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Specific Induction of Protein Kinase Cδ Subspecies after Transient Middle Cerebral Artery Occlusion in the Rat Brain: Inhibition by MK-801

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Protein kinase C (PKC) consists of a family of closely related Ca^{2+}/phospholipid-dependent phosphotransferase isozymes, most of which are present in the brain and are differentially activated by second messengers. Calcium-dependent PKC activity may cause neuronal degeneration after ischemic insult. PKC is also involved in trophic-factor signaling, indicating that activity of some PKC subspecies may be beneficial to the injured brain. Therefore, we screened long-term changes in the expression of multiple PKC subspecies after focal brain ischemia. Middle cerebral artery occlusion was produced by using an intraluminal suture for 30 min or 90 min. In *in situ* hybridization experiments, mRNA levels of PKCα, -β, -γ, -δ, -ε and -ζ were decreased in the infarct core 4 hr after ischemia and were lost completely 12 hr after ischemia. In areas surrounding the core, PKCδ mRNA was specifically induced 4, 12, and 24 hr after ischemia in the cortex. At 3 and 7 d, the core and a rim around it showed increased mRNA levels of PKCδ. No other subspecies were induced. At 2 d, immunoblotting demonstrated increased levels of PKCδ protein in the perifocal tissue, and immunocytochemistry revealed an increased number of PKCδ-positive neurons in the perifocal cortex. In the core, PKCδ-positive macrophages and endothelial cells were seen. Pretreatment with MK-801, an NMDA antagonist, inhibited cortical PKCδ mRNA induction. The data show that focal brain ischemia induces PKCδ mRNA and protein but not other PKC subspecies through the activation of NMDA receptors and that the upregulation lasts for several days in neurons of the perifocal zone.

Key words: phosphorylation; protein kinase C; brain ischemia; gene expression; penumbra; cortex; striatum; glutamate

In most rat models, focal brain ischemia lesions introduced by middle cerebral artery (MCA) occlusion consist of the focus, comprising the lateral striatum and the overlying neocortex, and the perifocal "penumbra," which consists of the surrounding zone of tissue insufficiently supplied by collateral flow from the leptomeningeal and the anterior and posterior cerebral arteries (Koizumi et al., 1986; Longa et al., 1989; Memezawa et al., 1992). The densely ischemic focal tissue is destined to die, but the penumbra is salvageable by recirculation or pharmacological intervention within 2–3 hr after MCA occlusion. Within a week or less after MCA occlusion, retrograde neuronal degeneration takes place in the ipsilateral thalamus (Iizuka et al., 1990), and disinhibitory overexcitation is thought to cause neuronal death in the ipsilateral substantia nigra (Saji and Reis, 1987; Nagasawa and Kogure, 1990; Tamura et al., 1990) .

Calcium overload resulting from its influx through glutamate receptors and calcium channels and its release from endoplasmic reticulum is thought to be a key mediator of neuronal death in ischemic injury (Siesjö et al., 1995). High levels of intracellular Ca^{2+} trigger activation of various enzymes, resulting in altered protein synthesis and phosphorylation, increased proteolysis, DNA fragmentation, lipolysis, and production of free radicals (Siesjö et al., 1995). Phosphorylation of target proteins by protein kinases is a major event in the mediation of cellular responses to neuronal injury. Several protein kinases, including protein kinase C (PKC) and Ca/calmodulin-dependent protein kinase are activated by calcium. Both of these kinases have been implicated in ischemic neuronal death (Hara et al., 1990; Madden et al., 1990; Aranowski et al., 1992; Maiese et al., 1993; Hanson et al., 1994; Waxham et al., 1996).

PKC consists of a family of >10 closely related Ca^{2+}/phospholipid-dependent phosphotransferase isozymes, most of which are present in the brain and are differentially activated by second messengers (Tanaka and Nishizuka, 1994). The enzyme family is subdivided into three major classes: the classical PKCs comprising α, β, and γ isoforms, all of which are calcium-dependent; the novel calcium-independent PKCs (δ, ε, η, and θ isoforms); and the atypical group whose members (ζ, λ, and μ) are both calcium- and diacylglycerol-independent enzymes (Tanaka and Nishizuka, 1994). In neurons, PKC has been implicated in the regulation of cell growth and differentiation (Dekker et al., 1989, 1990; Tanaka and Nishizuka, 1994; Roivainen et al., 1995), release of GABA, glutamate, and other neurotransmitters (Tanaka and Nishizuka, 1994; Basudev et al., 1995), apoptosis (Mailhos et al., 1994; Zhang et al., 1995a), gene expression (Angel et al., 1987; Nishizuka, 1992; Hirai et al., 1994; Tanaka and Nishizuka, 1994), synaptic plasticity (Dekker et al., 1989, 1990; Nishizuka, 1992; Tanaka and Nishizuka, 1994), ion channels (Chen and Huang, 1991; Tanaka and Nishizuka, 1994), and activity of neuronal nitric oxide synthase (nNOS) (Maiese et al., 1993; Tanaka and Nishizuka, 1994). *In vitro*, downregulation or inhibition of PKC increases neuronal protection against excitotoxicity (Favaron et al., 1990; Mattson, 1991), and *in vivo* some PKC inhibiting agents

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reduce ischemic injury (Kharlamov et al., 1993), suggesting that PKC is involved in ischemic neuronal death. Even though some studies indicate transient activation of Ca^{2+}-dependent PKCs after global brain ischemia (Cardell et al., 1991; Wieloch et al., 1991; Cardell and Wieloch, 1993), several groups have reported decreased PKC activities after global and focal brain ischemia (Crumrine et al., 1990, 1992; Louis et al., 1991; Domanska-Janik and Zalewska, 1992; Busto et al., 1994). In addition, membrane translocation of PKC in cardiac muscle cells provides transient protection against ischemia (Speechly-Dick et al., 1994; Liu et al., 1995; Mitchell et al., 1995), suggesting that immediate PKC activation rather than inhibition may be beneficial for cells with compromised energy sources.

Under certain physiological circumstances, a long-lasting and persistent Ca^{2+}-independent activation of PKC involving increased gene expression can take place in the mammalian brain (Young, 1989; Thomas et al., 1994b). The maintenance of long-term potentiation in the hippocampus, for example, was recently reported to be associated with such PKC activation (Klann et al., 1991; Thomas et al., 1994b) and to be dependent on NMDA receptors (Thomas et al., 1994a). Other reports have shown that free radicals are able to induce persistent activation of PKC in cultured astrocytoma cells (Brawn et al., 1995) and hippocampal homogenates (Palumbo et al., 1992). Because both free radicals and NMDA glutamate receptors are likely to be involved in neuronal death in focal brain ischemia, we decided to examine whether long-term alterations in mRNA or protein expression of PKC subspecies representing different subclasses takes place after transient unilateral MCA occlusion.

MATERIALS AND METHODS

Ischemia induction and tissue dissection. Focal cerebral ischemia was produced by intraluminal nylon thread introduction. Male Wistar rats (250–300 gm) were anesthetized with 4% halothane (70% N_20/30% O_2); during the operation, halothane concentration was reduced to 0.5%. The rectal temperature of the animal was maintained between 37.0 and 37.5°C with a heating pad. The left common artery was exposed, and the external carotid artery was ligated. A 0.25 mm nylon thread was inserted into the internal carotid artery up to the anterior cerebral artery. After 30 or 90 min of ischemia, restoration of the MCA blood flow was performed by removing the suture. At intervals of 0, 1, 4, or 12 hr, or 3 or 7 d after ischemia, the animals (n = 4 in each group) were anesthetized with pentobarbital (Mebunat; 40 mg/kg, i.p.) and decapitated for *in situ* hybridization. For Western and Northern blotting, two control and four ischemic animals were decapitated 2 d after 90 min of ischemia, and tissues representing the striatal infarct core and cortical perifocal area were dissected out from both the ipsilateral and contralateral sides. For immunocytochemistry, four rats were perfusion-fixed with 4% paraformaldehyde (Pease, 1962) 2 d after 90 min of ischemia, and the brains were post-fixed for 3 hr in the same fixative. Four additional animals were used to study the effect of pretreatment with MK-801, an NMDA antagonist. Three milligrams/kilogram M-K801 (Research Biochemical International, Natick, MA) were injected i.p. 30 min before 90 min of ischemia, and after 12 hr of reperfusion the animals were anesthetized with pentobarbital and processed for *in situ* hybridization. Control animals underwent identical surgery except that the thread inserted into the internal carotid artery did not reach the cerebral arteries.

