Molecular mechanisms involved in development of cerebral vasospasm

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Object. Although the agents responsible for production of vasospasm have not yet been clearly identified, the author reviews the molecular mechanisms involved in development of vasospasm mainly based on the experimental data in a canine two-hemorrhage model.

Methods. The blood products after subarachnoid hemorrhage most likely stimulate many cell membrane receptors, such as G protein-coupled receptors and receptor tyrosine kinases, to activate the tyrosine kinase pathway of the vascular smooth muscle cells. The activation of the tyrosine kinase pathway is associated with continuous elevation of intracellular Ca^{++} levels and activation of μ -calpain; the former may result mainly not from Ca^{++} release but from Ca^{++} influx from outside the cells. The increased intracellular Ca^{++} concentrations stimulate Ca^{++} /calmodulin (CaM)-dependent myosin light chain kinase to phosphorylate myosin light chain continuously during vasospasm. A topical application of genistein, ethylene-glycol-bis(β -aminoethylether) N,N'-tetraacetic acid, or various L-type Ca^{++} channel blockers likely induces reversal of vasospasm as a result of a decrease in intracellular Ca^{++} levels. The blood products also activate the rho/rho-associated kinase pathway during vasospasm most likely via G protein-coupled receptors, and the activated rho-associated kinase inhibits myosin phosphatase through phosphorylation at its myosin-binding subunit to induce Ca^{++} -independent development of vasospasm. The enhanced generation of arachidonic acid during vasospasm may also contribute to inhibition of myosin phosphatase, at least in part, through the rho/rho-associated kinase pathway. The activity of myosin phosphatase in vasospasm can also be inhibited by activated protein kinase C independently of the rho/rho-associated kinase pathway, but the inhibition may play a minor and transient role in contractile regulation. The protein levels of thin filament-associated proteins, calponin and caldesmon, are progressively decreased in vasospasm, whereas their phosphorylation levels are increased. Both changes probably contribute to the enhancement of smooth muscle contractility. Contractile and cytoskeletal proteins appear to be degraded in vasospasm by proteolysis with activated μ -calpain, suggesting that the intracellular devices responsible for smooth-muscle contraction are severely degraded in vasospasm.

Conclusions. It remains to be determined the extent to which Ca^{++} -dependent and -independent contractile regulations, proteolysis and phosphorylation of thin filament-associated proteins, and degradation of contractile and cytoskeletal proteins are involved in the development of vasospasm.

KEY WORDS • vasospasm • myosin light chain • thin filament-associated protein • cytoskeleton • phosphorylation • proteolysis

Subarachnoid hemorrhage-induced cerebral vasospasm occurs in 20 to 40% of patients with ruptured intracranial aneurysm.⁴⁹ Cerebral vasospasm appears to be biphasic, with an initial acute phase of 1 to 3 days after SAH and a subsequent delayed phase.⁵ Initial-phase vasospasm after SAH is easily observed in animal models but seems to occur rarely in humans;⁷ delayed-onset cerebral vasospasm is responsible for serious neurological morbidity and mortality and may be refractory to treatment. It is now widely accepted that blood products, especially oxyhemoglobin,

contribute to cerebral vasospasm, but the agent(s) responsible for cerebral vasospasm have yet to be clearly identified. Because in vitro experimental data do not always reflect in situ conditions, the molecular mechanisms of cerebral vasospasm are reviewed mainly based on data obtained from in vivo molecular studies of experimental vasospasm in a two-hemorrhage canine model developed by Varsos, et al.⁷⁵ The Ca^{++} -dependent and -independent vasoconstriction, proteolysis and phosphorylation of CaP and CaD, and degradation of contractile and cytoskeletal proteins are shown to be involved in the spastic BA.

