Generation of the catalytic fragment of protein kinase C alpha in vasospastic canine basilar artery

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In previous studies of topical application of calphostin C, a specific inhibitor of the regulatory domain of protein kinase C (PKC), and calpeptin, a selective inhibitor of calpain, to spastic canine basilar artery (BA) researchers have suggested that the catalytic fragment of PKC (known as PKM) is probably formed by a limited proteolysis of continuously activated μ -calpain, but there has been no direct evidence for PKM formation in vasospasm. The present immunoblot study with anti-PKC-alpha antibody shows a significant decrease in cytosolic 80-kD PKC-alpha and a concomitantly significant increase in membrane PKC-alpha in the spastic canine BA. In addition, an immunoblot study in which cleavage site-directed antibodies were used demonstrated a significant increase in immunoreactive 45-kD PKM. The changes in membrane PKC-alpha and PKM were enhanced with the lapse of time after subarachnoid hemorrhage. The cleavage site-directed antibodies distinguish the proteolyzed from the unproteolyzed forms of PKC for in situ analyses of enzyme regulation mediated by proteolysis. The data indicate that PKC-alpha in spastic canine BA is translocated to the cell membrane, where PKC-alpha is rapidly cleaved into PKM as a result of proteolysis of the isozyme by μ -calpain but not by m-calpain. The authors hypothesize that μ -calpain is continuously activated in spastic canine BA and produces PKM by limited proteolysis of PKC-alpha.

Key Words * immunoblotting * protein kinase C alpha * catalytic protein kinase C * vasospasm * dog

The mechanism of involvement of protein kinase C (PKC) in cerebral vasospasm remains controversial. Membrane PKC activity has been reported to increase with a reciprocal decrease in cytosolic PKC activity,[28] that is, the activation of intact PKC. Another report showed a decrease of 40 to 45% in cytosolic PKC activity without any significant changes in membrane PKC activity, indicating absence of activation of intact PKC; levels of immunoreactive PKC-alpha and PKC-epsilon but not PKC-zeta were decreased in spastic arteries.[38] In addition, there was a discrepancy between PKC activity and arterial narrowing.[30] A previous study in our laboratory demonstrated that calphostin C, a specific PKC inhibitor interacting with the regulatory domain, induced a slight dilation of spastic canine basilar artery (BA) but was unable to reverse vasospasm,[21] suggesting that intact PKC is minimally involved in the

development of vasospasm. However, the vasodilatory effect of calphostin C on the spastic canine BA was greatly enhanced after a topical treatment with calpeptin,[21] a selective inhibitor of calpain, resulting in the reversal of vasospasm. Additionally, μ -calpain, a Ca^{++} -dependent neutral protease, was continuously activated in the cerebral artery during vasospasm.[40] These results indicate that the catalytic domain of PKC (known as PKM) is dissociated from the regulatory domain by a limited proteolysis with calpain, causing activation of PKC.[21]

Several studies that surveyed PKC isozyme expression in extracts of intact vascular smooth-muscle cells by immunoblotting with type-specific antibodies found that multiple PKC isozymes are expressed in various smooth muscles,[12,13,31] and in most cases one PKC isozyme from conventional, novel, and atypical PKCs (usually PKC- α , PKC- δ , and PKC- ζ , respectively) is expressed. The isozyme PKC β , which is abundant in swine carotid arteries, appears to be expressed in only a few other vascular tissues. Likewise, PKC- ϵ from the novel PKC group appears to be variably expressed. Consequently, to analyze in situ enzyme regulation mediated by proteolysis, we examined intracellular proteolysis of PKC- α in the vasospastic canine BA by using cleavage site-directed antibodies that discriminate particularly for the proteolyzed form of enzymes.

MATERIALS AND METHODS

Animal Preparation

The care of the animals in this study was in compliance with United States Public Health Service standards. Adult mongrel dogs, each weighing between 10 and 14 kg, were anesthetized by intramuscular injection of ketamine hydrochloride (10 mg/kg), then with intravenously administered sodium pentobarbital (15 mg/kg), and maintained with a 70% nitrous oxide/30% oxygen mixture. Muscle relaxation was assured by an intravenous injection of pancuronium bromide at 30-minute intervals, and PaCO_2 was kept at a mean of 32 ± 3 mm Hg by adjusting the respiratory pump or by adding CO_2 to the inspired gas. The dogs' body temperature was kept at 37°C with a heating blanket. The mean arterial blood pressure and pulse rate were monitored continuously in the femoral artery and showed no changes during the procedure. After injection of contrast medium via the femoral artery, vertebral angiography was performed as a prespastic control in all animals.