In situ *hybridization.* Ten micrometer sections were cut on a cryostat at −20°C, collected on SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA), and stored at −20°C until used. The synthetic oligonucleotides used were as follows: 5′-CGGGGCCCAGCTTGGCTTTCTCGAACTTCTGC CTG-3′ (PKCα); 5′-CCTTGGTACCTTGGCCAATCTTGGCTCTCT-3′ (PKCβ); 5′-GAATGGGAGAGGAAGAGGGGCCCATCCGCACTCTC-3′ (PKCγ); 5′-AGACAGCTGTCTTCTCTCGAATCCCTGGTATATT-3′ (PKCδ); 5′-TAGACGACGAGGCTCGGTGCTCCTCTCCTCGGTTG-3′ (PKCε); 5′-GTCTGG-GTGGCCAGCATCCCTCTCTGGCTGCTTGG-3′ (PKCζ). Oligonucleotides with the length and GC-ratio similar to corresponding antisense oligonucleotides but without homology to any known gene sequences were used as controls. The probes were end-labeled with ^{35}S-ATP using terminal deoxynucleotidyl transferase (New England Nuclear, Boston, MA) and purified over Nuctrap Push Columns (Stratagene, La Jolla, CA). The slides were hybridized overnight in the hybridization solution containing 10×10^6 cpm/ml probe, 40 μl of 5 M dithiothreitol, 50 μl of salmon sperm DNA (10 mg/ml), and 900 μl of hybridization cocktail (50 ml of formamide, 20 ml of 20× SSC, 2 ml of 50 × Denhardt's reagent, 10 ml of 0.2 M sodium phosphate buffer, pH 7.4, 10 gm of dextran, 4 ml of 25% sarcosyl). The sections were washed in 1× SSC at 55°C for 2 hr, rinsed 2 × 5 min in deionized water at room temperature, dehydrated for 30 sec in 60% and 90% ethanol, air-dried, and covered with Kodak XAR-5 film for 16 d.

Northern blotting. For Northern blotting, total RNA was isolated from frozen rat brain using the TRIzol TM reagent (Life Technologies, Gaithersburg, MD) according to the instructions of the manufacturer. Samples of 30 μg/lane were electrophoresed through a formaldehyde/1.2% agarose gel and transferred to a Hybond N (Amersham, Berkshire, England) nylon membrane by capillary blotting. The membrane was hybridized with the ^{32}P-labeled oligonucleotide probe (the same as in *in situ* hybridization experiments; specific activity, 3.8×10^8 cpm/pmol) in 5× SSC/5× Denhardt's/50% formamide/1% SDS at 42°C overnight, and washed twice at room temperature for 5 min each in 2× SSC/0.1% SDS and in 0.2× SSC/0.1% SDS for 5 min at room temperature, and for 15 min at 42°C. The blot was kept wet and exposed for 8 d to Fuji x-ray film at −80°C using intensifying screens.

Immunoblotting. Tissues of the striatal core and perifocal cortical regions from the ischemic hemisphere, and of corresponding regions from the contralateral side, were homogenized separately in a buffer containing 20 mM Tris, pH 7.5, 2 mM EDTA, 5 mM EGTA, and 10 μg/ml aprotinin. Concentrated Laemmli sample buffer was added to a final concentration of 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol, and samples were heated to 95°C for 5 min. Ten to twenty micrograms of each sample were electrophoresed in 10% polyacrylamide gels. Proteins were electrophoretically transferred to nitrocellulose membranes (Hybond-C extra, Amersham), which were blocked for 1 hr at 25°C with 2% bovine serum albumin diluted in 0.02 M PBS, pH 7.2, containing 0.2% Tween. Blots were incubated with rabbit PKCα, -γ, and -δ antibodies (Life Technologies) (1:300–600 in blocking solution), mouse PKCε and -ζ antibodies (Boehringer Mannheim, Mannheim, Germany) (1:1000), and mouse PKCβ antibody (Seikagaku America, Rockville, MD) (1:1000) overnight at 4°C. After incubation for 1 hr at 25°C with anti-rabbit or anti-mouse peroxidase-conjugated antibody (Amersham) (1:1000), blots were washed four times in PBS-Tween, incubated with ECL detection reagent (Amersham), and exposed to Kodak XAR-5 film.

Immunocytochemistry. The same polyclonal PKCδ antibody used in immunoblotting was diluted 1:1000–2000 in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.3% Triton X-100 and 1% bovine serum albumin. Fifty-micrometer-thick vibratome sections were incubated in the primary antibody for 36–72 hr at 4°C, and the bound antibody was visualized with the avidin–biotin–peroxidase method (Vectastain Kit, Vector Labs, Burlingame, CA) using 3,3′-diaminobenzidine as the peroxidase substrate. Control staining included incubations with the primary antibody preabsorbed with the antigen peptide and incubations without the primary antibody.

For double-staining studies, the fixed brains were immersed in 20–30% sucrose buffer for 48 hr, snap-frozen in liquid nitrogen, and cut at 10 μm thickness in a cryostat. The sections were incubated for 48 hr in the mixture of PKC-δ (1:250) and mouse monoclonal glial fibrillary acidic protein (GFAP) (Sigma) (1:300) antibodies or PKC-δ (1:250) and mouse monoclonal OX-42 (Serotec, Oxford, UK) (1:200) antibodies. The OX-42 antibody is against complement C3 receptor and detects activated microglia and macrophages. Fluorescein isothiocyanate (FITC)-conjugated anti-rabbit (Jackson ImmunoResearch, West Grove, PA) and lissamon rhodamine-conjugated anti-mouse (Jackson ImmunoResearch) IgGs were used as secondary antibodies. The sections were coverslipped in glycerol/PBS (3:1) and examined in a Leica DMRB microscope equipped with FITC and rhodamine filter sets.

RESULTS

Expression of PKC mRNAs

In situ hybridization experiments showed distribution of the PKC subspecies similar to that reported previously (Young, 1989) (Figs. 1 and 2). Four hours after 30 or 90 min of ischemia, the mRNA levels of all PKC subspecies showed significant decrease or loss in the infarct core (Fig. 1–3). In the perifocal striatum and

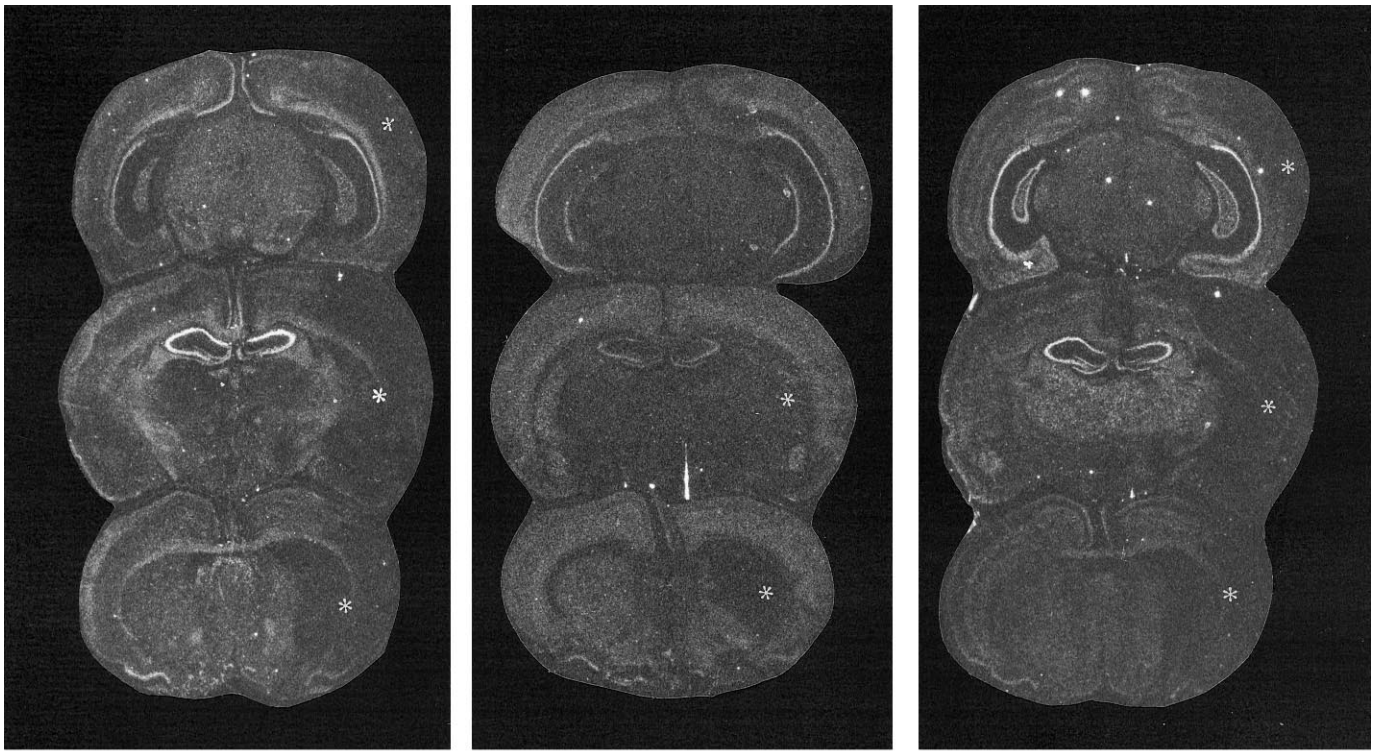

Figure 1. *In situ* hybridization autoradiographs showing the distribution of PKCα, PKCβ, and PKCγ mRNAs in the rat brain at posterior hippocampal (*top*), dorsal hippocampal (*middle*), and caudate (*bottom*) levels 24 hr after 90 min of MCA occlusion. The loss of the signal is seen in the infarct core (left hemisphere). In this particular brain, the expression PKC-β mRNA has not changed in the cortex. No changes in the mRNA expression of PKCα, PKCβ, and PKCγ subspecies are seen in the perifocal region. *Asterisks* indicate infarcted areas. Magnification, 3.5×.

