Full Paper

Comparative Study of Calcium Ion Dynamics and Contractile Response in Rat Middle Cerebral and Basilar Arteries

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Abstract. The objective of this study was to compare intracellular calcium concentration ($[Ca^{2+}]_i$) and contractile responses in isolated rat middle cerebral artery (MCA) with those in basilar artery (BA) employing real-time confocal laser microscopy. KCl elicited transient $[Ca^{2+}]_i$ elevation and sustained contraction in both arteries; moreover, nearly equal responses were evident in both arteries. Application of 5-hydroxytryptamine (5-HT), vasopressin (VP), and α,β -methylene adenosine 5'-triphosphate (α,β -me ATP) also induced elevation of $[Ca^{2+}]_i$ and contraction in both arteries. The maximum response of 5-HT and VP necessary to increase $[Ca^{2+}]_i$ and to constrict the MCA was less in comparison to the BA; however, a linear relationship emerged between the maximum response of $[Ca^{2+}]_i$ and that of contraction. Additionally, the slope of the correlation regression line of MCA was nearly identical to that of BA. On the other hand, cyclopiazonic acid (CPA)-induced Ca^{2+} release from store sites following contraction of MCA was distinct from that of BA. In MCA, velocity of $[Ca^{2+}]_i$ elevation in smooth muscle cells and Ca^{2+} -wave propagation along smooth muscle cells induced by 5-HT were slower than those in BA. These observations revealed that different regions of arteries along the same cerebral tissue may display distinct $[Ca^{2+}]_i$ response; moreover, this difference may be one reason for the distinct contractile response.

Keywords: confocal microscopy, intracellular calcium, basilar artery, middle cerebral artery

Introduction

Cerebrovascular disease is the third leading cause of death in Japan, trailing only cardiovascular disease and cancer. Cerebral vasospasm following subarachnoid hemorrhage (SAH) is a serious complication; however, factors that lead to this disturbance are poorly understood. Hypersensitivity to vasopressin (1) or hydroxyl radical (2), up-regulation of vascular receptors against vasoactive mediator (3), and impaired vasodilator response (4) may contribute to vasospasm after SAH. Therefore, abnormal responses of the cerebral artery such as increased vasoconstriction and impaired vasodilatation may induce cerebral vasospasm. This vasospasm is believed to be a main cause of secondary brain ischemia following SAH.

Numerous studies have employed the basilar artery (BA) in order to examine the mechanisms underlying cerebral vasospasm. However, approximately 60% – 70% of cerebral infarcts occur at sites associated with the middle cerebral artery (MCA). Additionally, not all arteries exhibit common responses to various agonist effectors (5, 6). In consideration of these points, questions arose as to whether the MCA displays responsibility similar to that of the BA. If the MCA demonstrates distinct responsibility in comparison to the BA, then the MCA is more appropriate for analysis of pathophysiological mechanisms of cerebrovascular dysfunctions.

Calcium acts as a universal signal for various cellular functions, including vasoconstriction. Therefore, intracellular calcium concentration ($[Ca^{2+}]_i$) modifications in smooth muscle or endothelial cells represent a key element in the pathogenesis of cerebrovascular dysfunctions. Previously, we documented the determination of contractile and $[Ca^{2+}]_i$ responses of BA employing real-time confocal laser microscopy (7). This approach

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can estimate [Ca²⁺]_i response at the whole tissue as well as at the individual cell level. Therefore, the current investigation compared [Ca²⁺]_i and contractile responses in MCA and BA by utilizing confocal laser microscopy. The results indicated that the [Ca²⁺]_i response of the MCA differs from that of the BA; moreover, the difference in the [Ca²⁺]_i response between MCA and BA may be attributable to differential responsibility to agonists in the MCA and BA.

Materials and Methods

Animals

All experiments were conducted in accordance with the regulations of the Committee of Animal Care and Welfare of Showa University.

Experimental animals consisted of male Wistar rats (Saitama Jikken Co., Ltd., Saitama) weighing 300–400 g. The rats were maintained in an air-conditioned animal room at 20 ± 2°C, with 50 ± 20% relative humidity and a 12-h light–dark cycle (lights on 8:00 to 20:00). Rats received a standard laboratory diet; additionally, water was available *ad libitum*.