Ca^{++} -DEPENDENT VASOCONTRACTION IN VASOSPASM

Central to the regulation of smooth-muscle contractility is Ca^{++} activity in the cytosol.⁷⁷ Smooth-muscle contraction is regulated by phosphorylation and dephosphorylation of MLC by Ca^{++} /CaM-regulated MLCK and myosin phosphatase, respectively. Analysis by pyrophosphate gel

Abbreviations used in this paper: BA = basilar artery; CaD = caldesmon; CaM = calmodulin; CaP = calponin; EGTA = ethylene-glycol-bis(β -aminoethylether) N,N'-tetraacetic acid; ERK = extracellular signal-regulated kinase; $\text{Ins}(1,4,5)\text{P}_3$ = inositol 1,4, 5-trisphosphate; MEK = mitogen-activated protein kinase kinase; MLC = myosin light chain; MLCK = myosin light chain kinase; ML-9 = 1-(5-chloro-naphthalenesulfonyl)-1H-hexa-hydro-1,4-diazepine; PiP_2 = phosphatidylinositol-bis-phosphate; PKC = protein kinase C; PLC- γ = phospholipase C- γ ; SAH = subarachnoid hemorrhage.

electrophoresis demonstrates three MLC bands in vasospasm as well as in KCl- and serotonin-induced vasoconstriction,²¹ suggesting that vasospasm-related MLC is phosphorylated by MLCK, because pyrophosphate gel electrophoresis resolves smooth-muscle MLC into three bands in the MLCK-mediated phosphorylation and into a single band in the PKC-mediated phosphorylation based on the phosphorylation state.⁶⁸⁻⁷⁰ It has been reported that μ -calpain but not m-calpain in the BA is continuously activated in vasospasm,^{56,79} which indicates a continuous rise of intracellular Ca^{++} levels, because μ - and m-calpains are activated at 1 μM and 1 mM Ca^{++} levels, respectively.⁶⁵ On the other hand, μ -calpain was observed to be transiently activated only in the beginning of KCl- or serotonin-induced vasoconstriction of the BA,⁷⁹ which is indicative of a transient rise of intracellular Ca^{++} levels and their return toward baseline despite maintained force. The phosphorylation of MLC in vasospasm, although reported to be unchanged after SAH in one study,⁶⁴ is increased in the anterior spinal artery⁶ and the BA⁵⁵ with the passage of time after SAH. In addition, late-onset vasospasm is reversed by a transclival topical application of ML-9,³⁹ a selective inhibitor of MLCK. Thus, the continuously elevated intracellular Ca^{++} during vasospasm activates MLCK, which leads to continuous phosphorylation of MLC that interacts with actin filament to induce contraction.⁷⁷ The intracellular tyrosine kinase pathway, including Shc, PLC- γ , Raf1, and ERKs in the BA, has been shown to be activated after SAH (Fig. 1), maximally on Days 0 and 2 and then gradually declined.²¹ The activation of Shc suggests a stimulation of signal transduction from G protein-coupled receptors, receptor tyrosine kinases, or both^{8,73,74} (Fig. 1). The activation of both G protein-coupled receptors and growth factor receptors generally leads to smooth-muscle contraction that is accompanied by activation of tyrosine phosphorylation of intracellular proteins and increase in intracellular Ca^{++} levels, both of which are suppressed by genistein,^{12,13} a tyrosine kinase inhibitor. The development of vasospasm is also inhibited by a transclival topical application of genistein,²¹ and the reversal of vasospasm by genistein is associated with the decrease in intracellular Ca^{++} levels.^{21,56} Thus, the enhancement of vasospasm-related tyrosine phosphorylation is closely associated with the increase in intracellular Ca^{++} levels.

Mechanisms that participate in the increase in intracellular Ca^{++} levels in smooth muscle generally include influx of extracellular Ca^{++} , release of intracellular Ca^{++} stored in the sarcoplasmic reticulum, extrusion of cytosolic Ca^{++} by a sarcolemmal Ca^{++} -adenosine triphosphatase, and several ion-exchange mechanisms allowing for bidirectional exchange of Ca^{++} and another cation (that is, Na^+) between the cytosolic and extracellular compartments.^{30,51} The major regulatory mechanism, however, is modulation of Ca^{++} release and influx. Phospholipase C- γ mediates hydrolysis of PiP_2 to $\text{Ins}(1,4,5)\text{P}_3$ and diacylglycerol, with $\text{Ins}(1,4,5)\text{P}_3$ stimulating release of intracellular Ca^{++} from Ca^{++} store sites (Fig. 1),^{4,63} and is activated by tyrosine phosphorylation in vasospasm more intensely in the early than late stage (Fig. 2). Phospholipase C- β is not detected in the BA, and PLC- δ is not activated by tyrosine phosphorylation during vasospasm (unpublished data). A potent inhibitor of $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{++} release, 2A-

