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Protein kinase Cδ and α are involved in the development of vasospasm after subarachnoid hemorrhage

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Abstract

We have previously shown the enhanced activity of protein kinase C in the membrane fraction of the canine vasospastic artery after subarachnoid hemorrhage, which increased with progression of angiographic vasospasm. This study examined identification of protein kinase C isoforms in the canine basilar artery, and the changes in expression and/or translocation of each isoform during the development of vasospasm. Vasospasm was produced by using the ''two-hemorrhage'' canine model in the basilar artery, and angiographic progression of vasospasm was assessed consecutively. Two isoforms, protein kinase Cα and δ were identified in basilar arteries by Western blotting. Densitometric analysis showed that the expression of protein kinase Cδ in the membrane fraction was significantly increased in the earlier stage, and protein kinase Cα was increased later as vasospasm progressed. These results indicate that protein kinase Cδ and α isoforms may play a significant role in the development and maintenance of vasospasm.

Keywords: Protein kinase C isoform; Subarachnoid hemorrhage; Cerebral vasospasm

1. Introduction

Cerebral vasospasm is a prolonged pathological tonic contraction of the cerebral vasculature after subarachnoid hemorrhage and seriously affects the prognosis of a patient, but the pathophysiological mechanisms of cerebral vasospasm remain unclear.

Investigations as to the mechanisms of vascular contraction have shown that phosphorylation of the 20-kDa myosin light chain (MLC_{20}) is a key event in the activation of Ca^{2+}-induced contractions (Horowitz et al., 1996). However, vascular smooth muscle cells may also contract via a process in which there is no detectable increase in the MLC_{20} phosphorylation level (Andrea and Walsh, 1992; Collins et al., 1992). Protein kinase C has been proposed to be involved in the regulation of vascular smooth muscle contraction (Andrea and Walsh, 1992; Katsuyama and Morgan, 1991; Khalil et al., 1992; Nishizuka, 1984, 1988).

We have been investigating the role of protein kinase C in the cerebral artery in the development and maintenance of vasospasm after subarachnoid hemorrhage (Nishizawa et al., 1992a,b, 1995) and have shown that administration of phorbol esters or a combination of diacylglycerol and phosphatidyl serine induces tonic contraction of the canine basilar artery (Nishizawa et al., 1992a). Measurement of protein kinase C activity by radioimmunoassay showed that the kinase activity was significantly enhanced in the membrane fraction of the cerebral vasospastic arteries in a canine subarachnoid hemorrhage model (Nishizawa et al., 1992b). Using enzyme immunoassay, protein levels of protein kinase C in the vasospastic canine basilar artery after subarachnoid hemorrhage increased compared to those of the control artery, and that the time course of the development of angiographic vasospasm and increased

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protein kinase C protein levels in the membrane fraction were closely correlated with time (Nishizawa et al., 1995). The protein levels of protein kinase C were not significantly increased in a mild vasospasm model compared to the control, but the increase was significant in a severe vasospasm model (Nishizawa et al., 1995). These results strongly suggest that protein kinase C plays a pivotal role in the development of vasospasm following subarachnoid hemorrhage.

Protein kinase C was originally identified as a single serine/threonine kinase, but a family of protein kinase C enzymes has since been found (Nishizuka, 1992). Thus far, at least 12 isoforms of protein kinase C have been identified (Nishizuka, 1992; Hug and Sarre, 1993; Johannes et al., 1995; Togashi et al., 1997; Jamieson et al., 1999; Hayashi et al., 1999). We previously demonstrated that protein kinase C activation is involved in the development of vasospasm. However, little remains known about the isoforms expressed in canine vasospastic arteries after subarachnoid hemorrhage (Takuwa et al., 1993).

In this study, we identified the specific protein kinase C isoforms that are expressed in the canine vasospastic basilar artery by immunoblot analysis, and examined whether the chronological changes in angiographic vasospasm and enhanced expression of protein kinase C isoforms in the membrane fraction are consistent. Our results indicated that protein kinase Cδ translocated from cytosol to membrane in the earlier stage, and protein kinase Cα followed as the angiographic vasospasm progressed.

