Relationship Between Global Cytosolic Ca\textsuperscript{2+} Concentration and Ca\textsuperscript{2+}-activated K\textsuperscript{+} Current in Rabbit Cerebral Arterial Myocyte

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Abstract

In smooth muscle cells, the sarcoplasmic reticulum (SR) has been identified as the primary storage site for intracellular Ca\textsuperscript{2+}. The peripheral SR is in close proximity with plasma membrane to make a narrow subsarcolemmal space. In this study, we investigated the regulation of subsarcolemmal [Ca\textsuperscript{2+}] (\([\text{Ca}^{2+}]_s\)) and global cytosolic [Ca\textsuperscript{2+}] (\([\text{Ca}^{2+}]_c\)) of rabbit arterial smooth muscle using whole-cell patch clamp technique and microspectrofluorimetry. The Ca\textsuperscript{2+}-activated K\textsuperscript{+} current (I_{K(Ca)}) and the ratio of fura-2 fluorescence (R_{340/380}) were considered to reflect the [Ca\textsuperscript{2+}]\textsubscript{s} and [Ca\textsuperscript{2+}]\textsubscript{c}, respectively. At a holding potential of 0mV, extracellular application of 10mM caffeine, a well-known Ca\textsuperscript{2+}-releasing agent, induced transient increase of I_{K(Ca)} and R_{340/380} (I_{K(Ca)}-transient and R_{340/380}-transient, respectively). The increase and decay of I_{K(Ca)}-transient was faster than R_{340/380}-transient. By repetitive application of caffeine, when the refilling state of SR was supposed to be lower than the control condition, I_{K(Ca)}-transient and R_{340/380}-transient were suppressed to different levels; e.g. the second application 20 sec after the first could induce smaller I_{K(Ca)}-transient than R_{340/380}-transient. Dissociation of I_{K(Ca)}-transient and R_{340/380}-transient was removed by sufficient (>3min) washout of caffeine. Recovery from the dissociation was also dependent upon the membrane potential; faster recovery was observed at negative (−40 mV) holding potential than at depolarized (0 mV) condition. Dissociation of I_{K(Ca)} from [Ca\textsuperscript{2+}]\textsubscript{c} was also partially prevented by perfusion with Na\textsuperscript{+}-free (replaced by NMDG+) extracellular solution. These results suggest that, 1) there is prominent spatial inhomogeneity of [Ca\textsuperscript{2+}] in cerebral arterial myocyte, 2) [Ca\textsuperscript{2+}]\textsubscript{s} is preferentially affected by the interference from nearby plasmalemmal Ca\textsuperscript{2+} regulation mechanism which is partly dependent upon extracellular Na\textsuperscript{+}.

Key Words: Smooth muscle, Ca\textsuperscript{2+} concentration, Ca\textsuperscript{2+}-activated K\textsuperscript{+} current

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Introduction

Intracellular free Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_c\)) and its regulation are crucial determinants of smooth muscle contractility. The sarcoplasmic reticulum (SR) has been identified as the primary intracellular organelle which modulates or buffers [Ca\(^{2+}\)]\(_c\) through Ca\(^{2+}\) sequestration/storage or agonist-induced Ca\(^{2+}\) release (van Breeman & Saida, 1989). In vascular smooth muscle cells, the SR has both peripheral and central components (Devine et al., 1972). The peripheral SR is in close proximity with plasma membrane to make a narrow restrictive space (Devine et al., 1972). Based on the structural characteristics of peripheral SR, it has been suggested that cytoplasmic space can be divided into superficial and deep cytoplasmic spaces (van Breeman et al., 1995). From its subcellular location, the superficial SR in smooth muscle has been speculated as a regulated barrier to diffusion of Ca\(^{2+}\) from the superficial space to deeper myoplasmic space (van Breeman et al., 1995). Theoretical modeling suggested that such a structure would create steep Ca\(^{2+}\) gradients immediately below the cell membrane (Kargacin, 1991).

