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# Immunohistochemical localization of protease-activated receptors in cerebral and testicular arterioles of rats: their dependence on arteriole size and organ-specificity*

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**Summary. Protease-activated receptors (PARs) expressed in the endothelia and smooth muscles of vessels may play important roles in blood vessel function. Using intracellular calcium ion concentration ($[Ca^{2+}]_i$) imaging, we recently observed that small — but not large — arterioles of the brain responded to proteases, while testicular arterioles showed no response. The purpose of the present study was to examine the heterogeneity of the localization of PARs in arterioles using immunohistochemistry. Consistent with the $[Ca^{2+}]_i$ imaging results, neither the thrombin receptor nor PAR2 were evident in large arterioles of the brain. However, the small arterioles of the brain, vascular smooth muscles, and endothelia showed a distinct immunoreactivity against the thrombin receptor and PAR2. The immunoreactivity of PARs in testicular arterioles was faint. In conclusion, size-dependent and/or organ-specific responses of arterioles to proteases are due to the heterogeneous localization of PARs.**

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## Introduction

Protease-activated receptors (PARs) comprise a family of G protein-coupled receptors, which are activated by various proteases (e.g., thrombin, trypsin, or inflammatory proteases that are formed at sites of tissue injury) (Dery *et al.*, 1998). To date, four members of this class of receptors have been identified: PAR1, 2, 3 and 4. The individual PARs appear to be involved in the regulation of a broad range of cellular functions, as follows: PAR1 and 2 can activate various intracellular signalling systems; PAR3 and 4 may function as modulators (Hollenberg, 1999, 2005; Cicala, 2002; Vergnolle *et al.*, 2003; Hirano and Kanaide, 2003, Hirano *et al.*, 2005; Saito and Bunnett, 2005; Stenberg, 2005; Legar *et al.*, 2006).

In a previous study, we examined the intracellular calcium ion ($[Ca^{2+}]_i$) dynamics of arterioles isolated from the rat brain and testis (Saino and Satoh, 2004). Smooth muscles of small arterioles in the brain (ca <50 $\mu$m in diameter) were responsive to proteases, but large brain arterioles were not (Misaki *et al.*, 2006). The response of testicular arterioles to proteases was limited. These findings indicate that the activation of arterioles by PARs is both dependent on arteriole size and is organ-specific. In general, bioimaging should be used to complement histochemical analyses (Satoh *et al.*, 1995), but bioimaging data for arteriole PARs do not exist.

While the precise mechanism for the different responses of brain arterioles to proteases remains an enigma, immunohistochemical data suggest that the size-dependent heterogeneous tissue localization of PARs in coronary arteries accounts for the differential response (Hamilton *et al.*, 2002). It is also possible that the PARs of both large

brain arterioles and testicular arterioles are not functional. In this study, we used immunohistochemistry to determine whether PAR-localization is either dependent on arteriole size or is organ-specific.

## Materials and Methods

All procedures were in accordance with guidelines established by the Laboratory Animal Care Committee at Iwate Medical University. Adult male Wistar rats (weighing 250–300 g) were euthanized by exposure to carbon dioxide gas. Three types of specimens were prepared as follows: 1) tissue slice specimens for immunohistochemical localization of PARs; 2) whole-mount specimens of arterioles for immunohistochemical localization of PARs; and 3) tissue slice specimens for the three-dimensional reconstruction of blood vessel arborization in the brain.