Materials

Materials used (and their sources) in this investigation were as follows: Fluo-4/AM (Molecular Probes, Inc., Eugene, OR, USA), Fura Red/AM (Molecular Probes, Inc.), 5-hydroxytryptamine (5-HT; Wako Pure Chemical, Osaka), α,β -methylene adenosine 5'-triphosphate (α,β -me ATP; Sigma, St. Louis, MO, USA), cyclopiazonic acid (CPA, Sigma), [Arg⁸]-vasopressin (VP; Peptide Institute, Osaka) and Cell-Tak[®] (non-toxic adhesive agent; BD Biosciences, Franklin Lakes, NJ, USA). Other reagents were of the highest grade commercially available.

Fluo-4/AM and Fura Red/AM were dissolved in dimethylsulfoxide and diluted with HEPES-Krebs solution (pH 7.4). HEPES-Krebs solution consisted of 135 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 2.5 mM CaCl₂, 10 mM glucose, and 8.4 mM HEPES. The remaining reagents were prepared in HEPES-Krebs solution (pH 7.4).

Preparation of cerebral artery and dye loading

Rats were anesthetized with diethyl ether, followed by exsanguination. Subsequently, brains were removed and the MCA (diameter: about 100 μ m) and the BA (diameter: about 150 μ m) were isolated. Each artery, which was cut into pieces of approximately 1 mm in length (before mounting on the plate), was mounted on a cover glass coated with Cell-Tak[®] in a chamber (volume: 3 ml), followed by immediate immersion in a

mixture of 10 μ M Fluo-4/AM and 10 μ M Fura Red/AM. After 30 min, arteries were washed three times with HEPES-Krebs solution (pH 7.4).

Determination of [Ca²⁺]_i and contractile responses in cerebral artery

[Ca²⁺]_i and contractile responses were determined according to the method of a previous report (7). A real-time confocal microscope (RTS-2000MP; Bio-Rad, Hercules, CA, USA) was utilized in this investigation. Specimens were irradiated with a 488-nm excitation beam generated with a diode laser. Fluorescence emission (Fluo-4/AM: 503–553 nm, Fura Red/AM: 580–700 nm) was guided through a water-immersion objective lens (10 $\times$, N.A. 0.3). In this system, the image frame was acquired continuously at 4.27-s intervals. Images were stored on a high-speed hard disk.

[Ca²⁺]_i and contractile responses of cerebral artery were observed 15 min after dye loading in order to permit complete de-esterification of the dye. The MCA and BA were soaked in HEPES-Krebs solution; subsequently, the fluorescent image of the resting state was acquired. Following acquisition of resting response, specimens were exposed to various agonists and image frames were acquired continuously. In the case of confocal imaging of arteriole smooth muscle cells, fluorescence emission was also guided through an oil-immersion objective lens (20 $\times$, N.A. 0.75); scanning images were acquired every 0.146 s.

Data analyses and statistics

The change in [Ca²⁺]_i in tissue levels was determined via ratiometry employing Fluo-4/AM and Fura red/AM as reported elsewhere (8) after subtraction of background fluorescence. Ratiometry eliminates moving artifacts and out-of-focus movements. The ratio of fluorescent intensity (Fluo-4/Fura red) was calculated from individual images utilizing Image J software (version 1.31; NIH, Bethesda, MD, USA). [Ca²⁺]_i response was evaluated as the change in fluorescent ratio. Furthermore, the fluorescence threshold of fluo-4/AM corresponding to 95% of the histogram area of the background was set to assess the contractile response; fluorescent area including the lumen of the artery was calculated with Image J software. Mean arterial diameter was defined as follows: mean arterial diameter = fluorescent area / arterial length. Contractile response was evaluated as the change in mean arterial diameter.