The Ca\(^{2+}\) gradient or inhomogeneity of [Ca\(^{2+}\)]\(_c\) within single myoplasmic space has been already suggested from simultaneous measurement of Ca\(^{2+}\)-activated K\(^+\) current (I\(_{K(Ca)}\)) and global [Ca\(^{2+}\)]\(_c\) using fluorescence ratiometry (Stehno-Bittel and Sturek, 1992). More recently, it was reported that strong stimulation of Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) by high concentration (10 mM) of caffeine, a representative agent inducing Ca\(^{2+}\) release via ryanodine receptor Ca\(^{2+}\) channels in SR membrane, induced phasic increase of both I\(_{K(Ca)}\) and [Ca\(^{2+}\)]\(_c\) and repetitive application of caffeine with short interval induced dissociation of \(\Delta I_{K(Ca)}\) and \(\Delta [Ca^{2+}]_c\), i.e. \(\Delta I_{K(Ca)}\) is sharply suppressed by second application while [Ca\(^{2+}\)]\(_c\) is relatively unaffected (Ganitkevich and Isenberg, 1996). They suggested that the dissociation of \(\Delta I_{K(Ca)}\) and \(\Delta [Ca^{2+}]_c\) indicates a gradient of [Ca\(^{2+}\)]\(_c\) during Ca\(^{2+}\) release directed from the subsarcolemma toward the center of the cell, which is reversed or dissipated by repetitive stimulation.

By the development of laser confocal microscopy combined with patch clamp, direct measurements of localized Ca\(^{2+}\) release (Ca\(^{2+}\)-spark) and related activation of I\(_{K(Ca)}\) (spontaneous transient outward current, STOC) were accomplished in the arterial (Nelson et al., 1996; Mirronnaue et al., 1997) or visceral smooth muscle cells (Imaizumi et al., 1998). Those results indicate that \(\Delta I_{K(Ca)}\) sharply reflects the localized change of subsarcolemmal [Ca\(^{2+}\)]\(_{si}\) (Ca\(^{2+}\)-hot spots). Those hot spots were mostly located near from the cell membrane (Imaizumi et al., 1998). The discrimination between subsarcolemmal and mean cytoplasmic Ca\(^{2+}\) concentration may be important since the control of I\(_{K(Ca)}\) by [Ca\(^{2+}\)]\(_{si}\) can regulate electrical excitability of vascular myocyte without interfering with the contractile state of smooth muscle which depends on [Ca\(^{2+}\)]\(_c\). (Nelson et al., 1995; Knot et al., 1998).

In our previous report, we found that the rabbit cerebral arterial myocyte contains both ryanodine-sensitive and insensitive Ca\(^{2+}\) pools (Kim et al., 1998). In the same myocyte, the ryanodine-sensitive Ca\(^{2+}\) pool plays a key role for the generation of caffeine (1 mM)-induced I\(_{K(Ca)}\) oscillation (Kang et al., 1995). In this report, we combined the whole-cell patch clamp technique with fluorescence ratiometry and compared the responses of I\(_{K(Ca)}\) and fluorescence ratio of rabbit cerebral smooth muscle to external application of caffeine. We could confirm
the dissociation of $\Delta I_{\text{K(Ca)}}$ and $\Delta [\text{Ca}^{2+}]_c$ which was partly prevented by the elimination of extracellular Na$^+$. Possible role of Na/Ca exchanger in $[\text{Ca}^{2+}]_c$ regulation will be discussed.

**Materials & Methods**

**Cell isolation and fura-2 loading**

Rabbits (New Zealand white rabbit, 1.5-2.0 kg) were intravenously anaesthetized with sodium pentobarbital (40 mg/kg) and exsanguinated. Both right and left middle cerebral arteries were dissected in a Ca$^{2+}$-free physiological salt solution (PSS). Isolated vessels were transferred to Ca$^{2+}$-free PSS containing collagenase (1.5 mg/ml, Wako), bovine serum albumin (2 mg/ml, Sigma) and dithiothreitol (DTT, 1 mg/ml) and incubated at 35°C for 20 min. After collagenase treatment, segments were transferred to modified Kraft-Bruhe (K-B) medium (Isenberg & Klöckner, 1982) and single myocytes were dispersed by gentle agitation with a fire-polished wide-bored glass pipette.

**Electrophysiological recordings**

Whole-cell membrane currents (Hamill et al., 1981) were measured with an Axopatch-1D patch-clamp amplifier (Axon Instrument, USA) filtered at 5 kHz. Glass pipettes with a resistance of 3-4 mega ohm were used to make a giga ohm seal. Axoscope v. 1.0 and Digidata-1200 (all from Axon Instrument, USA) were used for the acquisition of data and applying command pulses. For the continuous recording of $I_{\text{K(Ca)}}$ the data were sampled at 100 Hz using Axoscope and displayed on a computer monitor using Axoscope v. 1.0.

