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The role of protease-activated receptors on the intracellular calcium ion dynamics of vascular smooth muscles, with special reference to cerebral arterioles*

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Summary. Protease-activated receptors (PARs) mediate cellular responses to various proteases in numerous cell types, including smooth muscles and the endothelium of blood vessels. To clarify whether the stimulation of PARs induces responses in smooth muscle cells of cerebral arterioles, intracellular Ca^{2+} ($[Ca^{2+}]_i$) dynamics and nitric oxide (NO) production during PARs stimulation were investigated in the rat cerebral arterioles by real-time confocal microscopy, since $[Ca^{2+}]_i$ and NO are both key factors in the maintenance of strain in blood vessels. Testicular arterioles were also investigated for comparison. In smooth muscle cells of small cerebral arterioles (< 50 μm in diameter), thrombin and PAR1-activating peptide (AP) induced an increase in $[Ca^{2+}]_i$ and contraction. The response to PAR1 activation was caused by Ca^{2+} mobilization from intracellular Ca^{2+} stores. Trypsin and PAR2-AP induced a decrease in $[Ca^{2+}]_i$ in the cells which was considered to be mediated by endothelium-derived NO and/or by promoting a Ca^{2+} sequestration mechanism. PAR3- and 4-AP had little effect. In contrast to small cerebral arterioles, $[Ca^{2+}]_i$ dynamics in smooth muscle cells of large cerebral arterioles (< 150 μm in diameter) or testicular arterioles remained unchanged during PARs activation. The effects of PARs activation on the $[Ca^{2+}]_i$ dynamics and the contraction/relaxation of cerebral arterioles are also discussed in relation to the role of proteases in the regional tissue circulation of the brain.

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Introduction

Protease-activated receptors (PARs) represent a novel class of seven transmembrane domain G-protein coupled receptors, which are activated by proteolytic cleavage (Dery *et al.*, 1998). To date, four members of this class of receptors have been identified: PAR1, 2, 3, and 4 (Vu *et al.*,1991; Nystedt *et al.*, 1994; Ishihara *et al.*, 1997; Kahn *et al.*, 1998; Xu *et al.*, 1998; Hollenberg, 1999). The mechanism of PARs activation involves the proteolytic unmasking of an N-terminal sequence that acts as a tethered ligand (Dery *et al.*, 1998). Thrombin functions as an agonist for PAR1, 3, and 4 (Vu *et al.*, 1991; Ishihara *et al.*, 1997; Kahn *et al.*, 1998; Xu *et al.*,1998), and trypsin is a major agonist for PAR2 (Nystedt *et al.*, 1994). In addition, mast cell tryptase and coagulation factors VIIa and Xa are generally thought to serve as significant agonists of PAR2 (Nystedt *et al.*, 1994; Camerer *et al.*, 2000; Kawabata and Kuroda, 2000).

Recent studies have reported that PARs are present in a variety of cells and can be reading implicated in the regulation of a number of vital functions (Macfarlane *et al.*, 2001; Ossovskaya and Bunnett, 2004). In the vascular system, thrombin or trypsin not only function as a coagulation factor or digestive enzyme but also act as extracellular transmitters. Thrombin or trypsin induces the contraction and/or relaxation of vessels, enhancing

angiogenesis, the proliferation and hypertrophy of cells, and increases vascular permeability (Komuro *et al.*, 1997; Sobey and Cocks, 1998; Sobey *et al.*, 1999; Patterson *et al.*, 2001; Hirano and Kanaide, 2003; Major *et al.*, 2003; Moser and Patterson, 2003; Steinberg, 2005).

Still, the effect of PARs activation on vascular tone remains a controversial issue. Although a number of investigators have reported that the activation of PAR1 and 2 results in endothelium-dependent nitric oxide (NO)-cGMP-mediated relaxation of smooth muscle cells of large arteries (Ku and Zaleski,1993;Tesfamar iam,1994a,b; Ku and Dai, 1997; Mizuno *et al.*, 1998, 2000; Derkach *et al.*, 2000), few studies on the PARs of vascular smooth muscles have been performed. In previous studies, vascular contractions induced by thrombin in endothelium-free vessels were reported (Katusic *et al.*, 1984; White 1987), but the relationship between thrombin-induced contraction and PAR1 was not discussed. The significance of PAR3 and 4 in vascular systems remains unclear.

Tissue protease levels increase in certain pathological conditions (e.g., hemorrhage, infarction, or inflammation), suggesting that PARs-mediated action can be pathological rather than physiological (Cocks and Moffatt, 2000; Coughlin, 2000; Hirano and Kanaide,2003). Vasoconstriction of the brain after a severe hemorrhage results in serious additional neuronal damage, and thrombin and other proteases can also be activated in a hemorrhage or an ischemic lesion. In this sense, an evaluation of the effect of PARs activation on tissue circulation could lead to a better understanding of this process.

The aim of the present study was to determine whether PARs activation is involved in the mechanism of the contraction of cerebral arterioles. We also attempted to determine whether organ specificity and size-dependency were factors in responses to PARs activation. For this purpose, we analyzed the $[Ca^{2+}]_i$ dynamics of cerebral arterioles in response to PARs activation because it has been well established that changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) concentration are fundamental in the regulation of various cellular functions (Bootman *et al.*, 2001), including the contraction of smooth muscle cells (van Breemen and Saida, 1989; Somlyo and Somlyo, 1994). Thus, the $[Ca^{2+}]_i$ of vascular smooth muscle cells can be changed in response to the PARs activation. However, only a few studies on the effects of PARs activation on the $[Ca^{2+}]_i$ dynamics of vascular smooth muscle cells have been performed (Komuro *et al.*, 1997).

In the present study, we used rat cerebral arterioles in which the essential structural integrity was maintained because cultured smooth muscle cells are dedifferentiated, resulting in the loss of many muscle characteristics (Ueki *et al.*, 1987); as a result, alterations in their intracellular signaling system cannot be excluded. In this context, we developed a $[Ca^{2+}]_i$ imaging method for intact tissue specimens using real-time confocal microscopy, which has excellent characterization potential in terms of time and spatial resolution (Satoh *et al.*, 1997; Saino and Satoh, 2004).