the cortex, mRNA levels of PKCδ were increased slightly, especially after 90 min of ischemia (Fig. 3*A*). Although other PKC subspecies showed loss of mRNA expression in the infarcted area and no changes in perifocal regions, PKCδ mRNA was increased further in the perifocal area and in the neocortex, cingulate, and retrosplenial cortex at 12 and 24 hr (Fig. 3*B*). Occasionally, when the infarct area was very large, a slight PKCδ mRNA induction was detected at 12 hr in the hippocampus (Fig. 4*A*). Three days after the ischemia, expression of PKCδ mRNA tended to be increased in the core and was still high in a rim around the core. Seven days after the ischemia, PKCδ mRNA expression was increased further throughout the infarcted area (Fig. 3*C*). The lateral section of the ipsilateral thalamus showed a decreased signal of PKCδ both 3 and 7 d after 90 min of ischemia (Fig. 3*C*).

In brains treated with 3 mg/kg MK801 30 min before 90 min of ischemia, the PKCδ mRNA induction was inhibited in the perifocal cortex and also to a lesser extent in the perifocal striatum (Fig. 4).

To confirm the specificity of the RNA induced under ischemic conditions and recognized with the PKCδ oligonucleotide, hybridizations were performed in a 100-fold excess of unlabeled probe or with labeled oligonucleotides of the same length and GC-ratio but with the homology <80%. No PKCδ hybridization signal was detected in these experiments (not shown). In addition, Northern blotting with the PKCδ oligonucleotide revealed a single band of ~3.1 kb in the blot (Fig. 5).

PKC-immunoreactive proteins

Immunoblotting demonstrated protein expression of all the PKC subspecies in both the cortical and striatal regions supplied by the middle cerebral artery (Fig. 6). In samples representing the perifocal cortical area, the PKCδ-immunoreactive band was more prominent in the ischemic than in the contralateral side in three separate experiments. No such difference was seen in tissues representing the infarct core area. Similarly, no consistent differences were seen in other PKC subspecies in tissues representing either perifocal or infarct core areas.

In control brains, immunocytochemistry showed a high density of strongly PKCδ-positive cell bodies in the thalamus and septum (not shown). In the frontoparietal and cingulate cortex of control animals and in the contralateral side of ischemic brains (Fig. 7*A*), few immunolabeled neurons were observed. In addition, immunoreactive nerve fibers were seen throughout the brain and were stained most intensely in the striatum (Fig. 7*E*). Two days after 90 min of ischemia, an increased number of immunostained neurons were seen in the ipsilateral cortex around the infarcted core (Fig. 7*B–D*). In the perifocal striatum, the PKCδ-immunoreactive nerve fibers were stained more intensely than the fibers on the contralateral side (Fig. 7*E,F*). In the core, several immunoreactive cells were seen, especially around blood vessels, very likely representing endothelial cells (Fig. 7*G*). Many macrophage-like cells stained with the PKCδ antibody were also seen in the infarcted core (Fig. 7*H*).

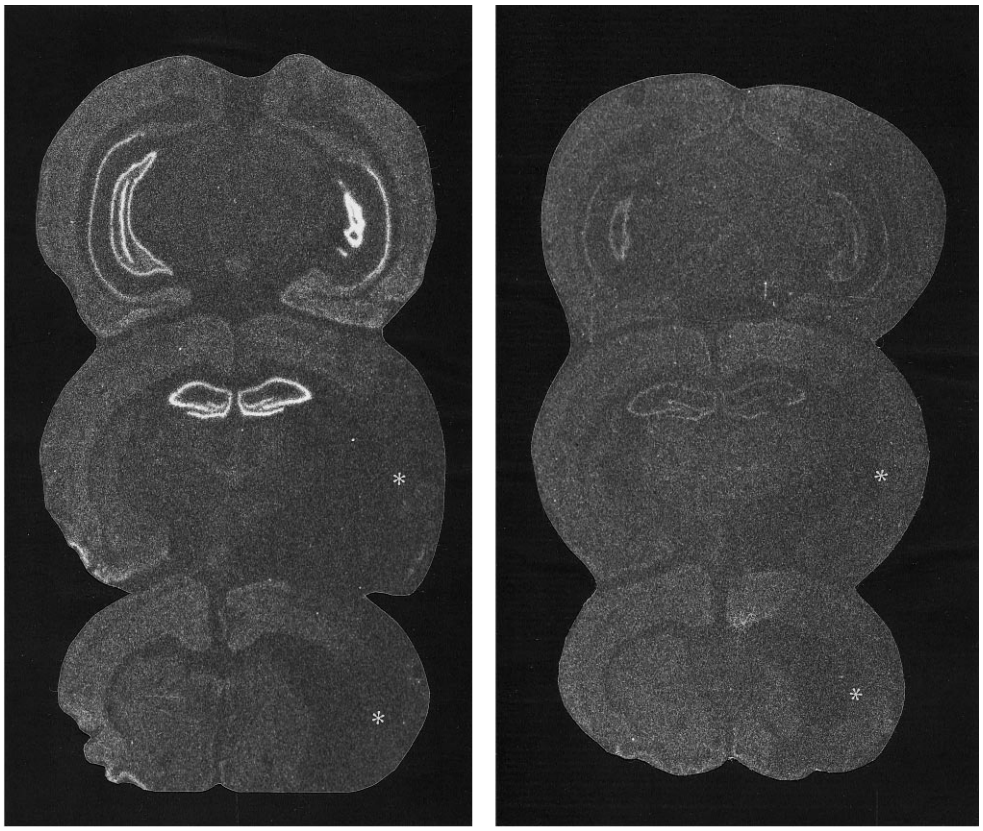

Figure 2. *In situ* hybridization autoradiographs showing the distribution of PKCε and PKCζ mRNAs in the rat brain at posterior hippocampal (*top*), dorsal hippocampal (*middle*), and caudate (*bottom*) levels 24 hr after 90 min of MCA occlusion. The loss of the signal is seen in the infarct core (left hemisphere). No changes in the mRNA expression of PKCε and PKCζ subspecies are seen in the perifocal region. *Asterisks* indicate infarcted areas. Magnification, 5×.

At 3 d, double-staining experiments showed that several PKCδ-immunopositive cells in the infarcted area (Fig. 8*A,B*) and in the thin perifocal rim were OX-42-positive (Fig. 8*C,D*). These cells were usually round and had only few if any processes (Fig. 8*A,B*), indicating that they represented invaded macrophages rather than activated microglia. Some PKCδ-immunoreactive structures seen around blood vessels were stained only faintly or not at all with the OX-42 antibody, supporting the conclusion that PKCδ is also expressed by endothelial cells in the infarct core. GFAP-immunoreactive cells were not seen in the infarct core. In the perifocal area, GFAP was not colocalized with PKCδ (Fig. 8*E,F*).

DISCUSSION

This study shows that from the multiple PKC subspecies expressed in the brain, transient focal brain ischemia specifically induces PKCδ mRNA and protein in neurons in the perifocal cortex and striatum, followed by long-lasting expression in macrophage-like cells and possibly in endothelial cells in the ischemic region. The early induction takes place in surviving neurons and involves the activation of NMDA receptors, suggesting that spreading depression (SD) accounts for the upregulation. Even though cortical SD is believed to exacerbate ischemic injury (Nedergaard and Astrup, 1986; Hansen and Nedergaard, 1988; Siesjö, 1991; Siesjö et al., 1995), recent studies have demonstrated that brief focal ischemia (Glazier et al., 1994) and SD cause cortical and hippocampal neurons to acquire tolerance to ischemia (Kawahara et al., 1993; Kobayashi et al., 1995). Altogether, the regulation of PKCδ subspecies differs remarkably from the other PKC subspecies examined and serves to adapt the energy-compromised perifocal tissue to altered requirements of protein phosphorylation during recovery from ischemic insult.