To analyze the [Ca²⁺]_i response in arteriole smooth muscle cells, an averaging window (20 μ m $\times$ 100 μ m) was placed along a smooth muscle cell in the Fluo-4 fluorescence images. In the analysis of the Ca²⁺ wave,

Fig. 1. The $[Ca^{2+}]_i$ and contractile responses induced by KCl, 5-HT, VP, and α,β -me ATP in middle cerebral and basilar artery. $[Ca^{2+}]_i$ response was evaluated as the change in fluorescent ratio (Fluo-4/Fura red). Contractile response was evaluated as the change in mean arterial diameter. A) Time course of $[Ca^{2+}]_i$ and contractile responses. B) Comparison of agonist-induced $[Ca^{2+}]_i$ and contractile responses. Each value represents the mean $\pm$ S.E.M. of 5 experiments. * $P < 0.05$, ** $P < 0.01$: Statistically significant difference from the BA.

the averaging region ($7 \mu m \times 7 \mu m$) was placed in this window at intervals of $20 \mu m$. The relative fluorescent intensity (F/F_0) served as an indicator of $[Ca^{2+}]_i$ response.

KCl-induced $[Ca^{2+}]_i$ and contractile responses were

expressed as the percentage change from the resting states. Agonist-induced $[Ca^{2+}]_i$ and contractile responses were normalized to the response to 50 mM KCl. In studies of individual cells, data are presented as the F/F_0 . Statistical analyses were performed by ANOVA followed

Fig. 2. Dose–response curves of [Ca²⁺]_i and contractile responses induced by 5-HT (A) and VP (B) in the middle cerebral and basilar artery. [Ca²⁺]_i response was evaluated as the change in fluorescent ratio (Fluo-4/Fura red). Contractile response was evaluated as the change in mean arterial diameter. Each point represents the mean ± S.E.M. of 3–5 experiments. **P*<0.05, ***P*<0.01: Statistically significant difference from the BA.

by Student-Newman-Keuls test, and Student's *t*-test between two groups; a *P*-value less than 0.05 was considered significant.

Results

The [Ca²⁺]_i and contractile responses induced by KCl and various agonists

Figure 1A illustrates the time course of [Ca²⁺]_i and contractile responses induced by 50 mM KCl and various agonists. Application of 50 mM KCl led to an increase in [Ca²⁺]_i, which preceded the contractile response in MCA and BA. The magnitude of increase in the fluorescence ratio was $31.2 \pm 4.8\%$ relative to the resting state in MCA and $25.0 \pm 3.5\%$ relative to the resting state in BA. No significant changes in fluorescence were observed between MCA and BA. Despite the transient nature of the [Ca²⁺]_i response, contraction was

sustained; indeed, approximately 18%–19% contraction was evident until 120 s in both arteries. Moreover, no statistically meaningful differences were detected between MCA and BA. In accordance with these results, agonist-induced [Ca²⁺]_i and contractile responses were normalized to the response to 50 mM KCl. Application of 10 μM 5-HT, 10 nM VP, and 10 μM α,β-me ATP also induced an increase in [Ca²⁺]_i and contraction in both arteries. However, 5-HT- and VP-induced [Ca²⁺]_i and contractile responses differed markedly between MCA and BA, although α,β-me ATP-induced responses were almost same. Comparisons of agonist-induced [Ca²⁺]_i and contractile responses are shown in Fig. 1B. AUC values of the [Ca²⁺]_i response induced by 5-HT and VP in the MCA were $9.0 \pm 1.9\%$ and $8.0 \pm 2.8\%$, respectively; moreover, AUC values of the contractile response were $12.9 \pm 5.8\%$ and $16.4 \pm 7.3\%$, respectively. Meaningful differences were evident in comparison with the

Fig. 3. Correlation of $[Ca^{2+}]_i$ and contractile responses induced by KCl, 5-HT, and VP in the middle cerebral and basilar artery. A) KCl-induced $[Ca^{2+}]_i$ and contractile responses. B) 5-HT- and VP-induced $[Ca^{2+}]_i$ and contractile responses. Each value represents the mean $\pm$ S.E.M. of 3–5 experiments.