PB, is membrane penetrable and does not affect $[\text{H}]\text{Ins}(1,4,5)\text{P}_3$ binding to its receptor nor the Ca^{++} influx from the exterior of cells.⁴⁵ The maximum relaxation of the spastic BA on post-SAH Day 7 induced by a transclival topical application of 2APB is $32.2 \pm 3.4\%$, if the relaxation induced by 10 mM EGTA is taken as 100% (Fig. 3, Table 1); this suggests that the Ca^{++} release from the intracellular Ca^{++} stores is not enough to maintain continuous vasospasm and that some mechanisms other than the Ca^{++} release, such as the Ca^{++} influx, are essential to induce the continuous elevation of Ca^{++} levels during vasospasm.

Influx of Ca^{++} can occur through several types of voltage-dependent channels and through so-called receptor-operated and/or second messenger-operated channels that appear to be voltage independent. Voltage-dependent Ca^{++} channels have been extensively investigated in smooth-muscle cells, including those obtained in cerebral vessels,^{36,42,60} and it has been suggested that the L-type Ca^{++} channel is the predominant channel in the regulation of cerebral vascular smooth-muscle tone. On Day 7 the spastic BA is dilated dose dependently in vivo by a transclival topical application of gadolinium chloride, an L-type Ca^{++} channel blocker,⁴⁰ as well as organic L-type Ca^{++} channel blockers including dihydropyridines (for example, nifedipine), benzothiazepines (diltiazem), and phenylalkylamines (verapamil), and their maximum responses are shown in Fig. 3 and Table 1, if the relaxation obtained by 10 mM EGTA is taken as 100%, suggesting that the L-type Ca^{++} channel is substantially involved in the enhancement of intracellular Ca^{++} levels in vasospasm. Thus, the Ca^{++} influx through L-type Ca^{++} channel is a major source of the increased intracellular Ca^{++} levels whereas the Ca^{++} release from Ca^{++} store sites is a minor source in vasospasm.

The activity of L-type Ca^{++} channels may be regulated by posttranslational modifications including phosphorylation and proteolysis. There is emerging evidence that smooth-muscle contraction and Ca^{++} influx through voltage-dependent L-type Ca^{++} channels are regulated by tyrosine kinases (Fig. 1). For instance, the fact that Ca^{++} currents are attenuated by tyrosine kinase inhibitors and enhanced by growth factors in rabbit colonic smooth-muscle cells²⁷ suggests novel mechanisms for tyrosine kinases in the regulation of intracellular Ca^{++} levels. In colonic smooth-muscle cells, the α_{1C} subunit of the L-type Ca^{++} channel has been reported to be tyrosine phosphorylated and activated following the stimulation by platelet-derived growth factor.³² The fact that the intracellular tyrosine kinase system is activated in vasospasm and that genistein induces reversal of vasospasm in vivo concomitantly with a marked reduction of intracellular Ca^{++} suggests that the intracellular Ca^{++} levels in vasospasm are controlled substantially by tyrosine phosphorylation of L-type Ca^{++} channels.²¹

The authors of previous studies have indicated that calpain catalyzes the conversion of the long form of the α_{1C} subunit of L-type Ca^{++} channel to the short form in vitro and in vivo,^{10,28} and the short form of the α_{1C} subunit is formed by truncation at the C-terminal end.^{11,28,29} The elimination of the C terminus of α_{1C} subunits by truncation of the complementary DNA gives rise to Ca^{++} channel in which the conductance activity is four- or sixfold higher than the long form,^{28,38,76} suggesting that the C terminus of α_{1C} subunits exerts an inhibitory control over the