Production of Spastic Artery

Cerebral vasospasm was produced by an injection of 5 ml of fresh autogenous arterial blood into the cisterna magna, followed by another injection 2 days later. Vertebral angiography was repeated in three groups of dogs (five animals per group) at 30 minutes, 2 days, and 7 days after the first intracisternal injection of blood. The caliber of the BA was measured at its narrowest point on the magnified angiogram to confirm vasospasm and expressed as a percentage of the prespastic caliber. In a control group of five dogs, 5 ml of saline was injected into the cisterna magna instead of fresh blood, and the angiographic caliber of the BA was examined 2 days postinjection.

Antibodies Against PKM

The preparation procedures for the antibodies used in the present study against the calpain-cleavage site of PKC- α have been reported previously.[14] Briefly, the synthetic peptides used for preparation of antibodies against the catalytic fragment of PKC- α were the sequences of human PKC- α produced at the cleavage sites by m- and μ -type calpains (LGPAGNKV and

VISPSEDRKQPSNNDRVKLT, respectively), and they were designated as CF- α 2 and CF- α 4 in that order. After conjugation with keyhole limpet hemocyanin, each synthetic peptide was injected into a rabbit. The antibodies obtained specifically reacted with the catalytic fragment of PKC- α ; they did not cross react with other fragments.[14] The present method, in which synthetic peptides mimicking the newly generated NH₂-terminal region were used as immunogens, has made it possible to establish such special antibodies without necessitating isolation of naturally produced immunogens. These special antibodies specifically recognized the catalytic fragments of PKC- α but not the unproteolyzed PKC- α in the spastic canine BA.

Immunoblotting of PKC- α and PKM

The animals were killed by rapid intravenous injection of sodium pentobarbital (50 mg/kg) to avoid any influence of ischemia resulting from exsanguination or perfusion on the PKC activity,[18,26] and the BA was removed together with the entire brain. In the spastic group, the blood clot around the BA and its branches was carefully removed without any mechanical stimulation of the arteries and placed in an ice bath. After a brief washing with phosphate-buffered saline (PBS), the BA was quickly frozen in liquid nitrogen until used. The BAs in the control group underwent a similar procedure without the removal of the blood clot.

The frozen BA was pulverized in liquid nitrogen and sonicated in 50 mM Tris-HCl (pH 7.5) containing 0.25 M sucrose, 2 mM ethylenediamine tetraacetic acid, 10 mM ethyleneglycol-bis(β -aminoether)N,N'-tetraacetic acid, and protein inhibitors (1.2 mM phenylmethylsulfonyl fluoride, 120 μ M N-tosyl-L-lysyl chloromethyl ketone hydrochloride, 60 μ M N-tosyl-L-phenylalanyl chloromethyl ketone, 28 μ M E64, 50 μ M leupeptin, 5 mM diisopropyl fluorophosphate, and 25 μ M bestatin). The cell lysates were centrifuged at 900 G for 30 minutes, and the supernatants were used for immunoblotting of PKM. For immunoblotting of PKC- α , the cell lysates were centrifuged at 800 G for 5 minutes, and the supernatants were further centrifuged at 17,000 G for 20 minutes. The precipitates were suspended in 50 mM Tris-HCl (pH 8) containing 0.3% (wt/vol) sodium deoxycholate, 50% (vol/vol) glycerol, 1 mM NaN₃, and 1.7 μ M CaCl₂ and centrifuged at 146,000 G for 4 minutes. The supernatant was used as a membrane fraction. The low-speed supernatants (17,000 G for 20 minutes) were further centrifuged at 356,000 G for 8 minutes, and the resulting supernatant was used as a cytosolic fraction. The samples for immunoblotting of PKM or PKC- α were treated with 10% (vol/vol) trichloroacetic acid. The precipitated proteins were collected by means of centrifugation and dissolved in 0.5 M Tris-HCl (pH 6.8) containing 2% (wt/vol) lithium dodecyl sulfate, 6% (wt/vol) glycerol, and 5% (wt/vol) 2-mercaptoethanol and heated at 100°C for 5 minutes. The samples were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane. The membranes were rinsed with PBS containing 5% skim milk for 60 minutes at 37°C and then incubated at 4°C for 12 hours with 7 ml of PBS containing 0.05% Tween 20, 5% skim milk, and 0.1 to 0.5 μ g/ml antibodies against PKC- α or the cleavage sites of PKC- α . The membranes were washed five times with 0.05% Tween 20 in PBS and once with 5% skim milk in PBS and then incubated for 60 minutes at room temperature with 10 ml of PBS containing 0.05% Tween 20, 5% skim milk, and anti-rabbit immunoglobulin G-conjugated horseradish peroxidase. The membranes were washed five times with 0.05% Tween 20 in PBS, incubated for 1 minute with a chemiluminescent agent, and exposed to film. The protein content of the sample was determined by Bradford's technique.[4] Densitometry was performed using a scanner interfaced with a computer and monitor. Scans were imported into commercially available software for analysis.