BA ($[Ca^{2+}]_i$ response: $17.1 \pm 4.9\%$ and $19.0 \pm 2.9\%$, contractile response: $45.3 \pm 7.9\%$ and $50.9 \pm 5.8\%$). On the other hand, AUC values of the α, β -me ATP-induced $[Ca^{2+}]_i$ and contractile responses in the MCA were $51.5 \pm 10.5\%$ and $74.7 \pm 6.8\%$, respectively. No significant differences were observed in comparison with the BA ($[Ca^{2+}]_i$ response: $54.4 \pm 7.6\%$, contractile response: $67.9 \pm 4.0\%$).

The dose–response relationship of $[Ca^{2+}]_i$ and contractile responses was examined as indicated in Fig. 2. Dose-dependent increases in $[Ca^{2+}]_i$ and contractile responses were observed in the presence of 5-HT and VP in both arteries. Efficacy of both agonists in the MCA was less than the BA. However, PD_2 values of $[Ca^{2+}]_i$ and contractile response were 6.4 and 6.5 in the MCA, respectively; moreover, they were 6.2 and 6.3 in the BA, respectively. No significant changes were observed in

the MCA and BA. In the case of VP, PD_2 values in the MCA were also the same as those of the BA ($[Ca^{2+}]_i$ response: 8.5 in MCA, 8.4 in BA and contractile response: 8.4 in MCA, 8.5 in BA).

Figure 3A shows the correlation between KCl-induced $[Ca^{2+}]_i$ and the contractile response. Correlation coefficients in the MCA and BA were 0.86 and 0.95, respectively. A linear relationship was found between the $[Ca^{2+}]_i$ response and contractile response in both arteries. The slope of the correlation regression line in the MCA and BA were 0.90 and 0.96, respectively. No meaningful differences were observed between MCA and BA. There was also a linear relationship between 5-HT- and VP-induced $[Ca^{2+}]_i$ response and the contractile response (correlation coefficients in MCA: 0.99, in BA: 0.97 for 5-HT; correlation coefficients in MCA: 0.99, BA: 0.98 for VP). The slope of the correlation regression line in

Fig. 4. The $[Ca^{2+}]_i$ and contractile responses induced by CPA in the middle cerebral and basilar artery. The $[Ca^{2+}]_i$ response was evaluated as the change in fluorescent ratio (Fluo-4/Fura red). The contractile response was evaluated as the change in mean arterial diameter. A) Time course of $[Ca^{2+}]_i$ and contractile responses. B) Comparison of maximum responses. Each value represents the mean $\pm$ S.E.M. of 5 experiments. * $P < 0.05$: Statistically significant difference from the BA.

the MCA and BA was 1.29 and 1.21 for the 5-HT-induced responses and 1.60 and 1.40 for the VP-induced responses, respectively. The slope of the correlation regression line for the MCA was nearly identical to that of the BA in terms of both agonists (Fig. 3B).

Ca²⁺ release from intracellular stores induced by CPA

In order to confirm the difference in Ca^{2+} release from intracellular stores, MCA and BA were stimulated with 10 μ M CPA under the Ca^{2+} -free condition. CPA evoked an increase in $[Ca^{2+}]_i$ and contraction; however, they gradually decreased following the maximum response in the MCA and BA. The MCA responses were smaller than those of BA. AUC values of $[Ca^{2+}]_i$ were $14.1 \pm 2.2\%$ in the MCA and $27.1 \pm 5.4\%$ in the BA; moreover, contractile responses were $10.2 \pm 2.4\%$ in the MCA and $23.0 \pm 5.9\%$ in the BA. Meaningful differences between the MCA and BA were apparent in both responses ($P < 0.05$) (Fig. 4).

Analysis of $[Ca^{2+}]_i$ response in arteriole smooth muscle cells

Figure 5 illustrates the time course of the $[Ca^{2+}]_i$ response induced by 50 mM KCl and 10 μ M 5-HT in arteriole smooth muscle cells of the MCA and BA. The KCl-induced $[Ca^{2+}]_i$ response reached a maximum level at 3.0 s in arteriole smooth muscle cells of the MCA and BA. Nearly equal velocities of increase in $[Ca^{2+}]_i$ were evident. On the other hand, 5-HT-induced $[Ca^{2+}]_i$

Fig. 5. Window analysis of the $[Ca^{2+}]_i$ response along the smooth muscle cells of MCA and BA induced by KCl (A) and 5-HT (B). A window was positioned to obtain fluorescence signal from the smooth muscle cell in Fluo-4 images. $[Ca^{2+}]_i$ response was evaluated as the change in relative fluorescent intensity (F/F_0). Each value represents the mean $\pm$ S.E.M. of 5 experiments.