Fig. 1. Regulation mechanisms involved in development of vasospasm. A basic aspect of signal transduction pathways involved in development of vasospasm is essentially similar to that involved in agonist-induced vasoconstriction. The increase in intracellular Ca^{2+} (Ca^{2+}) levels in vasospasm is induced mainly not by Ca^{2+} release from sarcoplasmic reticulum (SR) but by Ca^{2+} influx through an L-type Ca^{2+} channel. The major Ca^{2+} -dependent pathway increasing MLC phosphorylation in vasospasm is induced by activation of Ca^{2+} /CaM-dependent MLCK as a result of elevation of intracellular Ca^{2+} levels. The major Ca^{2+} -independent pathway enhancing MLC phosphorylation in vasospasm is induced by activation of rho-associated kinase, resulting in inhibition of myosin phosphatase activity and enhanced MLC phosphorylation. Increase in arachidonic acid (AA) in vasospasm can also activate rho-associated kinase and, at least in vitro, also inhibit myosin phosphatase activity by dissociating the regulatory from the catalytic subunit. The third pathway shown that increases MLC activity in vasospasm is through phosphorylation of CPI-17 by PKC, leading to direct inhibition of catalytic subunit of myosin phosphatase by the phosphorylated CPI-17. In addition, the decrease in protein levels of CaP and CaD as well as the increase in phosphorylation of CaP and CaD in vasospasm increase smooth-muscle contractility. (PKC = PKC activated by production of diacylglycerol (DAG) and catalytic fragment phosphorylates CaP and inhibits myosin phosphatase. ERK = ERK activated by tyrosine kinase pathway through receptor tyrosine kinase and G protein-coupled receptors [GPCRs] phosphorylates CaD.) GDI = guanine nucleotide dissociation inhibitors; GEF = guanine nucleotide exchange factor; PLA₂ = phospholipase A₂; RTK = receptor tyrosine kinase.

activity of L-type Ca^{2+} channels. De Jongh, et al.,¹⁰ have reported that μ -calpain cleaves a 37-KD fragment from the C terminus of L-type Ca^{2+} channel in a time- and concentration-dependent manner and that proteolysis is independent of the α_{1C} phosphorylation state. Because μ -calpain is continuously activated in vasospasm, the truncation of α_{1C} subunit might be induced by μ -calpain and play a significant role for sustained increases in the activity of L-type Ca^{2+} channels. Thus, the sustained increase in intracellular Ca^{2+} levels in vasospasm is mainly caused by the increase in Ca^{2+} influx resulting from both the tyrosine phosphorylation and the proteolysis of the L-type Ca^{2+} channels. In contrast in agonist-induced responses the rapid transient increase in intracellular Ca^{2+} appears to be

due to the Ca^{2+} release from the Ca^{2+} store sites, accelerated by the tyrosine phosphorylation of PLC- γ , and the transient increase is followed by lower intracellular Ca^{2+} levels that appear to be due to the Ca^{2+} influx of extracellular Ca^{2+} mediated by the lower levels of tyrosine phosphorylation of L-type Ca^{2+} channel.

Ca^{2+} -INDEPENDENT VASOCONTRACTION IN VASOSPASM

After SAH the blood products also activate the rho/rho-associated kinase pathway progressively with the time lapse after SAH probably secondary to G protein-coupled receptors;⁵⁵ additionally the blood products contribute to

Fig. 2. Analysis of PLC- γ activation showing a marked increase in tyrosine phosphorylation of PLC- γ in spastic BAs on Days 0 (D-0), 2 (D-2), and 7 (D-7) after SAH. The increase in tyrosine phosphorylation of PLC- γ on Day 7 is markedly inhibited by a transclival topical application of 17 μ M genistein (G). *Left:* Basilar artery lysates were immunoprecipitated with anti-PLC- γ antibody, resolved by 10% sodium dodecylsulfate polyacrylamide gel electrophoresis, transferred onto polyvinylidene difluoride membranes, and immunologically probed with anti-phosphotyrosine-horseradish peroxidase conjugate. *Right:* The membranes were reprobed with anti-PLC- γ antibody, and no significant changes in protein levels of PLC- γ were shown. The results are from a representative study performed three times with comparable outcomes.