SD consists of repeated ionic transients involving release of K^+ and uptake of Ca^{2+}, Na^+, and Cl^-, and it is most likely initiated and propagated by massive presynaptic release of glutamate and activation of NMDA receptors subsequent to local brain injury, including focal brain ischemia (Kraig and Nicholson, 1978; Hansen, 1985; Nedergaard and Astrup, 1986; Hansen and Nedergaard, 1988; Marrannes et al., 1988). Because restoration of the ionic gradients consumes high-energy phosphates, SD may lower the threshold of neuronal death during and immediately after ischemia. The induction of PKCδ occurred several hours after the insult, indicating that PKCδ is not likely to have a causal role in immediate ischemic cell death. Li et al. (1995) reported apoptotic cell death to be an ongoing process from 30 min through 4 weeks after focal brain ischemia, peaking at 1–2 d, with a scattered distribution in the ischemic region. The temporal profile of apoptotic cell death covers somewhat the period of upregulated expression of PKCδ described in the present study; however, PKCδ expression peaked at 1 d in surviving perifocal neurons, stayed moderately high in a thin perifocal rim through 7 d, and was further increased in non-neuronal cells in the infarct core at 7 d, suggesting that cells expressing PKCδ included cells not going through apoptosis, or did not overlap with the dying cells at all. The immediate loss of PKCδ mRNA in the infarct core supports the hypothesis that induction of PKCδ gene does not contribute to the immediate ischemic neuronal death in focal brain ischemia.

At later time points, the neuronal PKCδ expression was re-

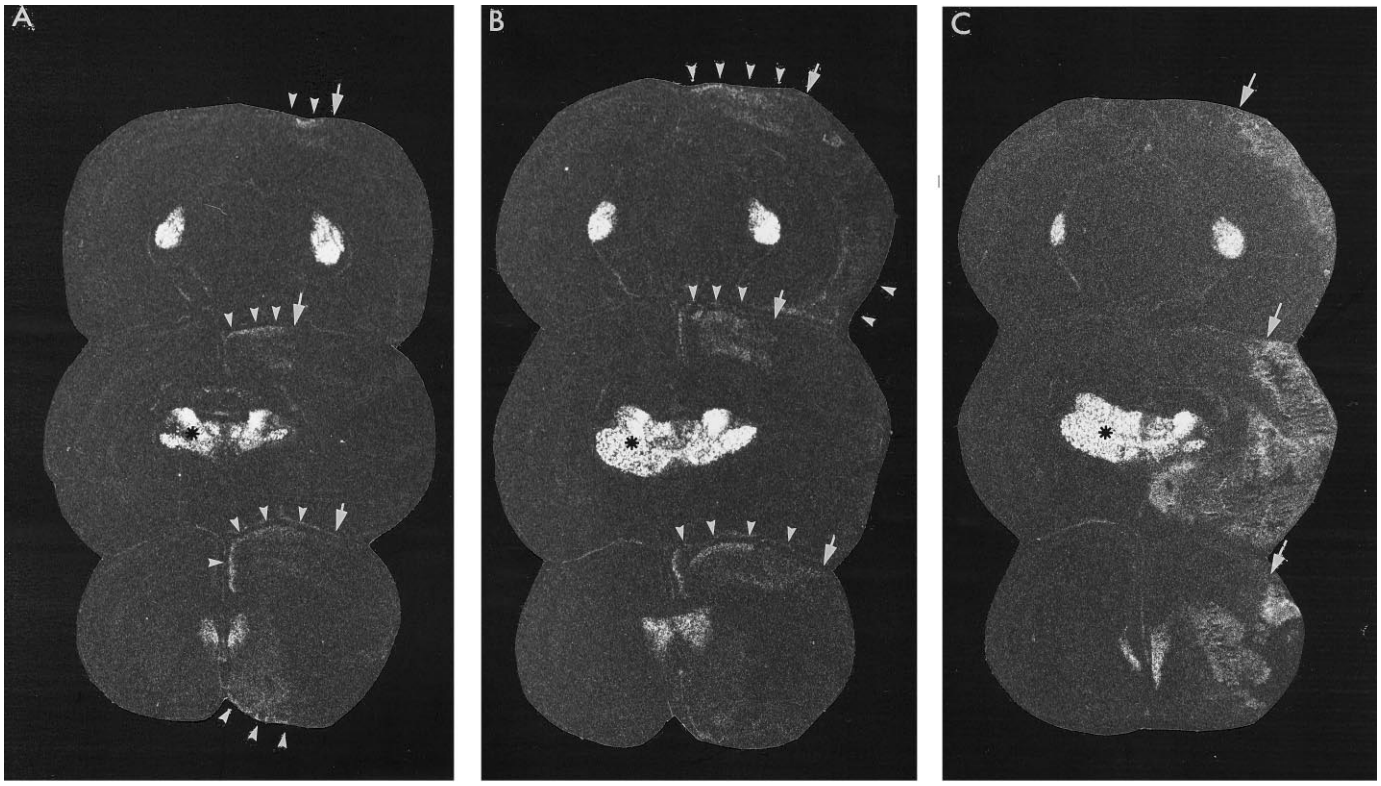

Figure 3. *In situ* hybridization autoradiographs showing the distribution and induction of PKCδ mRNA in the rat brain at posterior hippocampal (*top*), dorsal hippocampal (*middle*), and caudate (*bottom*) levels 4 hr (*A*), 24 hr (*B*), and 7 d (*C*) after 90 min of MCA occlusion. In the infarct core (*right*), the expression of PKCδ is decreased or lost at 4 hr (*A*), but is increased substantially 7 d (*C*) after 90 min of ischemia. Concomitantly, the perifocal and cortical expression is increased 4 hr (*A*) and 24 hr (*B*) (*arrowheads*) after the insult, but it is back to control levels 7 d (*C*) after the insult. The signal is decreased in the lateral section of the thalamus 7 d (*C*) after ischemia. *Arrows* (*A–C*) point to the upper margin of the infarcted areas, and *arrowheads* (*A, B*) point to the perifocal area with high expression of PKCδ mRNA. *Stars* (*A–C*) show the thalamic nuclei with high expression of PKCδ mRNA. Magnification, 8×.

stricted to the infarct core and a narrow zone surrounding it. This penumbral zone also shows upregulation of a number of other inducible genes, such as heat-shock proteins (Welsh et al., 1992; Kinouchi et al., 1993), amyloid precursor proteins (Pyykönen et al., 1995), cyclo-oxygenase-2 (our unpublished observations), heme oxygenase-1 (Koistinaho et al., 1996), many immediate early genes (An et al., 1993; Kinouchi et al., 1994a,b), and growth factors (Hsu et al., 1993; Iihara et al., 1994), indicating that cells at most immediate risk to die induce expression of a specific set of genes and their protein products. It is not known which proteins are involved in processes causing neuronal death and which proteins represent survival attempts by the neurons. Many of these cells are also expressed by non-neuronal cells. The expression of certain immediate early genes such as c-*fos* sometimes precedes programmed cell death (Schilling et al., 1993; Kasof et al., 1995), whereas it has been suggested that c-*jun* is a marker for surviving neurons (Sommer et al., 1995) but also necessary for apoptotic neuronal death (Estus et al., 1994; Schlingensiepen et al., 1994; Andersson et al., 1995; Ham et al., 1995). In certain non-neuronal cells, proteolytic activation of PKCδ has been associated with delayed cell death (Emoto et al., 1995). Because no proteolytic PKCδ fragments were detected 2 d after ischemia, the possibility that PKCδ induction is involved in delayed neuronal death after the insult is unlikely, but it cannot be excluded.

In the lateral part of the ipsilateral thalamus, which is not supplied by MCA, the expression of PKCδ mRNA declined 3 d after 90 min of ischemia, and 4 d later the expression was clearly below the control levels. The time course corresponds to the delayed degeneration of ipsilateral thalamic neurons, which is thought to be secondary to axonal damage in the cortical infarct (Iizuka et al., 1990). The reduction of PKCδ mRNA in the ipsilateral thalamus therefore is likely attributable to the degeneration of neurons expressing the gene.