2. Materials and methods

2.1. Animals

All experiments were performed according to the Rules of Animal Experimentation and the Guide for the Care and Use of Laboratory Animals of the Hamamatsu University School of Medicine. The basilar arteries of beagle dogs of either sex weighing 7–10 kg were used.

2.2. Angiography

An animal model of subarachnoid hemorrhage was produced using the ''two-hemorrhage'' canine model (Varsos et al., 1983), which has been approved and widely used as a standard experimental model of subarachnoid hemorrhage.

The dogs were anesthetized by intravenous injection of 25 mg/kg of pentobarbital sodium, and tracheal intubation was performed. A sterile catheter was inserted into a vertebral artery via a femoral artery under fluoroscopic control. The dog's head was fixed with a stereotactic frame, and 7 ml of Iopamirone 300® was injected to acquire an image of the basilar artery. End-tidal CO_2 was maintained at 38–42 mm Hg throughout angiography.

After obtaining a control image, 3 ml of autologous blood was manually injected into the cisterna magna, with the head of the dog maintained downward for 10 min so that it would collect around the basilar artery (day 1). Angiography was performed on day 4 before the second injection of blood, 1 h after the second injection, and on day 7. In our previous experiments, severe vasospasm was observed from 1 h after the second injection on day 4. Therefore, we took angiography 1 h after the second injection on day 4 for analysis of chronological progression of vasospasm, and used the basilar artery 1 h after the second injection on day 4 for further experiments. A total of six dogs were used to investigate the chronological progression of angiographic vasospasm. We also performed a sham-operated study, in which the same amount of sterile physiological saline was injected into the cisterna magna instead of autologous blood, and angiography was taken in the same way as for the ''two-hemorrhage'' model. A total of five dogs were used for the sham-operated study.

The basilar artery in the image was divided into three segments, and the diameter of the artery at the midpoint of each segment was measured under a microscope (mm). Arterial diameter was expressed as the mean of these three values. The diameter of the basilar artery on each day was expressed as a percentage of its diameter on the control angiogram (control = 100%).

2.3. Identification of PKC isoforms by Western blot analysis

Protein kinase C isoforms were identified by Western blot analysis according to a modified method by Obara et al. (1999). A total of 16 dogs were randomly divided into four groups: a control group, a group in which the dogs were sacrificed on day 4 before the second injection, a group in which the dogs were sacrificed on day 4, 1 h after the second injection, and a group sacrificed on day 7. The dogs were sacrificed by intravenous injection of an overdose of pentobarbital sodium (50 mg/kg). After sacrifice, the basilar artery with the brainstem and a piece of the cerebellum were excised as soon as possible. The specimens were immersed in ice-cold phosphate-buffered saline (PBS, consisting of 136.9 mM NaCl, 2.7 mM KCl, 10.1 mM $Na_2HPO_4 \cdot 12H_2O$, 1.8 mM KH_2PO_4) for dissection. The cerebellar portion was frozen in liquid nitrogen and kept at −80°C. The artery was dissected from the brainstem and arachnoid membrane under a microscope, and blood around the basilar artery was meticulously removed. Blood inside the lumen was flushed with PBS and the endothelium was mechanically removed (Nakayama, 1988). The artery was also frozen in liquid nitrogen and kept at −80°C.