Ca⁺⁺-independent development of vasospasm by inhibiting myosin phosphatase through phosphorylation at its myosin-binding subunit^{22,55} (Fig. 1). A transclival topical application of 10 μ M Y-27632, a specific inhibitor of rho-associated kinase,⁷² to the spastic BA on post-SAH Day 7 has been shown to induce a dose-dependent dilation and simultaneous decrease in phosphorylation of both the myosin-binding subunit of myosin phosphatase and MLC.⁵⁵ Analysis of those results indicates that rho-associated kinase is involved in the enhancement of vasospasm in addition to Ca⁺⁺/CaM-dependent MLCK. It remains to be determined to what extent rho-associated kinase and MLCK are involved in the development of vasospasm. The rho/rho-associated kinase pathway is continuously activated in both vasospasm and agonist-induced vasoconstriction, more markedly in the former than in the latter.⁵⁵ The activated rho-associated kinase also stoichiometrically phosphorylates directly MLC;⁴⁶ however, direct *in vivo* phosphorylation of MLC by rho-associated kinase plays no significant role *in vivo*. This is because, in the absence of intracellular Ca⁺⁺ to activate MLCK even

massive stimulation of the rho/rho-associated kinase pathway by glutamyl transpeptidase- γ induces minimal and very slow (or no) MLC phosphorylation and contraction of smooth muscle.^{34,62,66}

The increased production of prostaglandin E₂⁴³ and leukotrienes^{58,80} in vasospasm suggests that the generation of arachidonic acid is enhanced. Arachidonic acid can activate rho-associated kinase^{17,20} and may contribute an ancillary pathway of rho-associated mediated Ca⁺⁺ sensitization (Fig. 1). *In vitro*, arachidonic acid dissociates the regulatory from the catalytic subunit of myosin phosphatase,²⁶ leading to a several-fold reduction in myosin phosphatase activity. Protein kinase C is activated by diacylglycerol, which is produced by PLC- γ -mediated hydrolysis of PIP (Fig. 1),⁶³ and has been shown to translocate from the cytosol to the plasma membrane as well as to generate catalytic fragment μ -calpain-induced proteolysis during vasospasm.⁵⁶ The catalytic fragment of PKC is produced continuously in vasospasm and transiently in agonist-induced vasoconstriction by proteolysis with μ -calpain.⁵⁶ Activated PKC can enhance contraction at constant intracellular Ca⁺⁺ by inhibiting myosin phosphatase directly or by phosphorylating an inhibitor.^{35,62} This inhibition is mediated by phosphorylation of CPI-17.^{15,37,57} This smooth-muscle-specific myosin phosphatase inhibitor exerts its phosphatase inhibitory effect and hence Ca⁺⁺ sensitizing action in permeabilized preparations only when phosphorylated by PKC. Thus, PKC can inhibit myosin phosphatase independently of rho-associated kinase but play a minor and transient role in contractile regulation,^{21,34} with its extent possibly depending on the agonist and/or cell involved. The activities of myosin phosphatase are decreased in vasospasm,²² but it remains to be determined to what extent rho-associated kinase and PKC are involved in the inhibition of myosin phosphatase.

PROTEOLYSIS AND PHOSPHORYLATION OF CAP AND CAD IN VASOSPASM

The primary mechanism involved in activating smooth-

Fig. 3. Graphs demonstrating a log dose-response curve for dilation effect of inhibitors used. The spastic BA on Day 7 exposed via a transclival route was treated by a topical application of gadolinium chloride, diltiazem, and 2APB, and the relaxation induced by 10 mM EGTA was taken as 100%. The mean values of relaxation percentage of five samples in each agent group are shown. Vertical bars represent standard deviations.

TABLE 1

Maximum response of spastic BA to 2APB and Ca⁺⁺ channel blockers*

Factor	2APB	Gadolinium Chloride	Diltiazem	Nicardipine	Verapamil
response (%)	32.2 ± 3.4	70.5 ± 3.2	82.3 ± 3.5	84.3 ± 4.2	80.3 ± 3.4

* All values represent the mean ± standard deviation. All measurements were obtained on Day 7 post-SAH when relaxation induced by 10μM EGTA is taken as 100%. Five samples were tested with each of the agents.