Crumrine et al. (1992) reported a 50% reduction in PKC activity 6 hr after permanent focal brain ischemia, which is in agreement with many other studies measuring PKC activity after spinal cord (Kochar et al., 1989) or global brain ischemia (Crumrine et al., 1990; Louis et al., 1991; Domanska-Janik and Zalewska, 1992; Busto et al., 1994). Some studies have shown that after global ischemia there is a rapid translocation of cPKC subspecies, which is not associated with increased PKC activity (Cardell et al., 1991; Wieloch et al., 1991; Cardell and Wieloch, 1993). It has been suggested that the discrepancy can be explained by the activation of a PKC inhibitor or alternatively by a rapid downregulation of PKC during ischemia (Kochar et al., 1989; Cardell and Wieloch, 1993). It is also possible that total PKC activity decreases when specific subspecies, such as PKCδ in the present study, are increased concomitantly with a decrease in

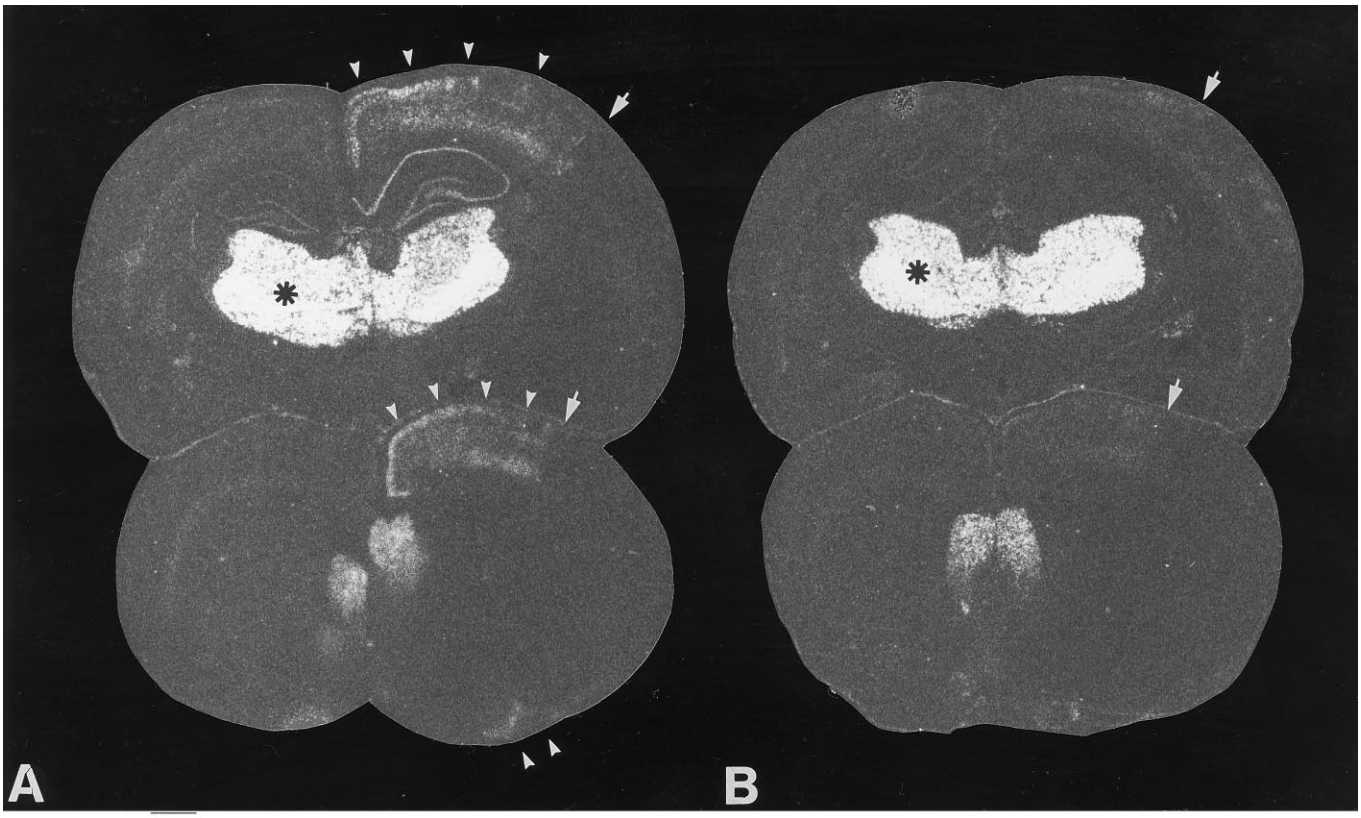

Figure 4. *In situ* hybridization autoradiographs showing inhibition of ischemia-induced perifocal PKCδ mRNA by administration of MK-801 (3 mg/kg) 30 min before 90 min of ischemia and followed by 12 hr of reperfusion. Sections at dorsal hippocampal (*top*) and caudate levels are shown. The ischemia-induced expression of PKCδ mRNA (*arrowheads*) is reduced significantly by MK-801 in perifocal regions, especially in the cortical area including the cingulate cortex. *A* shows an ischemic animal pretreated with 0.9% NaCl; *B* shows an ischemic animal pretreated with MK-801. In the brain shown in *A*, the lesion-induced PKCδ mRNA also encompasses the hippocampus. *Arrows* point to the infarct margins, and *asterisks* point to the thalamus that shows a high basal level of PKCδ mRNA expression. Magnification, 5×.

other subspecies. Interestingly, decreased expression of any PKC subspecies mRNA was not followed by an immediate reduction in the protein levels. The discrepancy may be attributable to the increased expression of PKC subspecies in endothelial (Krizbai et al., 1995) and other non-neuronal cells (Nishizuka, 1992; Gott et al., 1994) and to the fact that neuronal PKC proteins are not completely degraded within 2 d after transient focal ischemia.

There are several intracellular functions that could be influenced by the observed long-term induction of PKCδ in focal

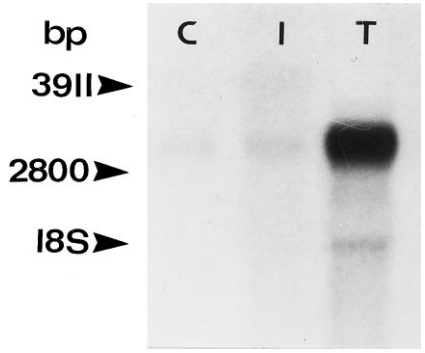

Figure 5. Northern blotting analysis of total RNA from the rat brain 2 d after ischemia. A ^{32}P-dATP-labeled PKCδ-oligonucleotide probe was used. The blot shows a band of ~3.1 kb. *C*, Contralateral cortex; *I*, ischemic perifocal cortex; *T*, thalamus. The signal is weak in the cortical tissues but is increased in the ischemic cortex.

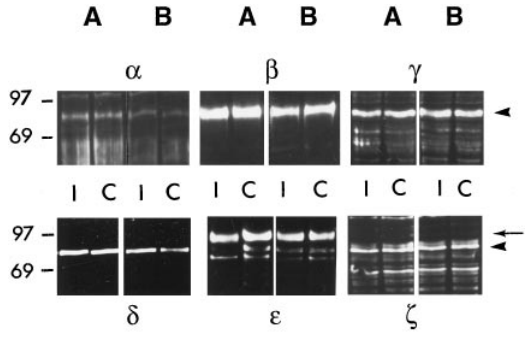

Figure 6. Western blots of PKC subspecies in homogenates of tissues from the striatal core (*A*) and perifocal cortical (*B*) regions from the ischemic hemisphere (*I*) and of the corresponding region on the contralateral side (*C*). *Arrowheads* indicate positions of PKCα, -β, -γ, -δ, and -ζ subspecies, and a *small arrow* points to PKCε, visible in the blots. The only clear change seen consistently in three separate experiments is the increase of PKCδ in the perifocal tissue from ischemic hemispheres (*B*, *I,* and *C*).