To detect immunoreactivity for PARs, the animals were perfused transcardially with Ringer's solution followed by 4% paraformaldehyde (PFA) in a phosphate buffer (PB). The brain was removed and soaked in the same fixative for 4 h at room temperature. After washing with PB, tissue sections of the brain (ca. 150 $\mu$m thick) were prepared using a vibrating blade microtome (Leica VT1000S, Nussloch, Germany). The sections were then incubated for 6 days at 4℃ with either the primary rabbit polyclonal antibody against the human thrombin receptor (i.e., antibody against PAR1; H-111, Santa Cruz Biotech., Santa Cruz Biotech, CA, USA) or the rabbit polyclonal antibody against human PAR2 (H-99; Santa Cruz Biotech) at a dilution of 1:50. After rinsing with PBS, the sections were incubated with Alexa Fluor 488-labeled anti-rabbit IgG (Invitrogen-Molecular Probes, Eugene, OR, USA) for 12 h at 4℃. Actin filaments were stained with Alexa 594-labeled phallodin (Invitrogen-Molecular Probes) to identify smooth muscle cells. The specificity of the immunohistochemical staining was confirmed by substitution of the primary antibody with normal serum. When normal serum was used, no immunostaining was observed.

Intact arteriole specimens for living cell imaging were obtained from the brain and testis (Misaki *et al.*, 2006) and were fixed in 4% PFA. Because the testis could not be sectioned using a vibrating blade microtome, only whole-mount specimens of isolated arterioles were obtained from the testis in the present study. PARs were localized in these whole-mount arteriole specimens using immunohistochemistry, as descried above.

The arterial arborization of brains was visualized using fluorescein-labeled *Lycopersicon esculentum* (tomato) lectin (FL-1171, Vector Lab., Burlingame, CA, USA), which is an effective marker of blood vessel endothelia (Ezaki *et al.*, 2006). The animals were injected with 0.5 m*l*/100 g of body weight of a lectin solution (500 $\mu$g/m*l*) via the tail vein. Thirty minutes after the injection, the animals were sacrificed and perfused, and the brain was sectioned, as described above. Smooth muscle cells were stained with Alexa 594-labeled phallodin, to distinguish between arteries/arterioles and veins/venules.

The specimens were mounted using Aquapolymount (Polyscience, Warrington, PA, USA). They were then visualized using laser scanning confocal microscopy (Zeiss-510, Jena, Germany). To avoid fluorescent cross-talk, which causes false-positive co-localization, a multi-track system of microscopy was used. It was difficult to keep the arteriole specimens attached to the cover slip of the chamber after the $[Ca^{2+}]_i$ dynamics experiment; therefore, in the present study, $[Ca^{2+}]_i$ dynamics and immunohistochemical staining could not be performed with the same specimen.

## Results and Discussion

Arteriole specimens were mainly obtained from the vascular network under the arachnoidea that covers the cerebral cortex. Arborization of rat brain arteries showed a characteristic pattern. Small arterioles (< ca. 50 $\mu$m in diameter) branched out from arteries (ca. 200–400 $\mu$m in diameter) or large arterioles (ca. 70–100 $\mu$m in diameter) at obtuse angles in the subarachnoid space. The branched arterioles ran in the subarachnoid space for less than a few millimeters, passed through the pia mater, and then entered the cerebral cortex perpendicularly. In the cerebral cortex, many capillaries exhibited perpendicular branching. Based on vessel diameter and localization, three types of arteries/arterioles were morphologically distinguishable. For convenience, these vessels are designated here as follows: subarachnoid arteries (SA); small branched arterioles (BrAl); and intracerebral arterioles (Fig. 1).

It is conceivable that unique structural features may reflect functional differences. Recently, we reported that thrombin and PAR1-activating peptide (AP) can elicit an increase in $[Ca^{2+}]_i$ in some smooth muscles of small arterioles in the brain. We hypothesized that the response was mediated *via* the PAR1 of smooth muscles (Misaki *et al.*, 2006). Conversely, both trypsin and PAR2-AP induced a decrease in $[Ca^{2+}]_i$ in smooth muscles in small arterioles. We postulated that the decrease was either a direct effect of PAR2 in smooth muscles and/or an indirect effect mediated *via* the endothelium possessing