muscle contraction is the phosphorylation of MLC. Phosphorylation–dephosphorylation of the thin filament–associated proteins, CaP and CaD, shows the secondary potential features of the regulation of the contractile system in smooth-muscle cells. The levels of protein and phosphorylation at serine/threonine residues of CaP are not significantly changed in vasospasm on Day 0, but the former progressively decreases and the latter continuously increases in vasospasm on Days 2 and 7.²¹ In contrast, the levels of protein and phosphorylation of CaP are not changed in KCl- and serotonin-induced vasoconstriction.²¹ Protein levels of CaD are not changed in vasospasm on Day 0 but decrease progressively thereafter, whereas phosphorylation at serine 759 of CaD is increased in vasospasm, maximally on Day 0 followed by a gradual decline (Fig. 4). In KCl- and serotonin-induced vasoconstriction, protein levels of CaD are not changed whereas phosphorylation levels of CaD are increased less markedly compared with those in vasospasm on Days 0 and 2 (Fig. 4). Thus, involvement of CaP phosphorylation in vasospasm may occur more in the late stage than in the early stage, whereas CaD phosphorylation in vasospasm may participate more in the early stage than in the late stage. The decrease in protein levels of CaP and CaD in vasospasm could be secondary to μ-calpain induced proteolysis.

There has been growing physiological support for the role of CaP and CaD in inhibiting the actin-activated Mg²⁺-adenosine triphosphatase activity of smooth muscle through its binding to actin^{31,59,61} (Fig. 1). Although the significance of phosphorylation of CaP and CaD in vivo remains controversial, the phosphorylation of CaP and CaD has been shown to lower markedly the affinity of CaP and CaD for actin and to alleviate the inhibition of

actin-activated myosin Mg²⁺-adenosine triphosphatase.^{24,53} Because CaP is phosphorylated in vivo by PKC,^{48,54} PKC activated in vasospasm may phosphorylate CaP (Fig. 1). It remains to be determined, however, to what extent PKC is involved in phosphorylation of CaP^{57,67} and inhibition of myosin phosphatase^{35,62} for the enhancement of smooth-muscle contractility in vasospasm. Caldesmon is phosphorylated in situ by ERK,^{1,2,19} and the molecular sites of CaD phosphorylated in situ are identical to those phosphorylated in vitro by ERK, but not by PKC or other kinases.^{1,2,24} The PD-98059, an MEK1 inhibitor, significantly inhibits myogenic tone in rat middle cerebral arteries and reduces vasopressin- and KCl-induced tone in vitro.⁴¹ Angiographically demonstrated vasospasm on Day 7 is reduced approximately 59% by the intracisternal injection of PD-98059 once a day on Days 3 through 6, and PD-98059 has been shown to abolish ERK immunoprecipitation in spastic BAs.⁷¹ These findings suggest that CaD is phosphorylated by ERK activated during vasospasm (Fig. 1). The decreased activities of smooth-muscle PP1 and PP2A in vasospasm²² may also enhance further the phosphorylation of CaP and CaD, because PP1 and PP2A function as CaP phosphatase^{33,78} and PP2A as CaD phosphatase.⁵² Thus, CaP and CaD may be involved in a significant increase of smooth-muscle contractility in vasospasm through μ-calpain-induced proteolysis as well as through phosphorylation of CaP and CaD by PKC and ERK, respectively.

DEGRADATION OF CONTRACTILE AND CYTOSKELETAL PROTEINS IN VASOSPASM

The extent of degradation of contractile and cytoskeletal proteins examined by immunoblot analysis in vasospasm and KCl- and serotonin-induced vasoconstriction⁴⁷ is shown in Table 2. The contractile and cytoskeletal elements in avian gizzard smooth-muscle cells in vitro are well organized in the resting state,¹⁴ as shown in Fig. 5. The actin filaments are linked to the plasma membrane at the subplasmalemmal dense plaques containing talin and vinculin as well as within the cell to the cytoplasmic dense bodies containing α-actinin.¹⁴ The myosin filament shows a plaitlike and interdigitating arrangement and is pre-

TABLE 2

Degradation of contractile and cytoskeletal proteins*

	Vasospasm		Vasoconstriction	
	Day 2	Day 7	KCl-Induced	Serotonin-Induced
actin	±	±	±	±
myosin	+	++	+	±
desmin	±	±	—	—
filamin	±	++	±	±
talin	++	+++	±	+
-actinin	++	+++	±	±
vinculin	—	++	—	—

* Degradation of contractile and cytoskeletal proteins in five spastic BAs in each group (vasospasm, KCl, and serotonin) were examined by immunoblotting. Symbols: — = intact; ± = slight; + = moderate; ++ = substantial; +++ = marked.