**Fluorescence measurements**

$[\text{Ca}^{2+}]_c$ was measured with a microfluorimeter consisting of an inverted fluorescence microscope (Diaphot 300, Nikon, Japan) with a 40 x 0.85 NA objective, a photomultiplier tube (type R 1527, Hamamatsu, Japan) and PTI deltscan illuminator (monochromator system with 2 nm bandpath, Photon Technology International Inc, USA). Light was provided by a 75 W xenon lamp (Ushino, Japan) through a chopper wheel (frequency of 4 or 10 Hz) which alternated the light path to monochromators (340 and 380 nm, respectively). A 425 nm short-pass excitation filter reduced background fluorescence. A 570 nm short-pass dichroic mirror passed emission light onto the photomultiplier. A mechanical diaphragm situated at an image plane in the emission path limited the measurement to a single cell. Both data acquisition and control of light application were done by using a computer software (Felix v. 1.1, PTI, USA). K$_5$ fura-2 was dissolved in the distilled water to make a 10 mM of stock solution and was diluted by pipette solution to a final concentration of 80 μM. After the whole-cell configuration was achieved, the myocyte was dialyzed for 4~5 min before the start of the experiment. The calibration of fluorescence ratio to $\text{Ca}^{2+}$ concentration was not done in this study but just displayed as the ratio of fluorescence intensity (340/380).

**Solutions and drugs**

The physiological salt solution contained (in mM) NaCl 135, KCl 5, CaCl$_2$ 2, MgCl$_2$ 1,
glucose 5, HEPES (N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid]) 10, and pH was adjusted to 7.4 by NaOH. CaCl₂ was just omitted to make “Ca²⁺-free PSS”. Modified ‘K-B medium’ contained L-glutamate 50, KCl 50, taurine 20, KH₂PO₄ 20, MgCl₂ 3, glucose 10, HEPES 10, EGTA (ethyleneglycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid) 0.5 and pH was adjusted to 7.3 by KOH. The pipette solution consisted of (in mM) KCl 140, MgATP 5, MgCl₂ 1, HEPES 10, K₅ fura-2 0.08; pH was adjusted to 7.3, using KOH. Cells were allowed to settle down in the experimental chamber (0.2 ml) and thereafter superfused by gravity at a constant flow of 2 ml/min. K₅ fura-2 was purchased from Molecular Probes, Inc. (USA) and all other drugs were purchased from Sigma (USA).

Statistics
In this report, statistical analysis was not used generally because the resultant analysis was mainly a qualitative comparison within the same myocyte. The figures shown in this article are representative examples selected after confirming the same results.

Results

Dissociation between the caffeine-induced Iₖ(Ca) transient and Ca²⁺ transient

The membrane current and cytoplasmic Ca²⁺ concentration ([Ca²⁺]c) of rabbit cerebral arterial myocytes were recorded simultaneously using KCl-rich pipette solution containing 80 µM fura-2. The membrane potential was held at 0 mV to inactivate voltage-dependent K⁺ current and thereby the recorded outward current was predominantly Iₖ(Ca). The spontaneous transient outward currents (STOCs) were usually observed in first few minutes after making a whole-cell configuration but were not prominent during the prolonged experiment (see the control trace of Fig. 1A and other figures). We interpret this because of the presence of Ca²⁺ indicator, fura-2 which also has a high Ca²⁺-buffering activity (Ganitkevich, 1998). In those cells where STOCs were consistently observed, corresponding changes in fluorescence ratio (R₃₄₀/₃₈₀) was not discernible (see the control trace of Fig. 1B).