Materials and Methods

Preparation of arterioles

Experiments were conducted according to the Guidelines of the Ethics Committee for Animal Treatment of Iwate Medical University. Adult male rats (Wistar, 8–12 weeks old, weighing 200–400 g) were killed by carbon dioxide gas followed by exsanguination. They were then perfused *via* the left cardiac ventricle with Ringer's solution (147 mM NaCl, 4 mM KCl and 2.25 mM $CaCl_2$) at 25℃ at a hydrostatic pressure of approximately 1 m of H_2O. A brain and testes were removed and placed in Hepes-buffered Ringer's solution (HR). This solution contained 118 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.13 mM $MgCl_2$, 1 mM NaH_2PO_4, 5.5 mM D-glucose, MEM amino acids solution (Gibco,Grand Island,NY,USA), 1% bovine serum albumin (Sigma, St. Louis, MO, USA), and 10 mM Hepes; pH 7.4, adjusted with NaOH. Cerebral and testicular arterioles were isolated under a binocular, connective tissues were then carefully removed with forceps, and the surrounding connective tissues were digested with collagenase (100 U/m*l*; Elastin Products, Owensville, Missouri , USA) in HR for 2 h at 37℃. After a brief washing with HR, the residual connective tissues were removed with a forceps. Ca^{2+}-deficient solutions were prepared by replacing $CaCl_2$ with EGTA (1 mM; Sigma). Some specimens were fixed and processed for electron microscopy (Saino *et al.*, 2002a, b; Matsuura *et al.*, 2004)

Dye loading for $[Ca^{2+}]_i$ and intracellular nitric oxide (NO) concentration measurement

Spatiotemporal changes in $[Ca^{2+}]_i$ in the arterioles were determined by ratiometry using Indo-1, a fluorescent dye for the measurement of Ca^{2+}. DAF-2 was used to estimate intracellular nitric oxide (NO) production (Kojima *et al.*, 1998; Nakatsubo *et al.*, 1998). Dye loading was facilitated by the use of either acetoxymethyl esters (Indo-1/AM; Dojindo, Kumamoto) or diacethyl esters (DAF-2/DA; Daiichikagaku, Tokyo). Both dyes are cell permeable compounds that are respectively converted to Indo-1 and

DAF-2, by intracellular esterases.

For the measurement of $[Ca^{2+}]_i$ levels, the specimens were transferred to HR that also contained 5 μM Indo-1/AM and 0.02% cremophor® EL (Nacalai Tesque, Kyoto), followed by incubation for 12 h at 4℃. To estimate NO production levels, samples were transferred to HR that contained 10 μM DAF-2/DA and 0.02% cremophor® EL followed by incubation for 1 h at room temperature. After incubation, they were placed on coverslips coated with Cell-Tak®, a nontoxic adhesive reagent, (Collaborative Biomedical Products, Bedford, MA, USA) in modified Sykus-Moor chambers and then continuously perfused with HR that also contained selected stimulants.

A real-time confocal microscope (RCM/Ab; revised version of RCM-8000, Nikon, Tokyo) was used to measure $[Ca^{2+}]_i$ dynamics and NO production. Cells loaded either with Indo-1 or DAF-2 were respectively exposed to an ultraviolet-beam (351 nm) or to a blue-beam (488 nm) for the respective measurement of $[Ca^{2+}]_i$ dynamics or NO production. An argon-ion laser was equipped with an inverted microscope (TE-300, Nikon), and the fluorescence emission was passed through a water-immersion objective lens (Nikon C Apo 40×, N.A. 1.15) to a pinhole diaphragm. The digital images in laser scanning microscopic imaging were composed of 512×480 pixels with a density resolution of 8 bits/pixel. Each pixel gave a spatial resolution of approximately 0.3 μm. A region of interest (ca. 1 μm in diameter) was set on each of the smooth muscle profiles or on the endothelium for a temporal analysis.

In this system, the minimum acquisition time per graphic frame was 1/30 sec. To obtain a confocal image with an acceptable signal/noise ratio, it was necessary to integrate 4–16 frames. Accordingly, in the present study, one confocal image was produced by the integration of 8 successively captured frames. To avoid photodamage and photobleaching, images were acquired intermittently (time interval for the $[Ca^{2+}]_i$ measurement, 2–5 sec, and that for the NO measurement, 10 sec). In preliminary examinations, we were able to confirm that the $[Ca^{2+}]_i$ dynamics of smooth muscle cells persisted for over five seconds. We therefore concluded that the time resolution in the present study was sufficient to permit the $[Ca^{2+}]_i$ dynamics of the cells to be recognized.

For the measurement of $[Ca^{2+}]_i$ dynamics by Indo-1, images were stored on a high-speed hard disk, and a ratio image from each pair was immediately computed: the fluorescence intensity of less than 440 nm ($F_{<440}$) to that greater than 440 nm ($F_{>440}$). A higher ratio ($F_{<440}$ / $F_{>440}$) is indicative of a higher $[Ca^{2+}]_i$, and the ratios were displayed in pseudocolor with 256 hues, red representing a high $[Ca^{2+}]_i$ and purple and blue a low $[Ca^{2+}]_i$. A few injured cells, which showed high $[Ca^{2+}]_i$ under resting conditions, were excluded from the subsequent analyses.

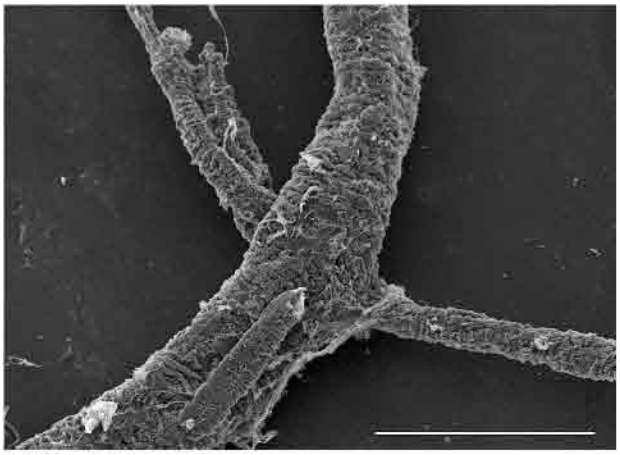

Fig. 1. Scanning electron micrograph of rat cerebral arterioles. Small (ca. 20 μm in diameter) and large (ca. 50 μm in diameter) arterioles are distinguished. These arterioles are surrounded by spindle-shaped smooth muscle cells in a circular fashion. Bar: 100 μm

In the measurement of NO production by DAF-2, the temporal fluorescent intensity of the dyes (F_t) was divided by the fluorescent intensity at the start (F_0). These relative values were assumed to represent the integrated intracellular NO.