Fig. 4. Analysis showing a marked increase in CaD phosphorylation in the spastic BAs on Day 0 (D-0) followed by gradual decline on Days 2 (D-2) and 7 (D-7). *Left:* Basilar artery lysates were resolved using 6% sodium dodecylsulfate polyacrylamide gel electrophoresis, transferred onto polyvinylidene difluoride membranes, and immunologically probed with anti-phospho-CaD antibody. *Right:* The membranes were reprobed with anti-CaD antibody after stripping each anti-phospho-CaD antibody off the membranes, showing a gradual fall after SAH. Similar results were achieved in three separate experiments.

Fig. 5. Schematic illustrations demonstrating the suggested arrangements of contractile and cytoskeletal elements in the resting state (A) and contraction (B) *in vitro* (data obtained from Draeger, et al). A: Actin filaments (long, thin lines) are linked to the plasma membrane at the subplasmalemmal dense plaques (closed triangles) and within the cell to the cytoplasmic dense bodies (closed circles). Myosin filaments (short, thick lines) are presumed to be side polar. The cytoplasmic dense bodies are also linked to the cytoskeleton (wavy lines) that forms longitudinal and transverse connections. B: Contractile units become uncoupled from the cytoskeleton because of partial degradation of the cytoskeleton (not depicted) and become more unhindered. Myosin filaments move to the center of the contraction bands (center and right), with actin either straddling the band (center) or sheared out on both sides (right). In the latter case the cytoplasmic dense bodies remain in the vicinity of the myosin filaments.

sumed to be side polar. The cytoskeleton, such as desmin and filamin, is composed of a mainly longitudinal, meandering, branched system of fibrils and linked to the cytoplasmic dense bodies. Thus, the proteins responsible for smooth-muscle contraction are actin and myosin as contractile units; desmin and filamin as cytoskeleton; and talin, vinculin, and α -actinin as attachment proteins for contractile and cytoskeletal elements. The contractile units become uncoupled from the cytoskeleton in contraction, because of partial degradation of the cytoskeleton, and more unhindered.¹⁴ Calpain is reported to initiate nonlysosomal degradation of actin, desmin, filamin, talin, vinculin, and α -actinin.^{3,9,16,23,25,50} We suggest, therefore, that the uncoupling of the contractile units from the cytoskeleton is more severe in vasospasm than in KCl- and serotonin-induced vasoconstriction, probably due to progressive proteolytic mechanism with μ -calpain after SAH, making relaxation difficult in response to vasodilator agents.

CONCLUSIONS

The continuous increase in intracellular Ca^{++} levels of vascular smooth-muscle cells is characteristic of cerebral vasospasm; its development is mainly the result of the increase in Ca^{++} influx through an L-type Ca^{++} channel by its tyrosine phosphorylation and proteolysis with μ -calpain. The continuous activation of Ca^{++} /CaM-regulated MLCK during vasospasm is the primary Ca^{++} -dependent mechanism of vasospasm. The inhibition of myosin phosphatase, which is induced by the activation of rho-associated kinase and PKC, is involved in the development of vasospasm as a Ca^{++} -independent mechanism. Both CaP and CaD are degraded and phosphorylated during vasospasm and are involved in a significant increase of smooth-muscle contractility. In addition, the marked degradation of contractile and cytoskeletal proteins also

contributes to the development of vasospasm, and this probably occurs by severe uncoupling of the contractile units from the cytoskeleton. Flamm, et al.,¹⁸ reported that the intravenous infusion of high doses of nicardipine had significantly beneficial effects on development of delayed cerebral ischemia after aneurysmal SAH. In our clinic similar results have been reported in early treatment with high doses of nicardipine as early as possible within 24 hours after SAH,⁴⁴ suggesting that the therapeutic reduction of intracellular Ca^{++} is an important measure to prevent development of vasospasm.

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