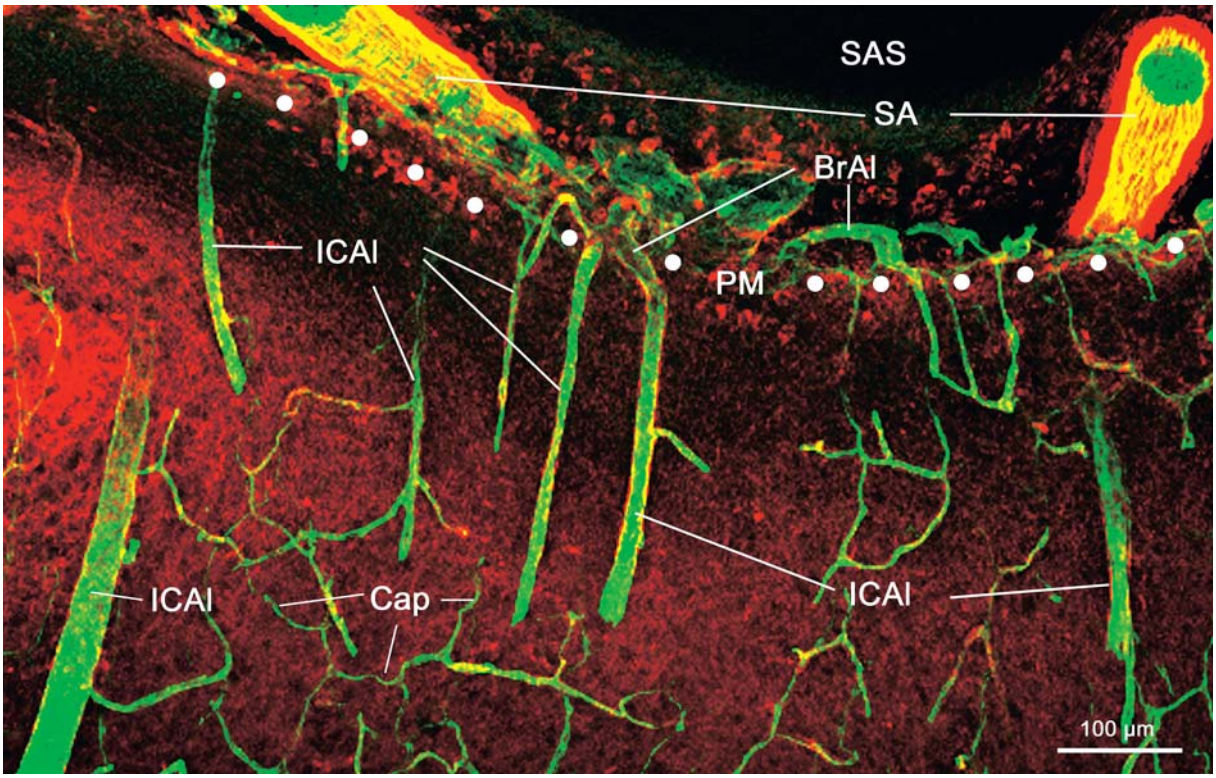


**Fig. 1.** Reconstructed image of consecutive, serially sectioned confocal micrographs showing the microvasculature of the rat cerebral cortex. The brain was cut perpendicular to the surface of the cerebral cortex. Endothelia and actin filaments were stained with fluorescein-labeled lectin (green) and Alexa 594-labeled phallodin (red), respectively. PM: piamater (white dots represent cerebral surface), SAS: subarachnoid space, SA: subarachnoid artery, BrAl: subarachnoid branched arteriole, ICAl: intracerebral branched arteriole, Cap: capillary. Bar: 100 $\mu$m

PAR2. The $[Ca^{2+}]_i$ imaging of small arteriole specimens corresponded to BrAl and ICAl. Large arterioles from the brain, which were classified as SA vessels, did not respond to either proteases or PARs-AP; moreover, testicular vessels were also unresponsive.