Fig. 6. KCl-induced $[\text{Ca}^{2+}]_i$ wave in the middle cerebral and basilar arteriole smooth muscle cells. A) Pseudocolor images of middle cerebral (MCA) and basilar (BA) arteriole smooth muscle cells. Color bar indicates fluorescent intensity of Fluo-4. B) Time course of $[\text{Ca}^{2+}]_i$ response in the area indicated by the white line. The $[\text{Ca}^{2+}]_i$ response was evaluated as the change in relative fluorescent intensity (F/F_0).

response reached a maximum level at 5.0 s in the MCA. Velocity of the $[\text{Ca}^{2+}]_i$ increase in the MCA was slower than that of the BA. In arteriole smooth muscle cells of the MCA and BA, 5-HT stimulation triggered a $[\text{Ca}^{2+}]_i$ wave that spread along the smooth muscle cells. However, KCl induced an increase in $[\text{Ca}^{2+}]_i$ throughout the smooth muscle cells simultaneously; no $[\text{Ca}^{2+}]_i$ wave was observed. Velocity of the 5-HT-induced $[\text{Ca}^{2+}]_i$ wave was approximately $80 \mu\text{m/s}$ in BA and $60 \mu\text{m/s}$ in MCA. Propagation of the $[\text{Ca}^{2+}]_i$ wave in the MCA was scarcely slower than that of the BA (Figs. 6 and 7).

Discussion

The current study compared $[\text{Ca}^{2+}]_i$ and contractile responses of the MCA and BA. The ratios of fluorescent intensity (Fluo-4/Fura red) in the resting state were $1.47 - 2.14$ (mean = 1.72) in the MCA and $1.43 - 2.45$ (mean = 1.66) in the BA. No significant differences in $[\text{Ca}^{2+}]_i$ level were observed between the MCA and BA in the resting state. These observations revealed nearly identical baseline status in the MCA and BA.

Treatment of the MCA and BA with KCl caused an

Fig. 7. 5-HT-induced [Ca²⁺]_i wave in middle cerebral and basilar arteriole smooth muscle cells. A) Pseudocolor images of middle cerebral (MCA) and basilar (BA) arteriole smooth muscle cells. Color bar indicates fluorescent intensity of Fluo-4. B) Time course of the [Ca²⁺]_i response in the area indicated by the white line. The [Ca²⁺]_i response was evaluated as the change in relative fluorescent intensity (F/F₀).

increase in [Ca²⁺]_i and contraction. These [Ca²⁺]_i and contractile response patterns were similar to one another; furthermore, the magnitude of both responses was similar. On the other hand, the effect of 5-HT and VP on the calcium response and subsequent contractile response was smaller in the MCA in comparison to the BA, although α,β -me ATP-induced [Ca²⁺]_i and contractile responses were nearly identical.

Based on studies of dose-response relationship of [Ca²⁺]_i and the contractile response induced by 5-HT and

VP, PD₂ values of [Ca²⁺]_i and contractile responses in MCA were almost the same as those of BA. Additionally, a linear relationship emerged between the [Ca²⁺]_i response and the contractile response; moreover, the slope of the correlation regression line of MCA was nearly identical to that of BA. On the basis of these results, we theorized that Ca²⁺ sensitivity does not differ between the MCA and BA. The difference in contractile response induced by both agonists may be caused by differences with respect to [Ca²⁺]_i dynamics. Mecha-