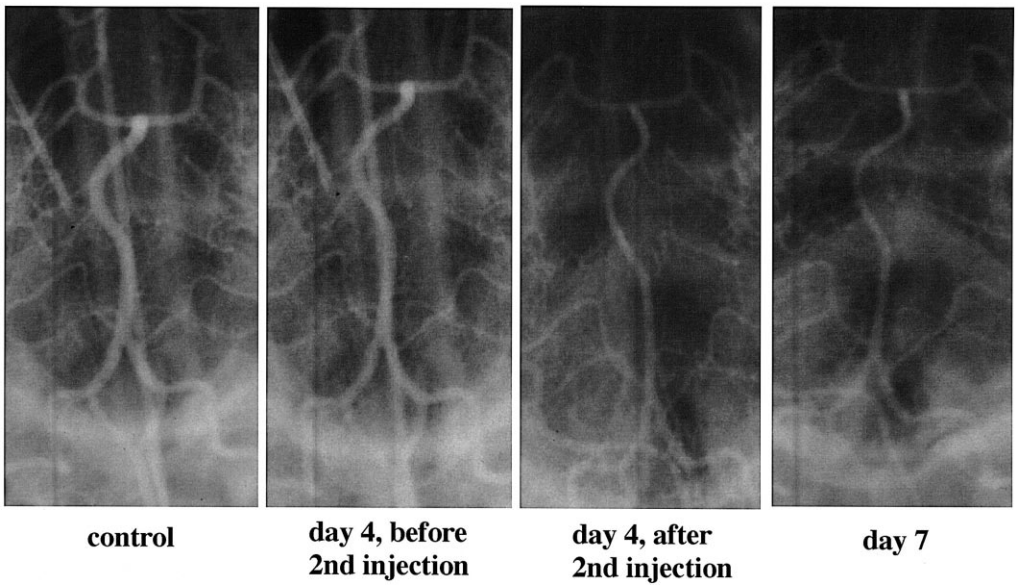

Fig. 1. Angiographic vasospasm of the basilar artery in the ''two-hemorrhage'' canine model.

The basilar artery and the cerebellar tissue were minced with scissors in the ice-cold homogenization buffer (consisting of 50 mM Tris–HCl, 5 mM ethylenediamine tetraacetic acid (EDTA), 10 mM ethyleneglycol-bis (β-aminoethylether)-*N*,*N*′-tetraacetic acid (EGTA), 1 mM phenylmethylsulfoxide, 5 mM dithiothreitol, 10 mM benzamidine, 25 mg/ml leupeptin, and 250 mM sucrose) and ultracentrifuged at $100{,}000 \times g$ for 30 min at 4°C. The cytosol and membrane fractions were obtained from the supernatant and pellet, respectively. Both fractions of the basilar artery and the cytosol fraction of the cerebellum were diluted with sodium dodecyl sulfate (SDS) sample buffer (pH 6.8, consisting of 62.5 mM Tris–HCl, 2% SDS, 10% glycerol and 8 mM dithiothreitol) (diluted 1:4 (vol/vol)). All samples were boiled for 5 min and used immediately. Tissue extracts were subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) on 10% polyacrylamide gels as described (Obara et al., 1999). The resolved proteins were electrophoretically transferred to a nitrocellulose membrane in 20% methanol, 25 mM Tris–HCl, and 192 mM glycine (pH 8.3) for 90 min at 120 mA. After transfer, the nitrocellulose membrane was incubated in 5.0% nonfat milk in Tris-buffered saline containing 150 mM NaCl, 20 mM Tris–HCl (pH 7.5) for 60 min, followed by incubation overnight at 4°C with the appropriate rabbit protein kinase C isoform-specific primary antibody (diluted 1:500 (vol/vol)) in Tris-buffered saline containing 3.0% horse serum.

The protein kinase C isoform-specific primary antibodies from Sigma (St. Louis, MO, USA) were raised in rabbits by using synthetic peptides corresponding to the C-terminal amino acid sequence of rat protein kinase Cα (659–672), protein kinase Cβ1 (658–671), protein kinase Cβ2 (660–673), protein kinase Cγ (684–697), protein kinase Cδ (662–673), protein kinase Cε (726–737), protein kinase Cη (670–683), and protein kinase Cζ (577–592). The membrane was then washed (three times, 5 min each) in Tris-buffered saline and incubated with 2 mg/ml of peroxidase-labeled goat anti-rabbit antibodies (diluted 1:500 (vol/vol)) in Tris-buffered saline containing 3.0% horse serum for 7 h. Next, the membrane was washed (three times, 5 min each) in Tris-buffered saline, and the