Previous immunohistochemical studies reported the presence of PARs on smooth muscles and the endothelium of large vessels in various tissues. However, these earlier studies did not examine arterioles, nor was the significance of PARs in smooth muscles discussed (D'Andrea *et al*., 1998; Sobey and Cocks, 1998; Hamilton *et al*., 2002; Hirano and Kanaide, 2003; Steinberg, 2005). In the present study, the immunohistochemical detection of PAR1 and PAR2 in brain slices was nearly identical to the results of $[Ca^{2+}]_i$ bioimaging of the microvasculature (Fig. 2). In the case of SA, i.e. large arteriole specimens were unresponsive to PAR1 and PAR2, the anti-thrombin receptor (i.e., anti-PAR1) and anti-PAR2 immunoreactivities were faint in most smooth muscles; still, some outer muscles and adventitia showed immunoreactivity. The endothelia consistently stained positive for PAR1, but PAR2-positive staining was unpredictable. In BrAl and ICAl, i.e. small arteriole specimens used in the $[Ca^{2+}]_i$ dynamics study, both smooth muscles and the endothelia showed the distinct immunoreactivity to PAR1 and PAR2.

In addition to slice specimens, we observed immunoreactivity to PARs in the isolated arterioles that were used in the $[Ca^{2+}]_i$ imaging study. Smooth muscles of small arterioles showed distinct immunoreactivity to PAR1 and PAR2 with that the immunoreactivity to PAR1 being fragmental, while that to PAR2 nearly uniform. Consistent with these findings, we previously reported that, in small arterioles of the brain, the increase in $[Ca^{2+}]_i$ during thrombin or PAR1-AP treatment was fragmental, and the decrease in $[Ca^{2+}]_i$ during trypsin and PAR2-