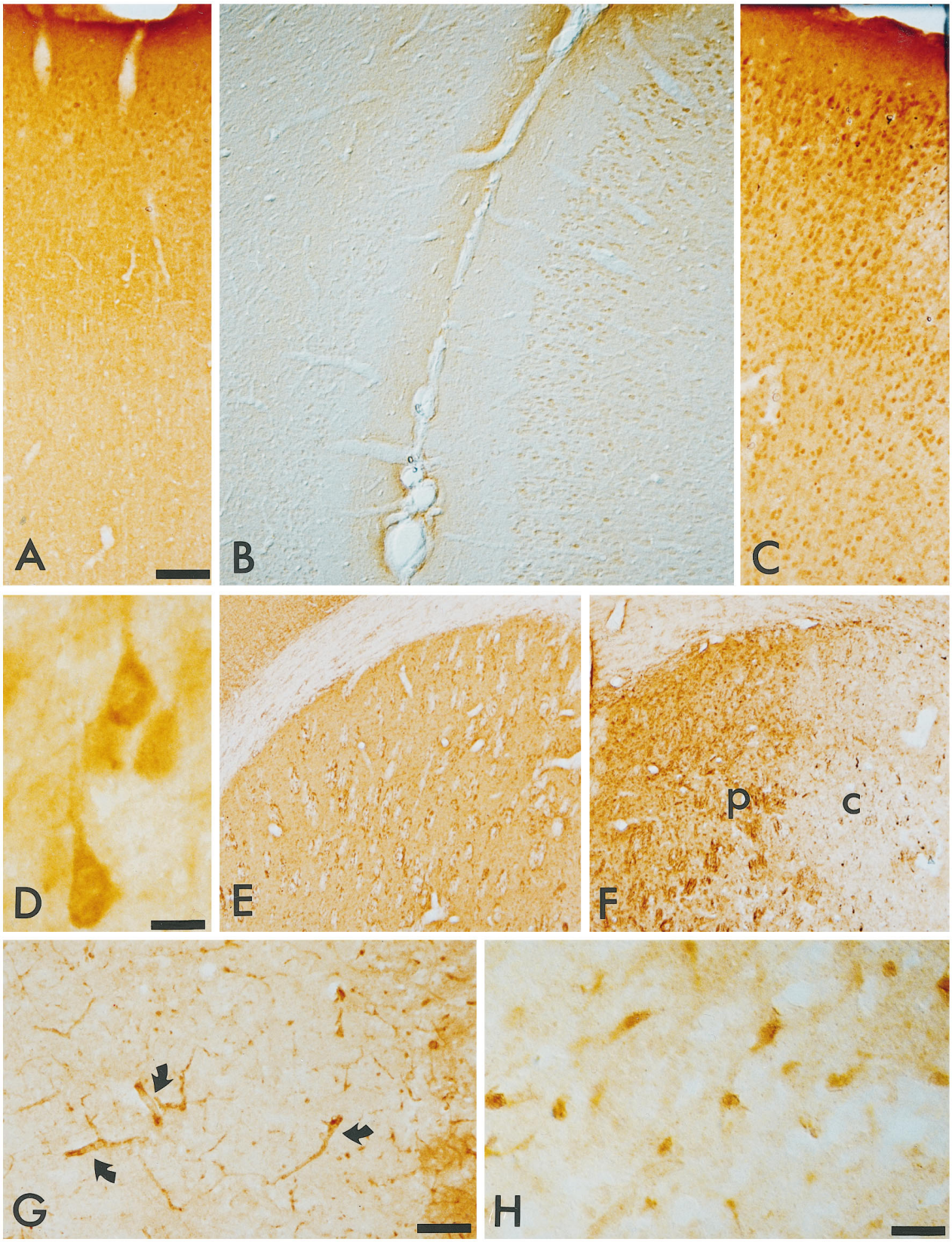

Figure 7. PKCδ-immunoreactive cells in the rat brain 2 d after 90 min of ischemia. On the contralateral side of the frontoparietal (*A*) and cingulate (*B*, *left*) cortex, only a few immunoreactive neurons are seen, whereas in corresponding areas on the ipsilateral side (*B*, *right*; *C*), a large population of cortical neurons are immunoreactive. In cortical neurons, immunolabeling is extranuclear (*D*). In the contralateral striatum, numerous immunoreactive bundles of nerve fibers are seen (*E*). In the ipsilateral striatum (*F*), these nerve fibers have disappeared in the ischemic core (*c*), but have become strongly immunoreactive in the perifocal zone (*p*). In the infarct core, immunoreactive material is seen around small blood vessels, presumably in endothelial cells (*G, curved arrows*) and in glial-like cells (*H*). Scale bars: *A–C, E, F*, 250 μm; *D*, 25 μm; *G*, 125 μm; *H*, 50 μm.

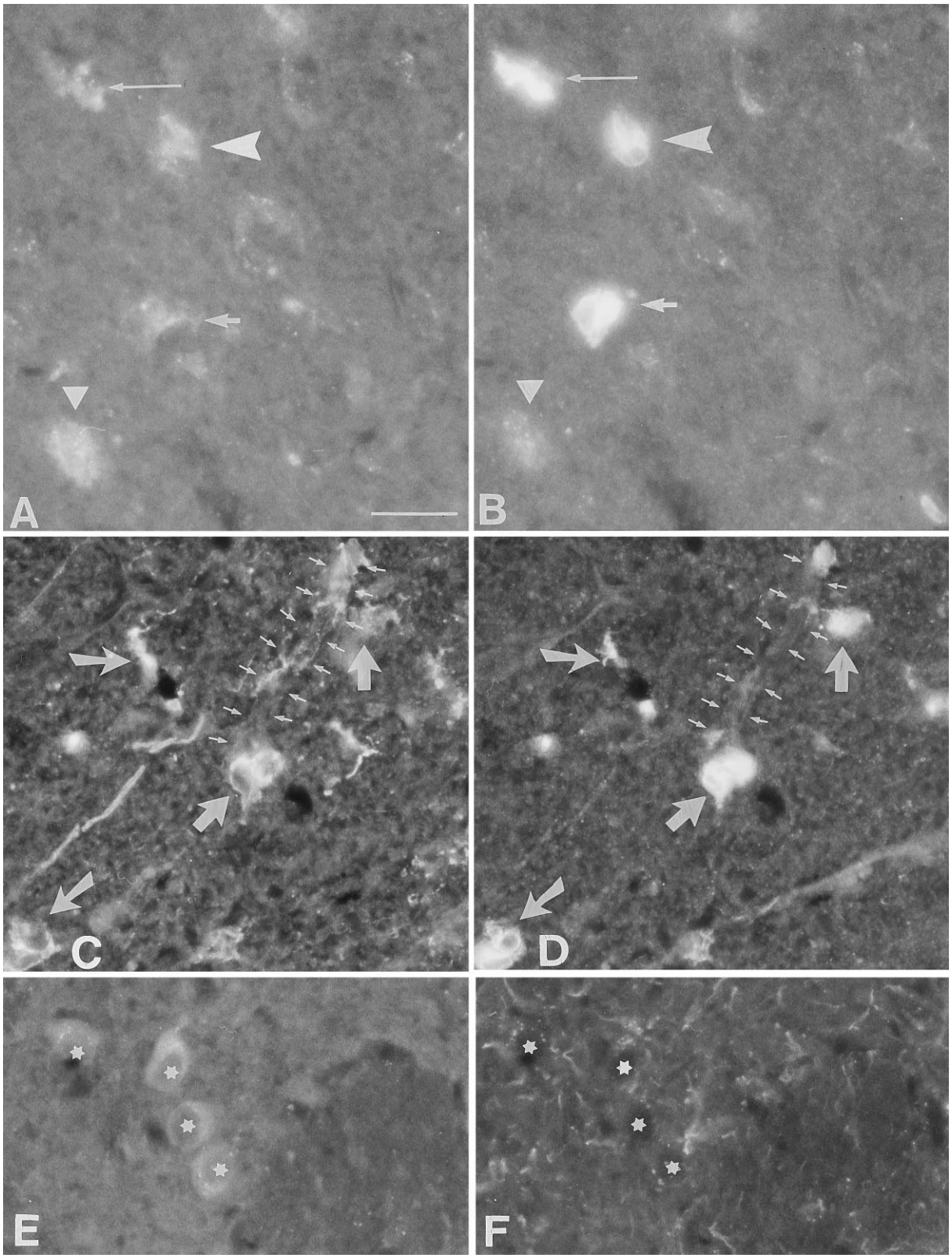

Figure 8. Immunofluorescence micrographs of focal (*A, B*) and perifocal (*C–F*) PKCδ immunoreactive cells double-stained with antiserum to complement C3 receptor, a marker for activated microglia and macrophages (*A–D*), and with an antiserum to GFAP (*E, F*), a marker for activated astrocytes, 3 d after 90 min of ischemia. Most of the PKCδ immunoreactive cells are macrophage-like cells with OX-42 immunoreactivity, round cell body, and few processes. GFAP-immunoreactive structures are not labeled with PKCδ antibody. *Large arrows* and *arrowheads* point to double-labeled macrophage-like cells. *Small arrows* show a small blood vessel surrounded by PKCδ-immmunoreactive cells and processes, some of which are also OX-42-immunoreactive. A field shown in *E* and *F* includes PKCδ-immunoreactive neuronal cell bodies (*white stars*) that are surrounded by GFAP-positive astrocyte processes (*F*). Scale bar (shown in *A*): 40 μm (*A, B, E, F*); 80 μm (*C, D*).

ischemia. In macrophages, PKCδ is the most abundant subspecies (Jun et al., 1994), and its expression may be relevant, for example, in the regulation of inducible nitric oxide synthase after ischemia-reperfusion injury. PKC also regulates neuron-specific nNOS (Maiese et al., 1993), but the wide distribution of the ischemia-induced expression of PKCδ seems not to be colocalized with scattered nNOS-expressing neurons (Dawson et al., 1991). In endothelial cells, PKC phosphorylation causes a 30% reduction in the activity of endothelial NOS (Hirata et al., 1995), which is localized also in hippocampal CA1 neurons (Dinerman et al., 1994). In addition to the functions in gene regulation, PKCδ expression may regulate specific glutamate receptor subtypes, release of excitatory or inhibitory neurotransmitters, or membrane-bound ionic pumps (Chen and Huang, 1991; Nishizuka and Tanaka, 1994; Basudev et al., 1995), all of which are evidently relevant for plastic changes after focal brain ischemia.