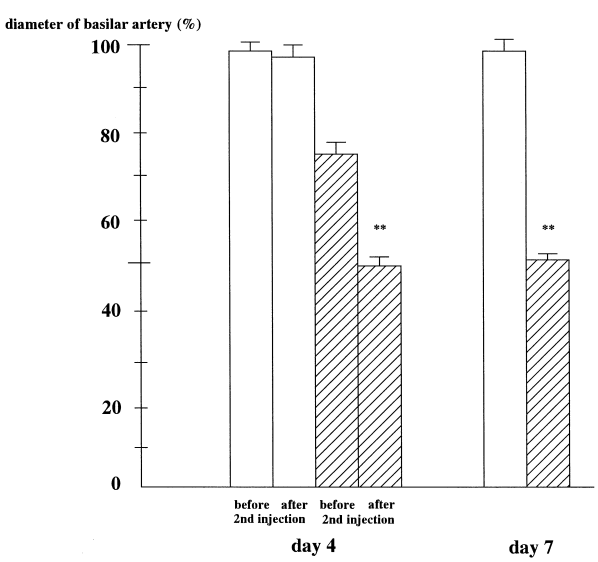

Fig. 2. Chronological changes in the angiographic diameter of the basilar artery in the ''two-hemorrhage'' canine model and in the sham-operated study. The diameter of the basilar artery on the control angiogram is 1.18 ± 0.12 mm ($n = 11$), and the diameter on each day was expressed as a percentage of the control (control = 100%). Data are expressed as the means ± S.E.M. $^{**}P < 0.01$, corresponding to day 4 before the second injection. Open bars, diameters in the sham-operated study, shaded bars, diameters before and 1 h after the second injection of blood on day 4, and on day 7.

peroxidase reaction was developed by enhanced chemiluminescence. The amount of protein kinase C was quantified by densitometric scans of immunostained nitrocellulose blots. The results were expressed as a percentage of the total amount of each protein kinase C isoform.

Rat brain was used as a positive control. Specificity of signal was shown by the absence or presence of isoform-specific synthetic peptide (5 μg of immunizing peptide). Isoform-specific synthetic peptides of protein kinase Cζ and η were not commercially available in Sigma products. Therefore, the presence of isoform-specific band of protein kinase Cζ and η was evaluated according to the position of the positive control band expressed in rat brain.

2.4. Total amounts of each protein kinase C isoform and α-smooth muscle actin in canine basilar arteries

In order to ensure that total amount of each protein kinase C isoform was not different among the groups of control, day 4 before and after the second injection, and day 7, we mixed the same amounts (40 μg) of protein from the cytosol and membrane fractions, and performed SDS–PAGE/Western blot analysis. We also carried out Western blot analysis using an antibody to α-smooth muscle actin to measure amounts of α-smooth muscle actin in the groups of control, day 4 before and after the second injection, and day 7. Results were expressed as arbitrary densitometric units (ADU).

2.5. Drugs

Iopamirone 300® was purchased from Japan Schering (Osaka, Japan). Pentobarbital sodium (Nembutal) was purchased from Dainabot (Osaka, Japan). Dithiothreitol, benzamidine and leupeptin were purchased from Wako (Osaka, Japan). Protein kinase C specific antibodies, peroxidase-labeled goat anti-rabbit antibodies, isoform-specific synthetic peptides of protein kinase Cα, β1, β2, γ, δ, and ε, the antibody to α-smooth muscle actin, and phenylmethylsulfoxide were purchased from Sigma. Enhanced chemiluminescence was purchased from Amersham Japan (Tokyo, Japan).