Perfusion

Intact arteriole specimens were selected and examined under the microscope. To reduce the possibility of the differential loading of tissues by Indo-1/AM, tissues which showed completely sufficient fluorescence signals were selected.

After a brief washing with HR, the specimens were continuously perfused with HR containing the following agonists: thrombin derived from human plasma (0.01 to 1 U/m*l*; Sigma), PAR1-activating peptide (AP; a synthetic agonist for PAR1; SFLLR-NH2; 100 μM; Bachem, Budendorf, Switzerland), trypsin derived the from porcine pancreas (1,000 U/m*l*; Sigma), PAR2-AP (a synthetic agonist for PAR2; SLIGLV-NH_2; 100 μM; Bachem), PAR3-AP (a synthetic agonist for PAR3; TFRGAP-NH_2; 100 μM; Bachem), PAR4-AP (a synthetic agonist for PAR4; GYPGKF-NH_2; 100 μM; Bachem), adenosine 5'-triphosphate (ATP; 10 μM; Kohjin, Tokyo), thapsigargin (5 μM; Alomone labs., Israel), $GdCl_3$ (10 μM; Wako,

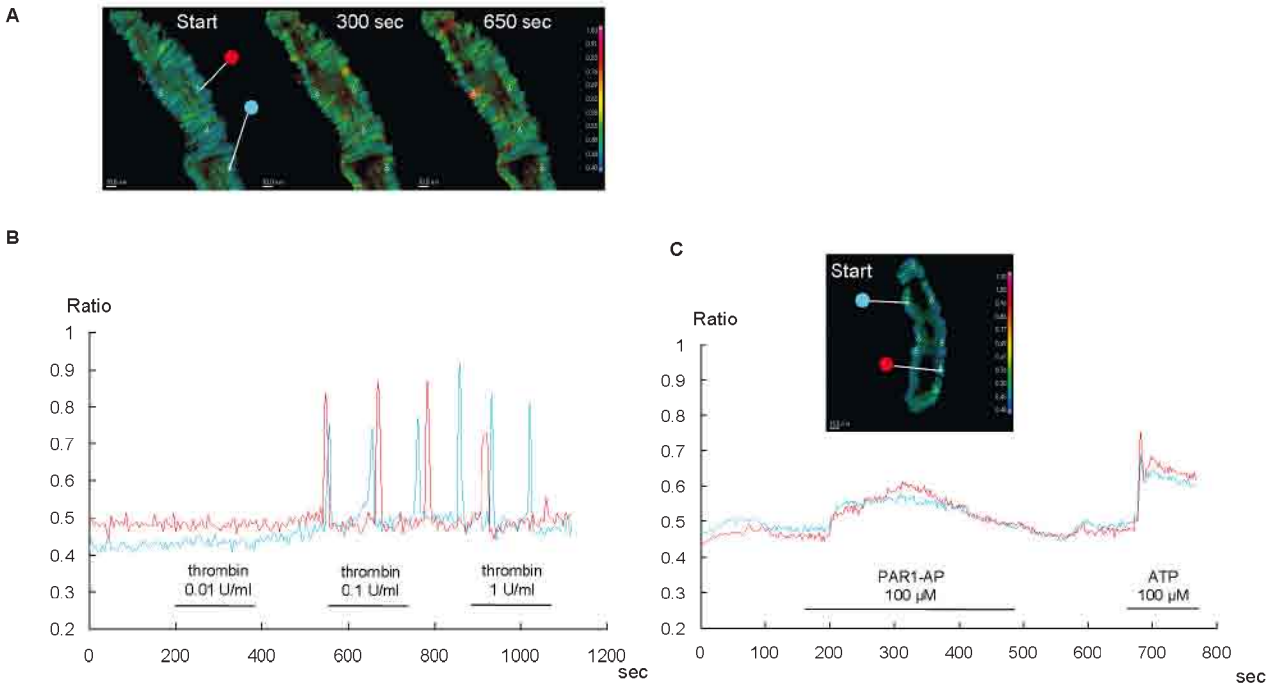

Fig. 2. $[Ca^{2+}]_i$ dynamics of a small cerebral arteriole (< 50 μm in diameter), induced by thrombin and PAR1-AP (synthetic activating peptide of PAR1). **A:** Pseudocolor images show spatial changes of $[Ca^{2+}]_i$ of arterioles. Color scale bar represents the ratio of fluorescences ($F_{<440}/F_{>440}$), representing $[Ca^{2+}]_i$. **B:** Temporal analysis showing the time course for $[Ca^{2+}]_i$ dynamics of a certain region of interest (ROI; red and blue lines correspond to red and blue solid circles in the pseudocolor images). **C:** Time course for $[Ca^{2+}]_i$ dynamics of small cerebral arterioles, induced by activating PAR1-AP. Red and blue lines correspond to red and blue solid circles in the image, respectively.

Osaka), N omega-nitro-L-arginine (L-NNA) (100 μM; Sigma), and 1*H*-[1,2,4]oxadiazolo [4,3,-*a*]quinoxalin-1-one (ODQ) (25 μM; Biomol, Hamburg, Germany). ATP was used as a positive control for the increase in $[Ca^{2+}]_i$ and the contraction of arterioles.

Results

The structural integrity of arterioles was maintained in the specimens prepared for the intracellular calcium imaging study. The arterioles consisted of smooth muscle cells arranged in a circular fashion around the endothelial lining. No significant ultrastructural damage (e.g. swollen mitochondria, or the vacuolation of the sarco endoplasmic reticulum) was detected by transmission electron microscopy in the specimens (Saino *et al.*, 2002a, b; Matsuura *et al.*, 2004).

Two sizes of the arterioles were distinguished in the arborization of blood vessels of the rat brain: small (less than ca. 50 μm in diameter) and large (from ca. 50 μm to less than ca. 150 μm in diameter) (Fig. 1). Small arterioles branched out at obtuse angles from the large arteriole. In testicular arterioles, the branching angle was relatively acute, compared with those of cerebral arterioles.

In digital images of laser confocal scanning microscopy, smooth muscle cells were observed as spindle shaped profiles, and the $[Ca^{2+}]_i$ dynamics of these cells could be analyzed (Fig. 2A, B). Because of poor dye-loading, no significant $[Ca^{2+}]_i$ dynamics of the endothelium could be determined in the present study.