REFERENCES

An G, Lin TN, Liu JS, Xue JJ, He YY, Hsu CY (1993) Expression of c-*fos* and c-*jun* family genes after focal cerebral ischemia. Ann Neurol 33:457–464.

Anderson AJ, Pike CJ, Cotman CW (1995) Differential induction of immediate early gene proteins in cultured neurons by beta-amyloid (A beta): association of c-Jun with A beta-induced apoptosis. J Neurochem 65:1487–1498.

Angel P, Imagawa M, Chui R, Stein B, Imbra RJ, Rahmstorf HJ, Jonart C, Herrlich P, Karin M (1987) Phorbol ester-inducible genes contain a common *cis* element recognized by a TPA-modulated *trans*-acting factor. Cell 49:729–739.

Aronowski J, Grotta JC, Waxham MN (1992) Ischemia-induced translocation of Ca^{2+}/calmodulin-dependent protein kinase II: potential role in neuronal damage. J Neurochem 58:1743–1753.

Basudev H, Romano-Silva MA, Brammer MJ, Campbell IC (1995) Effect of sodium on PKC translocation: relationship to neurotransmitter release. NeuroReport 6:809–812.

Brawn MK, Chiou WJ, Leach KL (1995) Oxidant-induced activation of protein kinase C in UC11MG cells. Free Radic Res 22:23–37.

Busto R, Globus MY-T, Neary JT, Ginsberg MD (1994) Regional alterations of protein kinase C activity following transient cerebral ischemia: effects of intraischemic brain temperature modulation. J Neurochem 63:1095–1103.

Cardell M, Wieloch T (1993) Time course of the translocation and inhibition of protein kinase C during complete cerebral ischemia in the rat. J Neurochem 61:1308–1314.

Cardell M, Boris-Moller F, Wieloch T (1991) Hypothermia prevents the ischemia-induced translocation and inhibition of protein kinase C in the rat striatum. J Neurochem 57:1814–1817.

Chen L, Huang L-YM (1991) Sustained potentiation of NMDA receptor-mediated glutamate responses through activation of protein kinase C by a μ opioid. Neuron 7:319–326.

Crumrine RC, Dubyak G, LaManna JC (1990) Decreased protein kinase C activity during cerebral ischemia and after reperfusion in the adult rat. J Neurochem 55:2001–2007.

Crumrine RC, Selman WR, LaManna JC, Lust WD (1992) Protein kinase C activity in permanent focal cerebral ischemia. Mol Chem Neuropathol 16:85–93.

Dawson TM, Bredt DS, Fotuhi M, Hwang PM, Snyder SH (1991) Nitric oxide synthase and neuronal NADPH diaphorase are identical in brain and peripheral tissues. Proc Natl Acad Sci USA 88:7797–7801.

Dekker LV, DeGraan PN, Oestreicher AB (1989) Inhibition of noradrenaline release by antibodies to B-50. Nature 342:74–76.

Dekker LV, DeGraan PN, Oestereicher AB (1990) Depolarization-induced phosphorylation of the protein kinase C substrate B-50 (GAP-43) in rat cortical synaptosomes. J Neurochem 54:1645–1652.

Dinerman JL, Dawson TM, Schell MJ, Snowman A, Snyder SH (1994) Endothelial nitric oxide synthase localized to hippocampal pyramidal cells: implications for synaptic plasticity. Proc Natl Acad Sci USA 91:4214–4218.

Domanska-Janik K, Zalewska T (1992) Effect of brain ischemia on protein kinase C. J Neurochem 58:1432–1439.

Emoto Y, Manome Y, Meinhardt G, Kisaki H, Kharbanda S, Robertson M, Ghayur T, Wong WW, Kamen R, Weichelbaum R, Kufe D (1995) Proteolytic activation of protein kinase C δ by an ICE-like protease in apoptotic cells. EMBO J 14:6148–6156.

Estus S, Zaks WJ, Freeman RS, Gruda M, Bravo R, Johnson EM Jr (1994) Altered gene expression in neurons during programmed cell death: identification of c-*jun* as necessary for neuronal apopotosis. J Cell Biol 127:1717–1727.

Favaron M, Manev H, Siman R, Bertolino M, Szekely AM, De-Erausquin G, Guidotti A, Costa E (1990) Down-regulation of protein kinase C protects cerebellar granule neurons in primary culture from glutamate-induced neuronal death. Proc Natl Acad Sci USA 87:1983–1987.

Glazier SS, O'Rourke DM, Graham DI, Welsh FA (1994) Induction of ischemic tolerance following brief focal ischemia in rat brain. J Cereb Blood Flow Metab 14:545–553.

Gott AL, Mallon BS, Paton A, Groome N, Rumsby MG (1994) Rat brain glial cells in primary culture and subculture contain the δ, ϵ and ζ subspecies of protein kinase C as well as the conventional subspecies. Neurosci Lett 171:117–120.

Ham J, Babij C, Whitfield J, Pfarr CM, Lallemand D, Yaniv M, Rubin LL (1995) A c-*jun* dominant negative mutant protects sympathetic neurons against programmed cell death. Neuron 14:927–939.

Hansen AJ (1985) Effect of anoxia on ion distribution in the brain. Physiol Rev 65:101–148.

Hansen AJ, Nedergaard M (1988) Brain ion homeostasis in cerebral ischemia. Neurochem Pathol 9:195–209.

Hanson SK, Grotta JC, Waxham MN, Aronowski J, Ostrow P (1994) Calcium/calmodulin-dependent protein kinase II activity in focal ischemia with reperfusion in rats. Stroke 25:466–473.

Hara H, Onodera H, Yoshidomi M, Matsuda Y, Kogure K (1990) Staurosporine, a novel protein kinase C inhibitor, prevents postischemic neuronal damage in the gerbil and rat. J Cereb Blood Flow Metab 10:646–653.

Hirai S, Izumi Y, Higa K, Kaibuchi K, Mizuno K, Osada S-I, Suzuki K, Ohno S (1994) Ras-dependent signal transduction is indispensable but not sufficient for the activation of AP1/Jun by PKCδ. EMBO J 13:2331–2340.

Hirata K, Kuroda R, Sakoda T, Katyama M, Inoue N, Suematsu M, Katayama M, Inoue N, Suematsu M, Kawashima S, Yokoyama M (1995) Inhibition of endothelial nitric oxide synthase activity by protein kinase C. Hypertension 25:180–185.

Hsu CY, An G, Liu JS, Xue JJ, He YY, Lin TN (1993) Expression of immediate early gene and growth factor mRNAs in a focal cerebral ischemia model in the rat. Stroke 24 [Suppl I]:I78–I81.

Iihara K, Sasahara M, Hashimoto N, Uemura Y, Kikuchi H, Hazama F (1994) Ischemia induces the expression of the platelet-derived growth factor-B chain in neurons and brain macrophages in vivo. J Cereb Blood Flow Metab 14:818–824.

Iizuka H, Sakatani K, Young W (1990) Neural damage in the rat thalamus after cortical infarcts. Stroke 21:790–794.

Jun CD, Choi BM, Lee SY, Kang SS, Kim HM, Chung HT (1994) Nitric oxide inhibits the expression of protein kinase C delta gene in the murine peritoneal macrophages. Biochem Biophys Res Commun 204:105–111.

Kasof GM, Mandelzys A, Maika SD, Hammer RE, Curran T, Morgan JI (1995) Kainic acid-induced neuronal death is associated with DNA damage and unique immediate-early gene response in c-*fos*-lacZ transgenic rats. J Neurosci 15:4238–4249.

Kawahara N, Penix L, Ruetzler CA, Wagner HG, Klatzo I (1993) Protective effects of spreading depression (SD) in cardiac arrest cerebral ischemia (CACI). Soc Neurosci Abstr 19:1666.

Kharlamov A, Guidotti A, Costa E, Hayes R, Armstrong D (1993) Semisynthetic sphingolipids prevent protein kinase C translocation and neuronal damage in the perifocal area following a photochemically induced trombotic brain cortical lesion. J Neurosci 13:2483–2494.