2.6. Statistical analysis

Data are expressed as the mean ± S.E.M. Data were analyzed by paired or unpaired Student's *t*-test, or Dun-

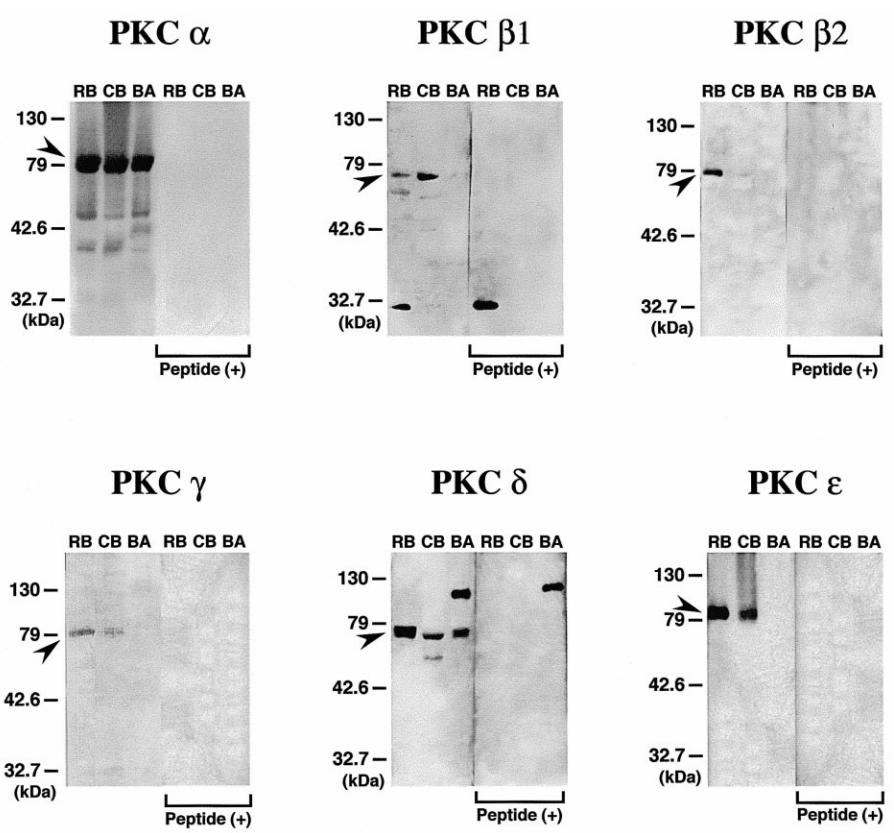

Fig. 3. Expression of protein kinase C isoforms in rat brain, canine cerebellum and canine basilar artery determined by Western blotting. The cytosol fraction of rat brain was used as a positive control. Isoforms of protein kinase C in the cytosol of the rat brain and the canine cerebellum, and those of the cytosol and the membrane fractions of the basilar artery were separated by SDS–PAGE and analyzed by Western immunoblotting. Immunoblots probed with antibodies against protein kinase Cα, β1, β2, γ, δ, and ε are shown. Arrowheads indicate protein kinase C isoform bands. The specificity was demonstrated by showing loss of the immunoreactive signal using isoform-specific synthetic peptides. Abbreviations: RB, rat brain, CB, canine brain (cerebellum), BA, canine basilar artery, peptide, isoform-specific synthetic peptide.