Effects of thrombin, PAR1, 3, 4-APs on $[Ca^{2+}]_i$ dynamics and the contraction on the small cerebral arterioles

Thrombin is known to activate PAR1, 3, and 4 (Vu *et al.*, 1991; Ishihara *et al.*, 1997; Kahn *et al.*, 1998; Xu *et al.*,

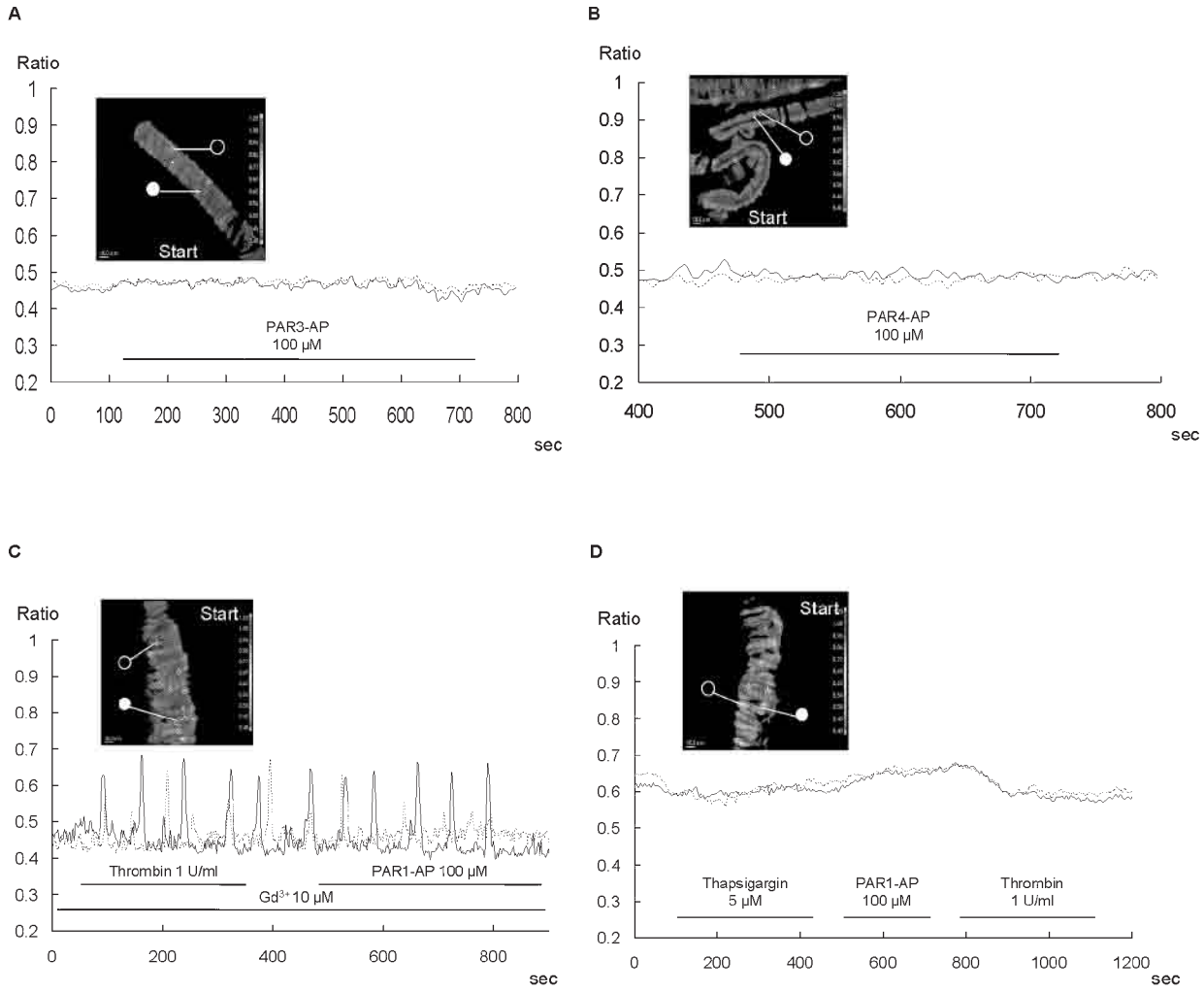

Fig. 3. A and **B:** Time course for $[Ca^{2+}]_i$ dynamics of small cerebral arterioles (< 50 μm in diameter) during PAR3-AP (**A**) and PAR4-AP (**B**) stimulation. Solid and dashed lines correspond to solid and open circles in the image, respectively. **C** and **D:** $[Ca^{2+}]_i$ dynamics of small cerebral arterioles (<50 μm in diameter), to observe the role of Ca^{2+} influx or Ca^{2+} mobilization during thrombin and PAR1-AP stimulations. **C:** Gd^{3+} did not inhibit thrombin (1 U/m*l*)- or PAR1-AP (100 μM)-induced $[Ca^{2+}]_i$ increases. **D:** After depleting the intracellular Ca^{2+} stores by thapsigargin, thrombin- and PAR1-AP-induced $[Ca^{2+}]_i$ increases were inhibited. Solid and dashed lines correspond to solid and open circles in the image, respectively.

1998); this reagent (0.1 and 1 U/m*l*, but not 0.01 U/m*l*) caused oscillatory fluctuations in $[Ca^{2+}]_i$ and persistent spasmoidic contractions of small cerebral arterioles (number of experiments = 7) (Fig. 2B). PAR1-AP induced an increase in $[Ca^{2+}]_i$, but oscillatory fluctuations were rare (n = 5) (Fig. 2C). Contractions of the arterioles induced by PAR1-AP were also faint and not spasmoidic. On the other hand, the sole administration of PAR3- or PAR4-APs did not induce any changes in $[Ca^{2+}]_i$ (n=3) (Fig. 3A, B). Thus, we concluded that the effect of thrombin on the $[Ca^{2+}]_i$ increase in smooth muscle cells is mainly mediated by PAR1.