Caffeine is widely used to induce Ca²⁺ release from SR through the ryanodine-sensitive Ca²⁺ release channel (RyR); the opening probability of RyR is normally regulated by the Ca²⁺ concentrations of cytoplasm and of SR, and caffeine is known to lower the threshold of RyR to Ca²⁺ (Iino, 1990). Previous report showed that relatively low concentration of caffeine (1 mM) could induce regular large oscillations of Iₖ(Ca) in rabbit cerebral artery that were considered to reflect the oscillatory release of Ca²⁺ from intracellular stores (Kang et al., 1995). In the present condition, however, external application of 1 mM caffeine usually induced single phasic increase of Iₖ(Ca) and [Ca²⁺]c (R₃₄₀/₃₈₀) and typical regular oscillations of Iₖ(Ca) were not observed. In rare cases (2 out of 10 cells tested) where caffeine-induced oscillations were observed, correspondent changes in R₃₄₀/₃₈₀ was indistinguishable (Fig. 1A & B). Above responses indicate the dissociation of Δ[Ca²⁺] responsible for respective changes.

Since the responses to 1 mM caffeine were irregular as shown above, concentration of caffeine was raised to 10 mM to ensure the full depletion of SR. Fig. 2 shows typical results from two different cells. On superfusing caffeine-containing solution, large transient activa-
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A. Under control condition, held at 0 mV, STOCs can be observed. External application of 1 mM caffeine induced initial increase of \(I_{K(Ca)}\) and fluorescence ratio (R\(_{340/380}\)) and oscillations of \(I_{K(Ca)}\) larger than STOCs followed. However, corresponding oscillations of R\(_{340/380}\) were not shown.

B. In another cell, application of 1 mM caffeine could not induce regular oscillations but induced bursts of spontaneous transient outward currents (STOCs). Some bursts appeared to be related with fluctuations of R\(_{340/380}\).

Fig. 1. Comparison of \(I_{K(Ca)}\) and R\(_{340/380}\) during the application 1 mM caffeine.

Faster activation and faster decay of \(I_{K(Ca)}\)-transient than those of R\(_{340/380}\)-transient were consistently observed (Fig. 2A(a)). When plotted on expanded time scale, \(I_{K(Ca)}\)-transient reached its peak faster than the R\(_{340/380}\)-transient and from its peak, \(I_{K(Ca)}\)-transient decayed faster than R\(_{340/380}\)-transient (Fig. 2A(b) & Fig. 2B). In some myocytes, \(I_{K(Ca)}\)-transient showed two peaks (split \(I_{K(Ca)}\)-transient) without corresponding decrease in Ca\(^{2+}\)-transient (Fig. 2A(b)). Faster activation and faster decay of \(I_{K(Ca)}\)-transient than those of R\(_{340/380}\)-transient were
Fig. 2. Comparison of the time-courses of $I_{K(Ca)}$ and $R_{340/380}$ change induced by 10 mM caffeine.

A. Short application (5 sec) of caffeine (10 mM) induced large $I_{K(Ca)}$-transient and concomitant $R_{340/380}$-transient. In A (b), the responses to first application of caffeine were shown on an expanded time-scale. The increase of $I_{K(Ca)}$ was split by transient decrease while $R_{340/380}$-transient did not show such fluctuation.

B. Another example of the response to 10 mM caffeine shown on expanded time-scale. Common in all other cells examined in this study although the extent of difference was variable depending on individual myocyte or experimental conditions (membrane potential, solution exchange rate, etc.).

To confirm that both caffeine-induced responses ($I_{K(Ca)}$-transient and $R_{340/380}$-transient) were due to the Ca$^{2+}$-release from SR, we applied cyclopiazonic acid (CPA), a SR Ca$^{2+}$-ATPase inhibitor (Seidler et al., 1989). Short pulses of (3–4 sec) of caffeine were repetitively applied (filled circles in Fig. 3). After confirming that similar amplitudes of $I_{K(Ca)}$-transient and $R_{340/380}$-transient were repeated, we continuously superfused 10 nM CPA. As was expected, CPA could suppress both responses and this effect was reversed by washout of CPA. However, the inhibition of $I_{K(Ca)}$-transient was relatively faster and more prominent than that of $R_{340/380}$-transient. Also, the recovery by washout was slower (Fig. 3). Similar responses were observed in two other cells tested. This result indicated that, 1) both responses were due to the
Fig. 3. Effects of CPA on $I_{K(Ca)}$-transient and $R_{340/380}$-transient. 10 mM caffeine was applied (2-3 sec) repetitively on the timing indicated by filled circles. 10 $\mu$M cyclopiazonic acid (CPA) was applied for 1 min as indicated in the figure. $I_{K(Ca)}$-transient was rapidly abolished by CPA while the corresponding $R_{340/380}$-transient remained about 30% of control.

simultaneous release of stored $Ca^{2+}$ by caffeine, and 2) the SR responsible for $I_{K(Ca)}$-transient appears to be more easily depleted by SERCA inhibitor.