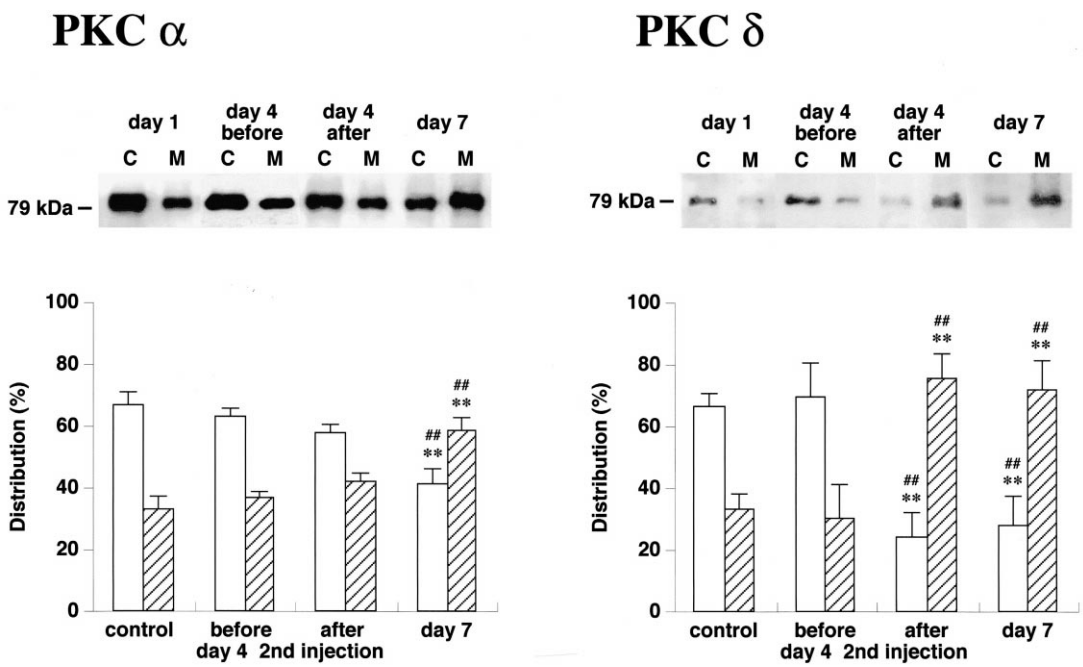

Fig. 4. Quantitative summary of chronological changes in the amounts of identified protein kinase C isoforms. The results are expressed as a percentage of the total amount of each protein kinase C isoform. The amounts of protein kinase Cα and protein kinase Cδ in the cytosol and the membrane fractions changed significantly as vasospasm developed ($n = 4$). Each value represents the mean ± S.E.M. Open bars, cytosol fraction, shaded bars, membrane fraction. Abbreviations: C, cytosol fraction, M, membrane fraction. In protein kinase Cα, $^{**}P < 0.01$, corresponding to the control, $^{\#\#}P < 0.01$, corresponding to the day 4 before and after the second injection. In protein kinase Cδ, $^{**}P < 0.01$, corresponding to the control, $^{\#\#}P < 0.01$, corresponding to the day 4 before the second injection.

nett's multiple comparison test after analysis of variance (ANOVA). P values less than 0.05 were considered significant.

3. Results

3.1. Chronological changes in the diameter of the basilar artery after subarachnoid hemorrhage

The chronological progression of angiographic vasospasm of the basilar artery is shown in Fig. 1, and the summarized data are illustrated in Fig. 2.

The diameter of the basilar artery on control angiogram was 1.18 ± 0.12 mm (= 100%, $n = 11$). The diameter of the basilar artery on each day was as follows: 76.3 ± 2.2% (number of dogs; $n = 6$) on day 4 before the second injection, 49.3 ± 2.1% ($n = 6$) on day 4, 1 h after the second injection, 50.1 ± 1.4% ($n = 6$) on day 7. There were statistically significant differences between the diameters before and after the second injection on day 4 ($P < 0.01$), and between the diameters before the second injection and on day 7 ($P < 0.01$), but not between the diameters on day 4 after the second injection and on day 7. In the sham-operated study, there were no statistically significant differences among the values of arterial diameter (Fig. 2).

Table 1
Total amounts of each protein kinase C isoform and α-smooth muscle actin in canine basilar arteries
Each value represents the mean ± S.E.M. of the total amount of each protein kinase C isoform and total amounts of α-smooth muscle actin. As for the total amount of each protein kinase C isoform, equal amounts (40 μg) of protein from both the cytosol and membrane fractions were mixed and SDS–PAGE/Western blot analysis was performed. Western blot analysis using an antibody to α-smooth muscle actin was also carried out for measurement of α-smooth muscle actin. In any protein kinase C isoform, total amount of protein kinase C isoform was not significantly different among groups of control, day 4 before injection, day 4 after injection and day 7 (ANOVA). Total amounts of α-smooth muscle actin among these four groups were not significantly different (ANOVA). Abbreviations: PKC, protein kinase C; ADU, arbitrary densitometric unit; *n*, number of preparations.