An extracellular Ca^{2+} deficient environment inhibited the thrombin-induced responses (data not shown), but this condition can empty intracellular Ca^{2+} stores as well as block Ca^{2+} influx. Gd^{3+} (10 μM), a nonspecific cation

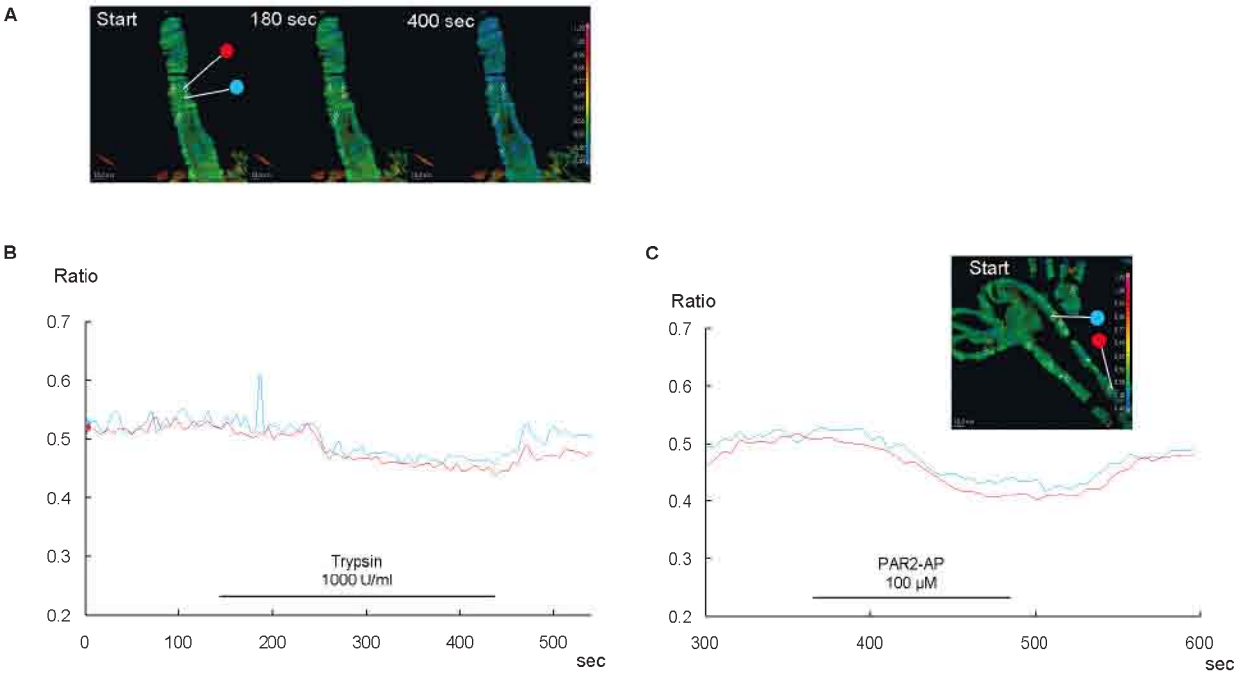

Fig. 4. $[Ca^{2+}]_i$ dynamics of a small cerebral arteriole (<50 μm in diameter) induced by trypsin and PAR2-AP. **A:** Pseudocolor images showing spatial changes of $[Ca^{2+}]_i$ of arterioles. **B:** Temporal analysis showing the time course for $[Ca^{2+}]_i$ dynamics of ROI (red and blue lines correspond to red and blue solid circles in the images). **C:** Time course for $[Ca^{2+}]_i$ dynamics of small cerebral arterioles during PAR2-AP stimulation. Red and blue lines correspond to red and blue solid circles in the image, respectively.

channel blocker, did not inhibit the thrombin- or PAR1-AP-induced $[Ca^{2+}]_i$ increases and contractions (n = 6) (Fig. 3C). The depletion of Ca^{2+} stores by thapsigargin (5 μM), an inhibitor of sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), suppressed thrombin- or PAR1-AP-induced responses (n = 4) (Fig. 3D). This indicates that the response to PAR1 activation was caused by Ca^{2+} mobilization from intracellular Ca^{2+} stores.

Effects of trypsin and PAR2-AP on $[Ca^{2+}]_i$ dynamics and contraction on the small cerebral arterioles

Extracellular trypsin caused a transient increase in $[Ca^{2+}]_i$ in a few smooth muscle cells in small cerebral arterioles, but no oscillatory fluctuations were induced and no evident contraction was observed. It is noteworthy that most smooth muscle cells showed a gradual $[Ca^{2+}]_i$ decrease due to the effect of trypsin. After the washing of trypsin, the decrease of $[Ca^{2+}]_i$ recovered (n = 5) (Fig. 4A, B). PAR2-AP elicited a similar $[Ca^{2+}]_i$ decrease (n = 15) (Fig. 4C).

To investigate NO production by PAR2 activation, the fluorescence intensity of DAF-2 was measured. NO production during the perfusion of HR containing trypsin or PAR2-AP was verified in the endothelium but not in smooth muscle cells (n = 4) (Fig. 5). A NO synthase (NOS) inhibitor, L-NNA (100 μM), inhibited trypsin- or PAR2-AP-induced $[Ca^{2+}]_i$ decreases of smooth muscle cells (n=6) (Fig. 6A,B). A specific inhibitor of soluble guanylyl cyclase, ODQ, did not inhibit the $[Ca^{2+}]_i$ decrease (n=3) (Fig. 6C, D), suggesting that the $[Ca^{2+}]_i$ decrease was cGMP-independent.

To determine whether PAR2 activation promotes the Ca^{2+} elimination/sequestration system of smooth muscles, we studied the effect of thapsigargin, which inhibits SERCA sequestering Ca^{2+} from the cytosol to the sarco/endoplasmic reticulum. Thapsigargin inhibited the PAR2-AP-induced decrease in $[Ca^{2+}]_i$ in smooth muscle cells (n = 2) (Fig. 7).

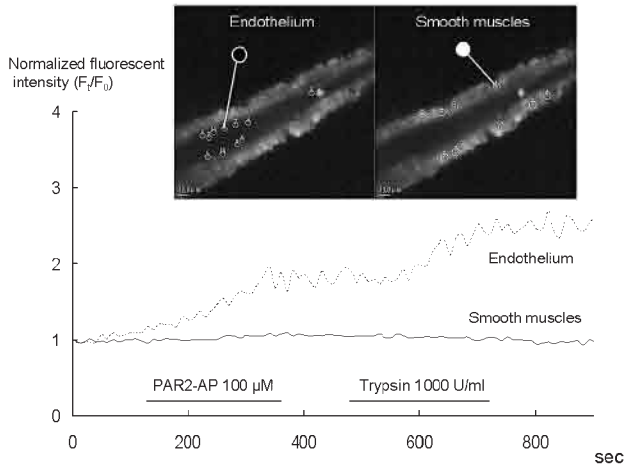

Fig. 5. Time course of DAF-2 fluorescent intensity, indicating NO production in the endothelium and smooth muscle cells of a cerebral small arteriole (< 50 μm in diameter) during trypsin and PAR2-AP stimulation. The dashed line represents NO production of endothelial regions (open circle), and the solid line shows that of smooth muscles (solid circle).