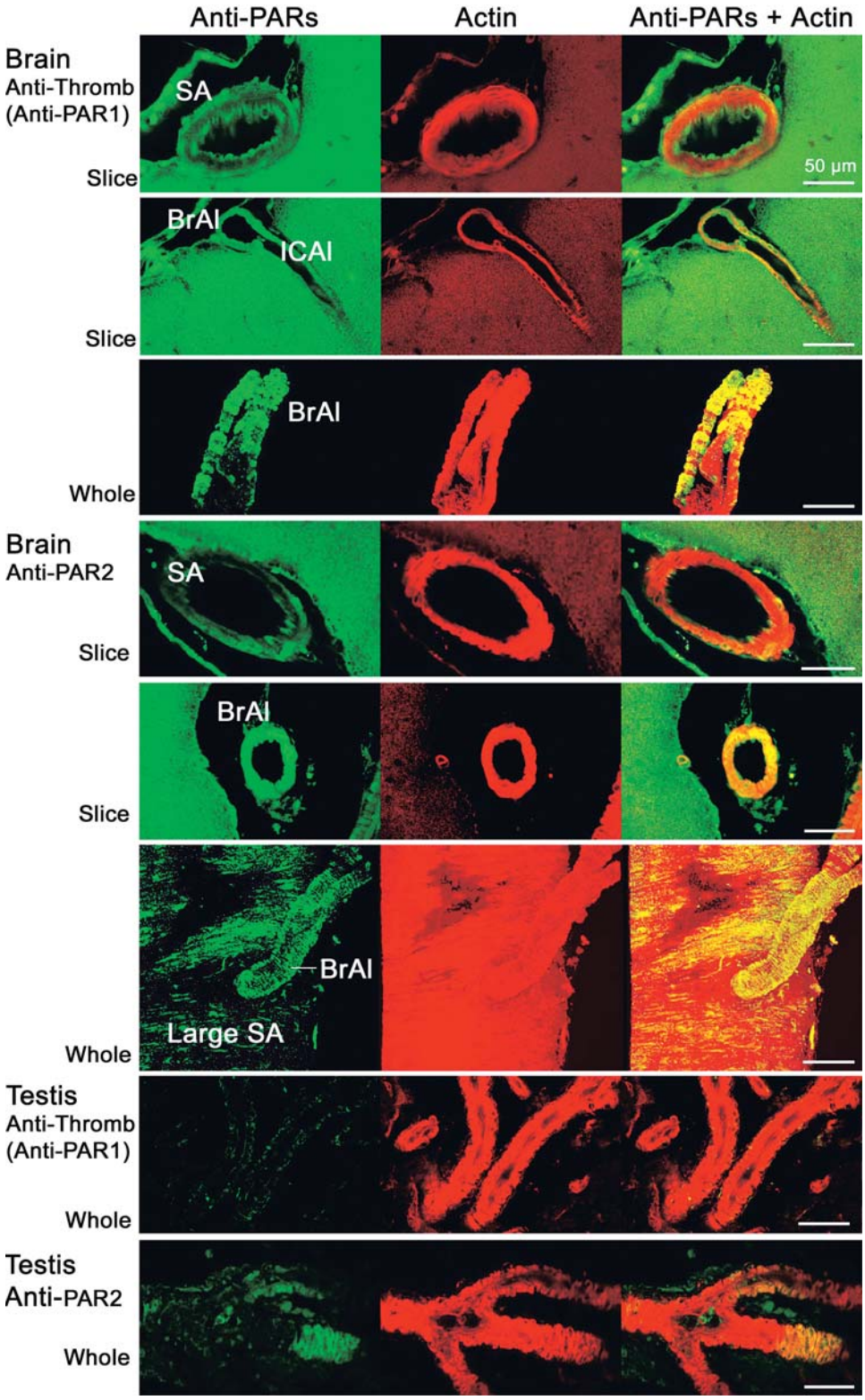


**Fig. 2.** Legend on the opposite page.

AP treatment was uniform (Misaki *et al*., 2006). PAR2 immunoreactivity of smooth muscles in SA was evident in the outer layer but was relatively (comment 3) faint in the deeper layers. In contrast, testicular arterioles showed no immunoreactivity to PAR1. Some of the testicular vessels occasionally showed segmental staining for PAR2. However, the regions were restricted so that, we were not able to detect a change in $[Ca^{2+}]_i$ during PAR2 stimulation.

Neurons and glia were stained with antibodies against PAR1 and PAR2. It is well known that neurons and glia cells of the nervous system possess PARs, and various proteases can activate different neuronal signaling systems (Saito and Bunnett, 2005). Thus, PARs control neural function both indirectly *via* blood circulation and directly by acting on nerve cells.

In the present study, immunohistochemistry was used to confirm the size-dependent functional heterogeneity of PARs in blood vessels of the brain, something previously suggested in $[Ca^{2+}]_i$ imaging studies. However, there are some discrepancies between the results of immunohistochemistry and $[Ca^{2+}]_i$ dynamics (e.g., PAR2 on large SA), demonstrating that the presence of a molecule does not always mean that it plays a functional role.

It is well-known that there are significant functional differences between brain vessels and those of other tissues/organs (e.g., vasospasms in various diseases, and the discrepancy between circulation volume and oxygen consumption of the brain), as documented by a large body of published research. Nevertheless, the function of the brain microvasculature remains enigmatic. Only a few studies on arterioles, which may play a pivotal role in tissue circulation, are available. The bioimaging method developed here, which uses real-time confocal microscopy, is a powerful tool for studying microvasculature function (Saino *et al*., 2002; Saino and Sato, 2004). In conclusion, the responses to proteases were dependent on arteriole size, and the differences can be extrapolated to other transmitters. These may explain some of the characteristics of blood circulation in the brain.

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**Fig. 2.** Confocal micrographs showing immunohistochemical localization of PARs (green, left column) and smooth muscles stained with Alexa 594-labeled phallodin (red, central column) in arteriole specimens (sliced and whole) of the brain and testis. Images of whole specimens consist of serially sectioned images. The yellow in the right column (mixed figures) indicates that smooth muscles possess PARs. E.g., the deep layer of large SA in the brain did not exhibit positive staining for anti-PAR2; therefore, not all smooth muscles showed the yellow. SA: subarachnoid artery, BrAl: branched arteriole, ICAl: intracerebral branched arteriole. Bars: 50 $\mu$m

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