	Amount of PKC (ADU)		α-Smooth muscle actin (ADU)	*n*
	PKCα	PKCδ		
Control	1648 ± 298	985 ± 96	1548 ± 38	5
Day 4 (before injection)	1557 ± 113	844 ± 87	1533 ± 116	6
Day 4 (1 h after injection)	1571 ± 239	840 ± 98	1521 ± 73	6
Day 7	1392 ± 177	800 ± 133	1522 ± 125	4

3.2. Identification of protein kinase C isoforms by Western blot analysis

We always used rat brain as a positive control in the identification of protein kinase C isoforms, and Western blot analysis consistently showed a band associated with protein kinase Cα at 80 kDa (Fig. 3, arrowhead). We examined eight protein kinase C isoforms (protein kinase Cα, β1, β2, γ, δ, ε, ζ, and η) with specific antibodies. Four protein kinase C isoforms were detected in canine basilar arteries in Western blot analysis: protein kinase Cα, δ, ζ and η. These bands showing protein kinase C isoforms nearly consisted with those in the rat brain (Figs. 3 and 4). In the present study, as isoform-specific synthetic peptides of protein kinase Cζ or η were not commercially available, we could not identify the specificity of the expression of these two protein kinase C isoforms. On the other hand, specificity of protein kinase Cα and δ, shown by loss of the immunoreactive signal, was demonstrated by inclusion of isoform-specific synthetic peptides during the primary antibody incubation stage (Fig. 3). The results of quantitative analysis of chronological changes in the amount of protein kinase C isoforms in the cytosol and the membrane fractions are summarized in Fig. 4 with statistical analysis.

As for protein kinase Cα, the value on day 7 in the cytosol fraction (number of the samples; $n = 4$) was significantly lower than the control and the day 4 (before and after the second injection) values ($n = 4$, respectively) ($P < 0.01$). In the membrane fractions, the difference among the values in the control group ($n = 4$) and in the groups on day 4 before ($n = 4$) and after the second injection ($n = 4$) was not statistically significant. However, the value on day 7 ($n = 4$) was significantly higher than the control and the day 4 (before and after the second injection) values ($P < 0.01$). As for the protein kinase Cδ values of the cytosol fractions, the values on day 4 after the second injection ($n = 4$) and those on day 7 ($n = 4$) were significantly decreased compared to the control group ($n = 4$) and the group on day 4 before the second injection ($n = 4$) ($P < 0.01$). Protein kinase Cδ values of the membrane fractions on day 4 after the second injection ($n = 4$) and those on day 7 ($n = 4$) were significantly higher than those in the control ($n = 4$) and day 4 before the second injection ($n = 4$) ($P < 0.01$). No statistically significant changes were noted in either the cytosol or the membrane fractions of protein kinase Cζ and η (each $n = 4$) during the study.

3.3. Total amounts of each protein kinase C isoform and α-smooth muscle actin

Total amount of each protein kinase C isoform and that of α-smooth muscle actin were summarized in Table 1. In any protein kinase C isoform, total amount of protein kinase C isoform was not significantly different among the groups of control, day 4 before and after the second injection, and day 7. Amount of α-smooth muscle actin in those groups was not significantly different either.

4. Discussion

We have summarized the results of this study and our conclusions as follows.

(1) At least four protein kinase C isoforms (protein kinase Cα, δ, ζ, and η) were detected in canine basilar arteries under control and vasospastic conditions.

(2) Among these isoforms, the specificity of the expression of protein kinase Cα and δ was identified using isoform-specific synthetic peptides. However, we could not identify the specificity of the expression of protein kinase Cζ and η because no isoform-specific synthetic peptides of protein kinase Cζ and η were commercially available.