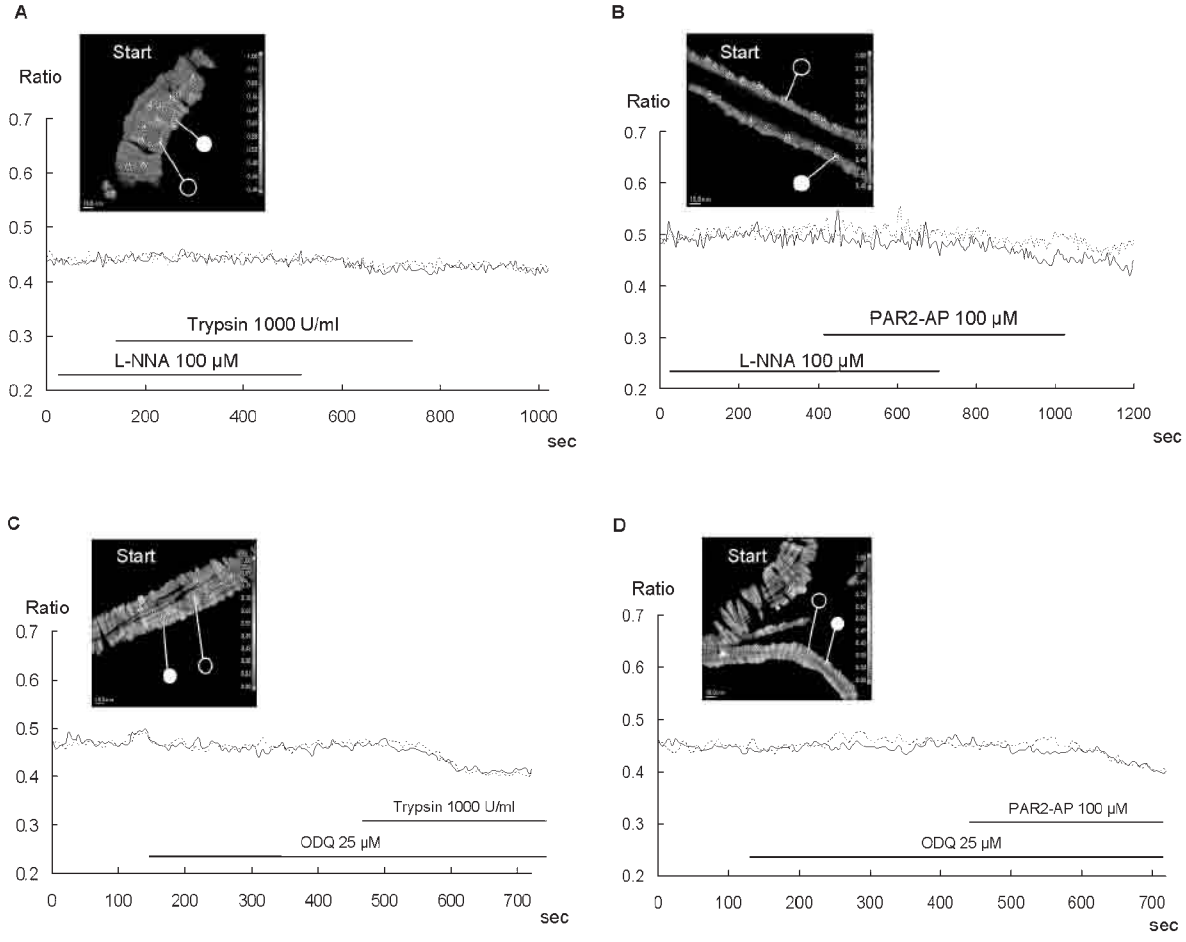

Fig. 6. A and **B:** Time course for $[Ca^{2+}]_i$ dynamics of small cerebral arterioles (< 50 μm in diameter) during trypsin (**A**) and PAR2-AP (**B**) stimulation with L-NNA. Solid and dashed lines correspond to solid and open circles in the image, respectively. **C** and **D:** Time course for $[Ca^{2+}]_i$ dynamics of small cerebral arterioles during trypsin (**C**) and PAR2-AP (**D**) stimulation with ODQ. Solid and dashed lines correspond to solid and open circles in the image, respectively.

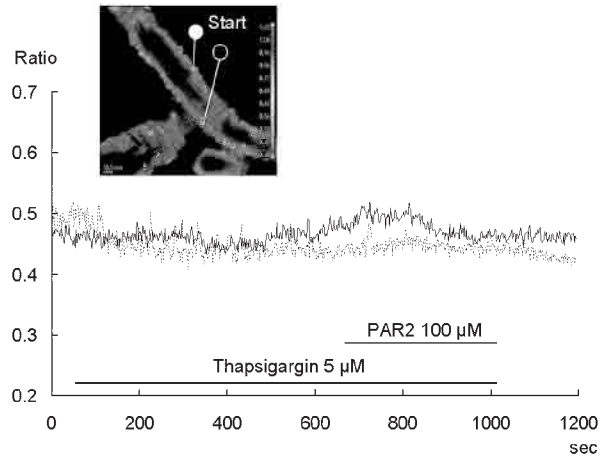

Fig. 7. Time course for $[Ca^{2+}]_i$ dynamics of small cerebral arterioles (< 50 μ m in diameter) during PAR2-AP stimulation with pretreatment of thapsigargin. Solid and dash lines correspond to solided and open circles in the image, respectively.