nisms that alter $[Ca^{2+}]_i$ can be influenced by the type and density of receptors stimulated, the involvement of these receptors in mechanisms underlying the regulation of Ca^{2+} influx, and release as well as association with mechanisms governing myosin light-chain phosphorylation and dephosphorylation (9). Saino et al. (10) reported that increased $[Ca^{2+}]_i$ due to 5-HT is the result of Ca^{2+} release from intracellular stores. Additionally, Ca^{2+} release from intracellular stores contributes to the contractile response to VP (11). On the other hand, P2X receptors are ligand-gated cation channels that are expressed by a variety of tissues including cerebral, mesenteric, and coronary artery (12–14). α, β -me ATP causes Ca^{2+} influx and vasoconstriction via the P2X receptors (15, 16). Based on these reports, we hypothesized that differences in Ca^{2+} release from intracellular stores contribute to distinct contractility in the MCA and BA. Therefore, the effect of CPA, an inhibitor of sarcoplasmic reticulum Ca^{2+} -ATPase (17), was examined. CPA has served as a valuable tool in terms of elimination of the Ca^{2+} -buffering function of sarcoplasmic reticulum.

Consequently, $[Ca^{2+}]_i$ and contractile responses were induced in the MCA and BA following application of CPA; the magnitude of these responses in the MCA was lower than that in the BA. Low et al. (18) noted that CPA alone leads to marked and sustained contraction in the aorta and transient or no contraction in resistance arteries; therefore, the role of the sarcoplasmic reticulum diminishes as the arteries become smaller. Asano et al. (19) also reported that CPA-induced contraction was extremely small in the mesenteric or coronary artery in comparison with the cerebral artery; moreover, a greater portion of the sustained Ca^{2+} influx is buffered by Ca^{2+} uptake into the sarcoplasmic reticulum in the resting state of the cerebral artery; therefore, the functional elimination of sarcoplasmic reticulum Ca^{2+} buffering by CPA causes potent contraction. The present results and the aforementioned reports suggest that the nature of the intracellular stores, for example, buffering function, changes as the artery becomes smaller. This difference may be one of the reasons accounting for the distinct $[Ca^{2+}]_i$ and contractile responses between the MCA and BA. On the other hand, it is well known that agonist-induced Ca^{2+} sensitization is responsible for a significant portion of the contractile tone. Recent studies also indicate that Ca^{2+} sensitization may play a pivotal role in the pathogenesis of abnormal contraction of the cerebral artery (20, 21). Akopov et al. (22) reported that Ca^{2+} sensitization by protein kinase C were inversely correlated with artery size. It is not clear from present studies whether Ca^{2+} sensitization is different between the MCA and BA. To clarify this point, further studies are

required.

Inositol 1,4,5-triphosphate (IP_3) is known to play a key role in agonist-induced Ca^{2+} release. IP_3 -induced Ca^{2+} release stimulates temporal and spatial Ca^{2+} release within the cell (23). Additionally, the noradrenaline-induced $[Ca^{2+}]_i$ response occurs within arterial smooth muscle in the form of a wave (24). The temporal and spatial distribution of the increase in $[Ca^{2+}]_i$ is an important factor in cellular signal transduction. In the current study, the velocity of $[Ca^{2+}]_i$ increase induced by 5-HT in the MCA was slower than that of the BA; however, in the case of KCl, no difference was evident between the MCA and BA. This discrepancy may be attributable to the spatiotemporal properties of the $[Ca^{2+}]_i$ response. Despite the increase in $[Ca^{2+}]_i$ throughout the smooth muscle cell in the presence of KCl, the $[Ca^{2+}]_i$ response in the form of a wave was observed following 5-HT application; moreover, the velocity of the $[Ca^{2+}]_i$ wave in MCA smooth muscle cells was scarcely slower than that of BA smooth muscle cells. Although a clear explanation has not been described regarding the involvement of the spread of $[Ca^{2+}]_i$ wave in the contractile response, this investigation indicated that spatiotemporal properties of the $[Ca^{2+}]_i$ response differ between the MCA and BA.

In summary, we concluded that different regions of arteries along the same cerebral tissue may display a distinct $[Ca^{2+}]_i$ response; moreover, this difference may be one reason for the distinct contractile response. Therefore, the MCA may afford important information in terms of the analysis of pathophysiological mechanisms of secondary brain ischemia that occur at the site of the MCA principally.

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