(3) Protein kinase Cδ was initially translocated from the cytosol to the membrane fractions, and protein kinase Cα was subsequently translocated.

These results indicate that protein kinase C isoforms, especially protein kinase Cδ and protein kinase Cα, play a pivotal role in the development of vasospasm after subarachnoid hemorrhage.

4.1. Chronological changes in the development of vasospasm and protein kinase C activity

In the ''two-hemorrhage'' canine model, severe vasospasm developed 1 h after the second injection on day 4, and continued until at least day 7. We previously reported that the canine basilar artery exhibited significant tonic vascular contraction when protein kinase C was activated by phorbol esters or by physiological activators such as a combination of diacylglycerol and phosphatidyl serine (Nishizawa et al., 1992a). Progression of angiographic vasospasm correlated with a progressive increase of protein levels of protein kinase C in the membrane fraction of the artery (Nishizawa et al., 1995). Therefore, protein kinase C activation in the basilar artery is not a simple result of subarachnoid hemorrhage, but can be considered to play a role in the development of vasospasm.

To more closely examine these events, we investigated the expression of protein kinase C isoforms in the development of vasospasm by Western blot analysis.

4.2. Protein kinase C isoforms expression in the canine basilar artery

At least 12 isoforms of protein kinase C have been identified thus far, and they have been classified into three groups: classical protein kinase C's (protein kinase Cα, β1, β2, and γ), novel protein kinase C's (protein kinase Cδ, ε, η, ν, θ) and atypical protein kinase Cs (ζ and ι/λ)

(Hug and Sarre, 1993; Johannes et al., 1995; Togashi et al., 1997; Jamieson et al., 1999; Hayashi et al., 1999).

In the canine basilar artery, we identified at least two protein kinase C isoforms (protein kinase Cα and δ) of which specificity was confirmed using isoform-specific synthetic peptides, and the bands corresponding to these protein kinase C's were expressed at an equivalent position to protein kinase C in the rat brain. Throughout these experiments, we always used rat brain as a positive control. In addition, we showed that protein kinase Cα and δ were significantly translocated from the cytosol to the membrane fractions. It is worth noting that protein kinase Cδ was initially translocated, followed by translocation of protein kinase Cα as angiographic vasospasm developed.

In a recent study of protein kinase C expression in the canine vasospastic artery after subarachnoid hemorrhage, the expression of protein kinase Cα, ε and ζ were shown and the amounts of these protein kinase C's were decreased during vasospasm (Takuwa et al., 1993). We identified the expression of protein kinase Cα and δ. Both protein kinase Cα and δ translocated from cytosol to membrane fractions. Because total amounts of protein kinase C isoforms and α-smooth muscle actin were not chronologically changed, we may say that translocation of protein kinase Cα and δ occurs. It is not clear at present why their results of protein kinase C isoform expression differ from the results of the present study. Furthermore, our previous study demonstrated that stimulation by low concentration of endothelin-1 (30–100 pM) combined with serotonin also induced translocation of protein kinase Cδ from cytosol to membrane fractions without any change in total amount of each isoform in the porcine coronary artery (Obara et al., 1999). Protein kinase Cδ may play a role as a Ca^{2+} sensitizer in the contractile activation. These previous results are consistent with our present results, and support that the present findings would be pertinent. Our present results indicate that protein kinase Cδ and α play a role in the development of vasospasm after subarachnoid hemorrhage.

Protein kinase Cδ was initially translocated from the cytosol to the membrane fractions on day 4, and then protein kinase Cα translocation followed on day 7. Protein kinase Cδ may also play a role as a Ca^{2+} sensitizer in the initiation/development of vasospasm, whereas protein kinase Cα may be mainly involved in the maintenance of severe vascular contraction. We are now investigating how these two isoforms interact with each other and contribute to the pathogenesis of vasospasm after subarachnoid hemorrhage.

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