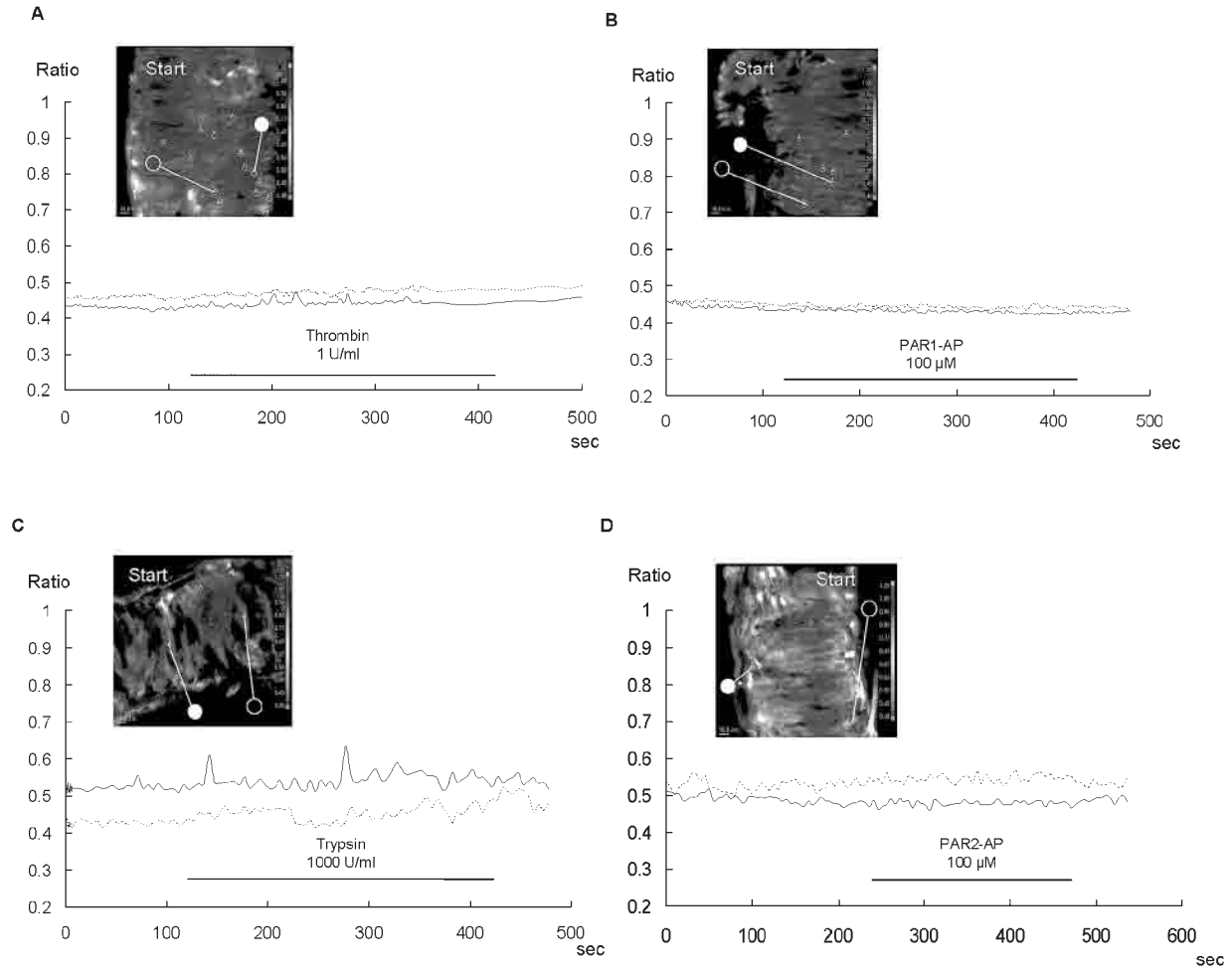

Fig. 8. A and **B:** Time course for $[Ca^{2+}]_i$ dynamics of large cerebral arterioles (<150 μm in diameter) during thrombin (**A**) and PAR1-AP (**B**) stimulations. Solid and dashed lines correspond to solid and open circles in the image, respectively. **C** and **D:** Time course for $[Ca^{2+}]_i$ dynamics of large cerebral arterioles during trypsin (**C**) and PAR2-AP (**D**) stimulations. Solid and dashed lines correspond to solid and open circles in the image, respectively.

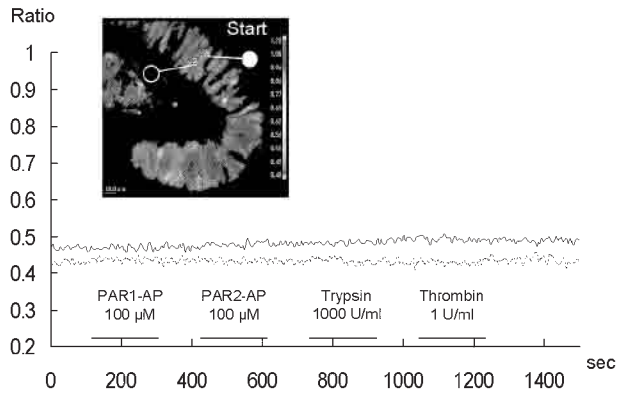

Fig. 9. Time course for $[Ca^{2+}]_i$ dynamics in testicular arterioles during PAR1-AP, PAR2-AP, trypsin and thrombin. Solid and dashed lines correspond to solid and open circles in the image, respectively.

Effects of proteases on $[Ca^{2+}]_i$ dynamics of the large cerebral arterioles

In contrast to small cerebral arterioles, the responses of large cerebral arterioles to extracellular thrombin or PAR1-AP were not conspicuous, although a few cells showed transient $[Ca^{2+}]_i$ increases (n =10) (Fig. 8A, B). ATP induced $[Ca^{2+}]_i$ the increase and contraction of the same specimens. Trypsin or PAR2-AP rarely elicited a transient increase in $[Ca^{2+}]_i$, but no decrease in $[Ca^{2+}]_i$ was found (n=11) (Fig. 8C, D).

Effects of proteases on $[Ca^{2+}]_i$ dynamics of the testicular arterioles

Different concentrations of thrombin or PAR1-AP did not elicit any $[Ca^{2+}]_i$ dynamics or the contraction of testicular arterioles (n = 8). No changes in $[Ca^{2+}]_i$ were observed during perfusion by HR containing different concentrations of trypsin or PAR2-AP (n =10) (Fig. 9).