Sources of Supplies and Equipment

Anti-PKC- α antibody was supplied by Transduction Laboratories, Lexington, KY; anti-rabbit immunoglobulin G-conjugated horseradish peroxidase by DAKO, Glostrup, Denmark; and the chemiluminescent agent by E. I. Dupont de Nemours and Co., Inc., Boston, MA. The HP Scan Jet IIcx scanner was manufactured by Hewlett-Packard Co., Palo Alto, CA; the Mac Performa model 6210 by Apple Computer Co., Ltd., Tokyo, Japan; and the hyperfilm-MP by Amersham International, Buckinghamshire, England. The National Institutes of Health Image software was supplied by Wayne Rasband, Bethesda, MD.

Fig. 1. A series of three representative vertebral angiograms showing canine BA before (left) and after (right) exposure to blood at 30 minutes (upper), 2 days (center), and 7 days (lower).

RESULTS

Caliber of the BA

No significant narrowing of the BA was shown on angiographic studies before the animals in the control group were killed. Representative angiograms of the BA in the spastic group at 30 minutes, 2 days, and 7 days after the first blood injection are shown in Fig. 1, and the percentage of caliber reduction, as seen in

Fig. 2, indicates the occurrence of vasospasm.

Fig. 2. Bar graph showing the mean percentage of caliber reduction in canine BA as measured on angiography. Vertical lines indicate the standard deviation of the mean. Statistical significance was determined using Student's t-test. n = number of dogs; 30 m = vasospasm 30 minutes after exposure to blood; 2 d = vasospasm 2 days after exposure to blood; 7 d = vasospasm 7 days after exposure to blood.

Immunoblotting of PKC-alpha and PKM

Representative immunoblots of PKC-alpha and PKM in the spastic BA are shown in Fig. 3. The immunoreactive 80-kD PKC-alpha showed a significant decrease in cytosolic fraction in spastic BA and a concomitantly significant increase in membrane fraction. The increase in membrane PKC-alpha was significantly enhanced with the lapse of time after subarachnoid hemorrhage (SAH), suggesting an increased translocation of PKC-alpha from cytosol to cell membrane. The immunoreactive PKM was not observed in the control BA but was identified by immunoblotting with anti-CF-alpha4 antibody as a 45-kD peptide in the spastic BA, indicating that 45-kD PKM is formed as a result of cleavage of PKC-alpha by μ -calpain. However, addition of m-calpain did not result in cleavage of PKC-alpha, and 46-kD PKM was not formed, as demonstrated by immunoblotting with anti-CF-alpha2 antibody.

Fig. 3. Representative immunoblots showing PKC-alpha and PKM in the canine BA. Equivalent amounts of lysates from the canine BAs were immunologically probed with antibody against PKC-alpha following separation into cytosolic (A) and membrane (B) fraction; or probed with two antibodies against cleavage sites of PKC-alpha, anti-CF-alpha2 (C) and anti-CF-alpha4 (D), without separation into cytosolic and membrane fractions. High-molecular-weight markers were used as the size standard. Lane 1 = control; lane 2 = vasospasm 30 minutes after exposure to blood; lane 3 = vasospasm 2 days after exposure to blood; lane 4 = vasospasm 7 days after exposure to blood.

These data indicate that PKM is produced as a result of cleavage of PKC-alpha by μ -calpain but not by m-calpain. In addition, the formation of PKM increased significantly with the passage of time after SAH. The mean densitometric values of cytosolic and membrane PKC-alpha (Fig. 4A and B) as well as 45-kD PKM (Fig. 4C) are shown.

Fig. 4. Bar graphs showing the mean percentage of densitometric values of cytosolic and membrane PKC-alpha as well as 45-kD PKM in canine BA, which reveals a decrease in cytosolic PKC-alpha (A) as well as an increase in membrane PKC-alpha (B) and PKM (C) after vasospasm. Because PKM was absent in control BAs, densitometric values of PKM in spastic BAs 2 and 7 days after exposure to blood were calculated as the percentage of densitometric values of PKM in spastic BA 30 minutes after exposure to blood. Statistical significance was determined using Student's t-test. Abbreviations as in Fig. 2.

