] PDBu binding sites and abnormal calcium accumulation, in both remote areas on the ischemic side preceded the histologic findings of delayed neuronal damage. Below, we discuss the mechanism of exo-focal delayed neuronal damage, focusing on the intra- and inter-cellular signal transducing systems.

Recent studies have suggested that the phosphatidylinositol second messenger system is severely disturbed during and/or after brain ischemia [27-28]. This system is initiated through activation of phospholipase C which hydrolyses phosphatidylinositol-biphosphate to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol [29]. It is well known that IP_3 can cause the release of intracellular calcium from non-mitochondrial intracellular stores [29]. On the other hand, diacylglycerol activates PKC, causing phosphorylation of receptors and calcium channels [30]. PKC also plays an important role in mediating the release of neurotransmitters and may be involved in the generation of long-term potentiation [31-34]. Furthermore, PKC is increased in the cell membrane and decreased proportionally in the cytosol after long-term

potentiation [31]. [^{3}H] PDBu binding activity, therefore, may mainly reflect the translocation of PKC to the cell membrane. Moreover, the excitatory amino acid released during and after ischemia may play a key role in the translocation of PKC, because the excitatory amino acid, glutamate, can translocate PKC from cytosol to the membrane fraction [35]. Cardell et al. [36] reported that the translocation of PKC to the cell membrane in the early recirculation phase may enhance the release of neurotransmitters including neurotoxic excitatory amino acids and that down-regulation of PKC in the late stage after ischemia may severely hamper the action of the trophic factor and the survival promoting factors.

We reported that significant increases of [^{3}H] IP_3 binding sites were observed in the thalamus on the ischemic side compared with the control values 2 weeks and 4 weeks after the ischemic insult using the same model of ischemia [10]. In that study, we speculated that increased [^{3}H] IP_3 binding activity could be due to decreased levels of IP_3 in the tissue rather than to any increase of binding sites themselves and that the increase of [^{3}H] IP_3 binding sites might reflect synaptic plasticity related to neurotransmission at the chronic stage of ischemia. In another communication, we speculated that the mechanism of the postischemic neuronal damage in the thalamus and the substantia nigra might be explained by transsynaptic degeneration resulting from the thalamo-cortical and striato-nigral pathways associated with the ischemic foci, respectively, and that neurotransmitters and receptors might play important roles in the pathogenesis of the exo-focal postischemic neuronal damage [4]. The present results indicate that an alteration of the second messenger system may be involved not only in the ischemic foci, but also in neuronal degeneration of the exo-focal postischemic brain areas. From these observations we may conclude that the second messenger systems (PKC and IP_3) as well as the disturbance of calcium ion balance may play important roles in the pathogenesis of the exo-focal postischemic neuronal damage, in the neurotransmitter release, and in the synaptic plasticity. Further detailed investigation about the second messenger system including PKC is required to clarify the mechanism of transsynaptic neuronal degeneration after ischemia.

It is of interest that marked deficits in the intracellular actions of second messengers have been reported in neuronal degeneration of Alzheimer's disease [37]. A recent study suggests that marked reduction of the PKC level and/or the number of IP_3 binding sites was seen in the neocortex and hippocampus of patients with Alzheimer's disease [38]. These observations are of particular interest in relation to the role of second messengers against ischemic neuronal damage as well as in dementia. Based on the results of the present study, we suggest that second messenger systems may be involved in neuronal degeneration and/or plasticity prior to the manifestation of histologic changes and that intracellular signal transduction may play an important role in neuronal degeneration of the exo-focal postischemic brain areas.

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Table 1. Time-course of (^{3}H)phorbol 12, 13-dibutyrate binding in each structure of the rat brain after 90 min MCA occlusion followed by different periods of recirculation

Structure	Control	90-min ischemia	3 hours	6 hours	1 day
Ischemic side					
FrPaM	1232 ± 56	1228 ± 25	1211 ± 57	1157 ± 50	1254 ± 38
FrPaSS	1130 ± 76	1192 ± 34	1012 ± 75*	1004 ± 35*	954 ± 16**
CPu(L)	1038 ± 58	1042 ± 40	946 ± 35*	864 ± 58**	748 ± 59**
CPu(M)	1022 ± 82	1031 ± 44	1033 ± 45	1019 ± 50	1041 ± 48
Hippocampus	1486 ± 92	1471 ± 43	1530 ± 27	1511 ± 55	1567 ± 39
Thalamus	813 ± 43	815 ± 30	786 ± 57	775 ± 47	799 ± 35
Substantia nigra	646 ± 39	605 ± 60	605 ± 21	665 ± 73	636 ± 50
Pons	267 ± 74	255 ± 34	269 ± 23	258 ± 23	263 ± 28
Non-ischemic side					
FrPaM	1192 ± 54	1244 ± 43	1209 ± 17	1220 ± 63	1232 ± 49
FrPaSS	1141 ± 40	1186 ± 51	1178 ± 44	1163 ± 96	1093 ± 28
CPu(L)	1032 ± 60	1039 ± 43	1046 ± 40	1075 ± 54	1004 ± 61
CPu(M)	1019 ± 57	1020 ± 52	1024 ± 44	1055 ± 37	1007 ± 51
Hippocampus	1489 ± 67	1485 ± 72	1506 ± 40	1519 ± 33	1541 ± 45
Thalamus	791 ± 48	788 ± 52	802 ± 19	762 ± 41	761 ± 58
Substantia nigra	592 ± 24	618 ± 57	649 ± 26	643 ± 61	660 ± 45
Pons	272 ± 71	260 ± 33	257 ± 36	264 ± 31	252 ± 27

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<0.05; **p<0.01, significant difference from control values using the Mann-Whitney U-test.