Discussion

The vascular tones of arterioles (resistance vessels, less than about 150 μm in diameter) are especially important in pathological conditions because they play an essential role in controlling regional tissue circulation. However, previous studies on the effect of PARs on the vascular system involved the use of large arteries (distributing vessels). To our knowledge, studies on the effect of PARs on arterioles have not been reported to date for any tissues. Most previous studies on PARs of the vascular system were focused on the effect on the endothelium (Sobey *et al.*, 1999; Hamilton *et al.*, 2002), but not smooth muscles (Damiano *et al.*, 1999), despite the fact that the presence in PARs of smooth muscles has been verified immunohistochemically (D'Andrea *et al.*, 1998). This is the first study to demonstrate the effects of PARs activation on the $[Ca^{2+}]_i$ dynamics of smooth muscles and the contraction/relaxation in cerebral arterioles as well as implicate the significant role of proteases in the regional tissue circulation of the brain. We also showed that the responses differed between small and large cerebral arterioles and also between cerebral and testicular arterioles.

Mechanism of the $[Ca^{2+}]_i$ increase of arteriole smooth muscle cells by thrombin

The present study showed that thrombin induces the mobilization of Ca^{2+} in smooth muscle cells and the contraction of cerebral small arterioles. This is reasonable because PARs are a G-protein-coupled family of receptors, and it is well documented in various cells that the activation of G-protein coupled receptors cause Ca^{2+} mobilization via inositol trisphosphate production (Berridge *et al.*, 2003; Jeng *et al.*, 2004). Our results also suggest that the response to thrombin is mainly mediated by PAR1. However, the oscillatory fluctuations and spasmoidic contractions observed during stimulation by thrombin were not elicited in PAR1-AP stimulation. Thus, the possibility that PAR3 and/or 4 enhanced PAR1-mediated responses can not be excluded in this study, although coordinated effects of PARs in smooth muscle cells have not been reported previously.

Mechanism of $[Ca^{2+}]_i$ decrease of arteriole smooth muscle cells by trypsin

We showed that trypsin and PAR2-AP cause a decrease in $[Ca^{2+}]_i$ in smooth muscle cells of small cerebral arterioles, and L-NNA inhibited this decrease. In endothelial cells, trypsin or PAR2-AP induced NO production; such was not the case in smooth muscle cells. These findings indicate that trypsin and PAR2-AP stimulate NO production in the endothelium via NOS activation, and endothelium-derived NO cause a decrease in $[Ca^{2+}]_i$ in the smooth muscle cells of cerebral small arterioles, resulting in relaxation of the vessels.

In addition to the well-established cGMP-dependent

relaxation of smooth muscle cells (Carvajal *et al.*, 2000), recent studies have shown that NO interferes with the $[Ca^{2+}]_i$ signaling mechanism of cells and consequently leads to the cGMP-independent relaxation of arteries (Soloviev *et al.*, 2004; Lehen'kyi *et al.*, 2005). According to these previous studies, the decrease in $[Ca^{2+}]_i$ of the smooth muscle cells during PAR2 stimulation was not inhibited by ODQ, the inhibitor of soluble guanylyl cyclase. Thus, the mechanism of cGMP-independent relaxation appears to function in smooth muscle cells of small cerebral arterioles.

Because PAR2 was observed in vascular smooth muscle cells (Sobey *et al.*, 1999), trypsin and PAR2-AP can directly affect on the Ca^{2+} signalling system in vascular smooth muscle cells. The fact that inhibition of SERCA by thapsigargin suppressed the PAR2-mediated $[Ca^{2+}]_i$ decrease suggests that the sarco/endoplasmic sequestration of Ca^{2+} in smooth muscle cells is causally involved in the decrease. It is probable that PAR2 activation may promotes the SERCA function in the cells.

Size-dependent and organ specific PARs activation

In this study, we demonstrated that the responses of smooth muscle cells of cerebral arterioles to proteases were size-dependent, in that PAR1- mediated contraction was more potent in small arterioles. The size-dependent responses can be explained by the Janus-faced nature of PAR1 on the vascular tone. To summarize the previous (Ku and Zaleski, 1993; Tesfamariam, 1994a, b; Ku and Dai, 1997; Mizuno *et al.*, 1998, 2000; Derkach *et al.*, 2000) and present data on PARs of the vascular system, PAR1 activation may indirectly cause vascular relaxation *via* endothelial NO production, being followed by vascular contraction *via* the $[Ca^{2+}]_i$ increase in smooth muscle cells. It is conceivable that, when PAR1 is activated, the contraction overcomes the relaxation in small arterioles, while both are balanced in large arterioles. Our results also suggest that PAR2 activation probably results in relaxation by endothelial NO-mediated and/or the enhanced sequestration of Ca^{2+} in smooth muscle cells. The effect of PAR2 activation is potent in smaller arterioles, indicating a higher level of expression of PAR2.

A previous immunohistochemical study on coronary arteries (ca. 200 μm to 2 mm in diameter) indicated that PAR1 and PAR2 were present in smooth muscle cells of small but not large vessels (Hamilton *et al.*, 2002). The size-dependent difference in PARs expression observed in the coronary artery may be applicable in cerebral arterioles, although further studies using immunohistochemistry are needed to clarify this point.

Compared with testicular arterioles, cerebral arterioles were found to be sensitive to PARs activation. Organ specificity, which may be dependent on the expression of PARs in the endothelium and in smooth muscle cells, should be taken into consideration in the pathophysiology of brain damages and developing novel therapies that target PARs.

Significance of PARs in cerebral blood flow

Under various pathological conditions, protease levels increase and PARs expression is enhanced in many cells (Wilcox *et al.*, 1994; Damiano *et al.*, 1999; Hirano *et al.*, 2005). If a hemorrhagic occurs in the brain, thrombin, which is involved in clot formation, induces the contraction of smooth muscle cells of small arterioles by PAR1 activation. This contributes to hemostatic functions and, in turn, disturbs tissue circulation.

In contrast to PAR1, PAR2 increases blood flow by causing small arterioles to relax. Various protease levels (e.g., coagulation factor VIIa or Xa, but trypsin may be implausible) that activate PAR2 increase in lesions of the brain, serving as a protective mechanism against ischemia (Sobey and Cocks, 1998). Even when an acetylcholin-induced endothelium-dependent relaxation mechanism is damaged, PAR2-mediated vasodilatation continues to function (Sobey *et al.*, 1999). Thus, the robustness of the PAR2-mediated vasodilatation mechanism is appropriate for preserving tissue circulation against various contractive factors.

In conclusion, the present study indicates that smooth muscle cells of cerebral arterioles undergo contraction or relaxation as the results of PAR1 or PAR2 activation, and that the responses to proteases are size-dependent. These findings shed new light onto the significance of PARs in the cerebral blood flow. PAR1 inhibitors or PAR2 activators will be target drugs to improve or preserve the cerebral blood flow.

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