Kinouchi H, Sharp FR, Koistinaho J, Hicks K, Kamii H, Chan PH (1993) Induction of heat shock hsp70 mRNA and hap70 kDa protein in neurons in the penumbra following focal cerebral ischemia in the rat. Brain Res 619:334–338.

Kinouchi H, Sharp FR, Chan PH, Koistinaho J, Sagar SM, Yoshimoto T (1994a) Induction of NGFI-A mRNA following middle cerebral artery occlusion in rats: in situ hybridization study. Neurosci Lett 171:163–166.

Kinouchi H, Sharp FR, Chan PH, Koistinaho J, Sagar SM, Yoshimoto T (1994b) Induction of c-*fos*, *jun*-B, c-*jun*, and hsp70 mRNA in cortex, thalamus, basal ganglia, and hippocampus following middle cerebral artery occlusion. J Cereb Blood Flow Metab 14:808–817.

Klann E, Chen S-J, Sweatt JD (1991) Persistent protein kinase activation in the maintenance phase of long-term potentiation. J Biol Chem 266:24253–24256.

Kobayashi S, Harris VA, Welsh FA (1995) Spreading depression induces tolerance of cortical neurons to ischemi in rat brain. J Cereb Blood Flow Metab 15:721–727.

Kochar A, Saitoh T, Zivin J (1989) Reduced protein kinase C activity in ischemic spinal cord. J Neurochem 53:946–952.

Koistinaho J, Miettinen S, Keinänen R, Vartiainen N, Roivainen R, Laitinen JT (1996) Long-term induction of heme oxygenase-1 (HSP32) in astrocytes and microglia following transient focal brain ischemia. Eur J Neurosci, in press.

Koizumi J, Yoshida Y, Nakazawa T, Ooneda G (1986) Experimental studies of ischemic brain edema. 1. A new model experimental model of cerebral embolism in rats in which recirculation can be introduced in the ischemic area. Jpn J Stroke 8:1–8.

Kraig RP, Nicholson C (1978) Extracellular ionic variations during spreading depression. Neuroscience 3:1045–1059.

Krizbai I, Szabo G, Deli M, Maderspach K, Lehel C, Olah Z, Wolff JR, Joo F (1995) Expression of protein kinase C family members in the cerebral endothelial cells. J Neurochem 65:459–462.

Li Y, Chopp M, Jian N, Yao F, Zaloca C (1995) Temporal profile of in situ DNA fragmentation after transient middle cerebral artery occlusion in the rat. J Cereb Blood Flow Metab 15:389–397.

Liu Y, Tsuchida A, Cohen MV, Downey JM (1995) Pretreatment with angiotensin II activates protein kinase C and limits myocardial infarction in isolated rabbit hearts. J Mol Cell Cardiol 27:883–892.

Longa EZ, Weinstein PR, Carlson S, Cummins R (1989) Reversible middle cerebral artery occlusion without craniotomy in rats. Stroke 20:84–91.

Louis J-C, Magal E, Brixi A, Steinberg R, Yavin E, Vincendon G (1991) Reduction of protein kinase C activity in the adult rat brain following transient forebrain ischemia. Brain Res 541:171–174.

Madden KP, Clark WM, Kochhar A, Zivin JA (1990) Effects of protein kinase C modulation on outcome of experimental CNS ischemia. Brain Res 547:193–198.

Maiese K, Boniece IR, Skurat K, Wagner JA (1993) Protein kinase modulates the sensitivity of hippocampal neurons to nitric oxide toxicity. J Neurosci Res 36:77–87.

Mailhos C, Howard MK, Latchman DS (1994) A common pathway mediates retinoic acid and PMA-dependent programmed cell death (apoptosis) of neuronal cells. Brain Res. 644:7–12.

Marrannes R, Willems R, DePrins E, Wauquier A (1988) Evidence for a role of the *N*-methyl-D-aspartate (NMDA) receptor in cortical spreading depression in the rat. Brain Res 457:226–240.

Mattson MP (1991) Evidence for the involvement of protein kinase C in neurodegenerative changes in cultured human cortical neurons. Exp Neurol 112:95–103.

Memezawa H, Smith M-L, Siesjö BK (1992) Penumbral tissues salvaged by reperfusion following middle cerebral artery occlusion in rats. Stroke 23:552–559.

Mitchell MB, Meng X, Ao L, Brown JM, Honken AH, Benerjee A (1995) Preconditioning of isolated rat heart is mediated by protein kinase C. Circ Res 76:73–81.

Nagasawa H, Kogure K (1990) Exo-focal postischemic neuronal death in the rat brain. Brain Res 524:196–202.

Nedergaard M, Astrup J (1986) Effect of hyperglycemia on direct current potential and (14C)2-deoxyglucose phosphorylation. J Cereb Blood Flow Metab 6:607–615.

Nishizuka Y (1992) Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science 258:607–614.

Palumbo EJ, Sweatt JD, Chen S-J, Klann E (1992) Oxidation-induced persistent activation of protein kinase C in hippocampal homogenates. Biochem Biophys Res Commun 187:1439–1445.

Pease PC (1962) Buffered formaldehyde as a killing agent and primary fixative for electron microscopy. Anat Rec 142:342.

Pyykönen I, Keinänen R, Koistinaho J (1995) Differential expression of β-amyloid precursor protein mRNAs following transient middle cerebral artery occlusion. J Cereb Blood Flow Metab 15[Suppl 1]:S345.

Roivainen R, Hundle B, Messing RO (1995) Ethanol enhances growth factor activation of mitogen-activated protein kinases by a protein kinase C-dependent mechanism. Proc Natl Acad Sci USA 92:1891–1895.

Saji M, Reis DJ (1987) Delayed neuronal death of substantia nigra neurons prevented by gamma-aminobutyric acid agonist. Science 235:66–69.

Schilling K, Robertson LM, Curran T, Morgan JI (1993) Continuous c-*fos* expression precedes programmed cell death in vivo. Nature 363:166–169.

Schlingensiepen KH, Wollnik F, Kunskt M, Schlingensiepen R, Herdegen T, Brusch W (1994) The role of Jun transcription factor expression and phosphorylation in neuronal differentation, neuronal cell death, and plastic adaptations in vivo. Cell Mol Neurobiol 14:487–505.

Siesjö B (1991) The role of calcium in cell death. In: Neurodegenerative disorders: mechanisms and prospects for therapy (Price D, Aguayo A, Thoenen H, eds), pp 35–59. Chichester: Wiley.

Siesjö BK, Katsura K-I, Kristian T (1995) The biological basis of cerebral ischemic damage. J Neurosurg Anesthesiol 7:47–52.

Sommer C, Gass P, Kiessling M (1995) Selective c-JUN expression in CA1 neurons of the gerbil hippocampus during and after acquisition of an ischemia-tolerant state. Brain Pathol 5:135–144.

Speechly-Dick ME, Moeaner MM, Tellon DM (1994) Protein kinase C: its role in ischemic preconditioning in the rat heart. Circ Res 75:586–590.

Tamura A, Kirino T, Sano K, Takagi K, Oka H (1990) Atrophy of the ipsilateral substantia nigra following middle cerebral artery occlusion in the rat. Brain Res 510:154–157.

Tanaka C, Nishizuka Y (1994) The protein kinase C family for neuronal signaling. Annu Rev Neurosci 17:551–567.

Thomas KL, Davis S, Laroche S, Hunt SP (1994a) Regulation of the expression of NR1 NMDA glutamate receptor subunits during hippocampal LTP. NeuroReport 6:119–123.

Thomas KL, Laroche S, Errington LM, Bliss TV, Hunt SP (1994b) Spatial and temporal changes in signal transduction pathways during LTP. Neuron 13:737–745.

Waxham MN, Grotta JC, Silva AJ, Strong R, Aronowski J (1996) Ischemia-induced neuronal damage: a role for calcium/calmodulin-dependent protein kinase II. J Cereb Blood Flow 161:1–6.

Welsh FA, Moyer DJ, Harris VA (1992) Regional expression of heat shock protein-70 mRNA and c-*fos* mRNA following focal ischemia in rat brain. J Cereb Blood Flow Metab 12:204–212.

Wieloch T, Cardell M, Bingren H, Zivin J, Saitoh T (1991) 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 56:1227–1235.

Young III WS (1989) Levels of transcripts encoding a member of the protein kinase C family in the paraventricular and supraoptic nuclei are increased by hyperosmolality. J Neuroendocrinol 1:79–81.

Zhang W, Lawa RE, Hintona DR, Su Y, Couldwell WT (1995) Growth inhibition and apoptosis in human neuroblastoma SK-N-SH cells induced by hypericin, a potent inhibitor of protein kinase C. Cancer Lett 96:31–35.