Table 1. Time-course of (^{3}H)phorbol 12, 13-dibutyrate binding in each structure 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	1207 ± 55	1051 ± 48	1143 ± 67	1148 ± 56
FrPaSS	219 ± 26**	208 ± 24**	217 ± 37**	246 ± 25**
CPu(L)	607 ± 68**	479 ± 82**	549 ± 91**	490 ± 95**
CPu(M)	1061 ± 96	1026 ± 52	1012 ± 50	1022 ± 54
Hippocampus	1497 ± 40	1509 ± 40	1490 ± 62	1519 ± 45
Thalamus	530 ± 47**	345 ± 62**	387 ± 64**	348 ± 64**
Substantia nigra	477 ± 38**	464 ± 26**	431 ± 44**	351 ± 37**
Pons	254 ± 17	261 ± 44	236 ± 16	262 ± 49
Non-ischemic side				
FrPaM	1140 ± 54	1057 ± 57	1081 ± 50	1109 ± 74
FrPaSS	1003 ± 78	1087 ± 21	1048 ± 63	1099 ± 29
CPu(L)	1012 ± 43	1001 ± 35	996 ± 32	1039 ± 41
CPu(M)	1076 ± 41	1030 ± 42	1025 ± 55	1016 ± 53
Hippocampus	1517 ± 50	1507 ± 40	1500 ± 34	1502 ± 52
Thalamus	777 ± 35	749 ± 33	745 ± 48	755 ± 49
Substantia nigra	608 ± 43	602 ± 68	619 ± 45	632 ± 32
Pons	260 ± 39	289 ± 49	241 ± 11	257 ± 35

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<0.05; **p<0.01, significant difference from control values using the Mann-Whitney U-test.

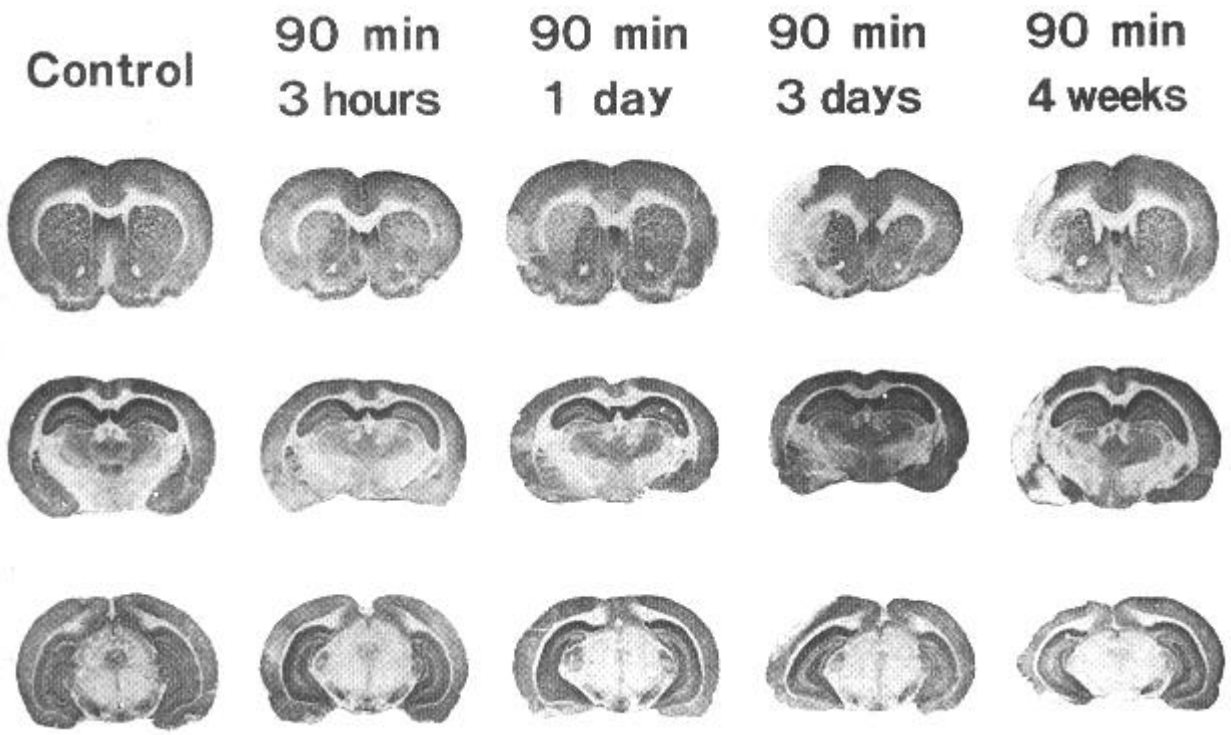

Fig. 1. [^{3}H] PDBu autoradiograms of the brains of sham-operated control rats and those obtained after 90 min of MCA occlusion followed by 3-hour, 1-day, 3-day, and 4-week recirculation. Representative autoradiograms show coronal sections at the level of the caudate putamen (top), the thalamus (middle),and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