DISCUSSION

The activity and location of PKC are regulated by Ca^{++} and phospholipids. When the concentration of intracellular Ca^{++} increases, cytosolic PKC associates with membranes and phosphorylates the substrates present in the membranes. Translocation of PKC is also observed when cells are treated with phorbol esters.[8,17,20] The immunoreactive PKC- α was significantly decreased in the cytosolic fraction of spastic canine BA and concomitantly increased gradually in the membrane fraction with the passage of time after SAH, suggesting translocation of PKC- α as a result of vasospasm. The membrane-bound PKC is known to be cleaved at a specific site by calpain to form PKM[30] and then released to cytosol to phosphorylate cytosolic substrates.[15,24,36] Calpain is classified into two homologous isozymes with different Ca^{++} requirements: μ - and m-calpains active at micro- and millimolar concentrations of Ca^{++} , respectively.[23,35] The isozyme μ -calpain particularly dissociates PKC into catalytic and regulatory domains by limited proteolysis and activates PKC irreversibly even in the absence of Ca^{++} and phospholipid.[16,36] The anti-CF- α 2 and anti-CF- α 4 antibodies used were shown to bind specifically to PKM proteolyzed by m- and μ -calpain, respectively, but not to the unproteolyzed proteins. The 45-kD PKM formed as a result of cleavage of PKC- α by μ -calpain was observed in the canine spastic BA by means of immunoblotting with anti-CF- α 4 antibody, whereas no 46-kD PKM formed because m-calpain did not cleave PKC- α , suggesting that μ -calpain is activated in vasospasm and m-calpain is not. Because μ - and m-calpain are activated at intracellular Ca^{++} concentrations of 1 μM and 1 mM, respectively,[23,24] the intracellular Ca^{++} concentration in the canine BA could be increased between 1 μM and 1 mM during vasospasm. The limited proteolysis of PKC is also thought to be an initial step in downregulation of this enzyme,[1,24] because PKM is unstable and degraded in cells. The results of our immunoblot studies are consistent with those of the previous pharmacological studies:[21] H-7 reversed vasospasm because H-7 is a potent inhibitor of PKC that interacts with the adenosine triphosphate (ATP)-binding site in the catalytic domain[9,25] and is therefore effective for inhibition of PKC- α and PKM. Calphostin C, which interacts with the regulatory domain of PKC, could not reverse vasospasm. However, it became effective for the reversal of vasospasm after the cleavage of PKC- α by μ -calpain was prevented by pretreatment with calpeptin, because calphostin C is effective for inhibition of PKC- α but not for inhibition of PKM formed during vasospasm.[21]

Once the activation of PKC is identified, it is necessary to detect its intracellular substrates and their role in the development of vasospasm. Recently, vasospasm of canine anterior spinal artery has been reported to be associated with an increased phosphorylation of myosin light chain (MLC).[5] Biochemical analysis reveals that PKC-catalyzed phosphorylation reduces by approximately 50% the actin-activated Mg^{++} -ATPase activity of myosin prephosphorylated by MLC kinase (MLCK).[11,27] In addition, the PKC-catalyzed phosphorylation of MLCK decreases its activity,[10] suggesting that the in vitro effects of PKC-catalyzed phosphorylation of MLC and MLCK would be consistent with relaxation rather than a contractile response. In addition, mechanical response in permeabilized gizzard smooth muscle phosphorylated at PKC-specific residues of MLC in vivo is an attenuation of Ca^{++} -induced contraction.[29] Phorbol ester-induced contractions of smooth muscles are not accompanied by significant phosphorylation of MLC on the PKC-specific residues.[32,34] Also, phorbol-12,13-dibutyrate was shown to increase MLC phosphorylation, albeit indirectly, at MLCK-specific but not PKC-specific residues of MLC obtained from rabbit femoral artery.[19] Furthermore, contraction of intact smooth-muscle fibers in response to physiological agents that activate PKC is not accompanied by MLC phosphorylation on PKC-specific residues, but is accompanied by MLC phosphorylation on

MLCK-specific residues.[32,33] Thus, a great deal of effort has been devoted to identifying the substrates of PKC that are involved in the contractile response, but the weight of evidence is against either MLC or MLCK involvement as a substrate of PKC.

Recently, attention has been focused on the possible roles of the thin filament-associated regulatory proteins caldesmon and calponin, to account for activation of arterial smooth muscle independently of MLC phosphorylation. Both proteins have been shown to inhibit actin-activated myosin ATPase by interacting with F-actin, tropomyosin, and/or myosin[7,37] and therefore have been attributed a role in the modulation of the contractility of smooth muscle. Both caldesmon and calponin can be phosphorylated in vitro by PKC, and once phosphorylated, they no longer inhibit actomyosin ATPase, resulting in enhancement of smooth-muscle contractility.[2,39] Furthermore, caldesmon is reported to be phosphorylated in situ by PKC-dependent activation of mitogen-activated protein kinase[2,3,6] and calponin is phosphorylated in situ by PKC.[22] Therefore, PKM generated during vasospasm may be at least partly involved in the further development of vasospasm by the indirect phosphorylation of caldesmon through PKM-dependent mitogen-activated protein kinase and by the direct phosphorylation of calponin.

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