Although the response amplitudes of $I_{K(Ca)}$-transient and $R_{340/380}$-transient were relatively constant in the same myocyte under experiment, we could also observe the dissociation of them by rapid reapplication of caffeine as was previously reported in guinea-pig coronary artery (Ganitkevich and Isenberg, 1996, see Introduction). In Fig. 4A, short perfusion (3~4 s) of 10 mM caffeine induced large and sharp $I_{K(Ca)}$-transient and $R_{340/380}$-transient. Upon second application of caffeine after a relatively short washout interval (20~30 s), the amplitude of $Ca^{2+}$-transient was suppressed to about 70% of initial control while the amplitude of $I_{K(Ca)}$-transient was suppressed to about 20% of first response (Fig. 4A). After the 1 min and 2 min of washout interval (third and fourth application of caffeine, respectively), $I_{K(Ca)}$ recovered to 50% and 85% while the corresponding amplitudes of $Ca^{2+}$ transients remained at 80 and 90%, respectively (Fig. 4A). In six other cells, same protocol was applied and the normalized values of all seven cells were plotted together (Fig. 4B(a) and (b)). Also, the ratio of normalized $I_{K(Ca)}$-transient over normalized $R_{340/380}$-transient were obtained at respective timing of caffeine application and plotted as dissociation index (Fig. 4B(c)).

Underlying mechanism of the dissociation between $I_{K(Ca)}$-transient and $Ca^{2+}$-transient

We hypothesized that the dissociation of $I_{K(Ca)}$-transient and $R_{340/380}$-transient was due to the relatively slower and less complete refilling of $Ca^{2+}$-pool that is mainly responsible for $I_{K(Ca)}$ activation i.e., the refilling procedure of the SR located just beneath the plasma membrane may be slower than those of deeper cytoplasmic SR. If this was the case, such dissociation would
Fig. 4. Dissociation of $I_{\text{K(Ca)}}$-transient and $R_{\text{340/380}}$-transient.

A. Held at 0 mV, short exposure to 10 mM caffeine is indicated by the closed circles. An initial caffeine application induced $I_{\text{K(Ca)}}$-transient of 800 pA. After 30 s washout, a second caffeine application induced sharply suppressed $I_{\text{K(Ca)}}$-transient of only 40 pA (5% of initial control) while the second $R_{\text{340/380}}$-transient was about half of initial one. The recovery from suppression was confirmed by a third, fourth and fifth application of caffeine with longer interval.

B. Results from seven cells are shown. Different symbols indicate results from different cells. $I_{\text{K(Ca)}}$-transient and $R_{\text{340/380}}$-transient were normalized against the responses to initial caffeine application (a) & (b). In (c), normalized values of $I_{\text{K(Ca)}}$-transients were divided by normalized $R_{\text{340/380}}$-transient at each timing of caffeine application and plotted as the dissociation index.
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Fig. 5. Dependence of the recovery from dissociation on the holding membrane potential.

A. Held at 0 mV, in the presence of verapamil (1 \(\mu\)M), the dissociation of \(I_{\text{K(Ca)}}\) and \(R_{\text{M(Ca)}}\)-transient was confirmed. Then, membrane potential was held at \(-40\ \text{mV}\) during the resting period between caffeine applications. During the caffeine application (closed circles), the membrane potential was depolarized to 0 mV.

B. In this example, the sequence of membrane potential condition was reversed. As a first step, complete recovery of \(I_{\text{K(Ca)}}\) and \(R_{\text{M(Ca)}}\)-transient was confirmed by clamping the membrane potential at \(-35\ \text{mV}\) during the resting period. Nextly, the same myocyte was held at 0 mV and caffeine was applied respectively.

C. The effect of holding potential on dissociation index was shown in 3 cells.
Fig. 6. Effect of external Na⁺ replacement on the dissociation of I_{K(Ca)}⁻ and R_{340/380}-transient. Firstly, dissociation of I_{K(Ca)}⁻ and R_{340/380}-transient was confirmed. Then, external Na⁺ was completely replaced with N-methyl-D-glucamate (NMDG) and 10 mM caffeine was applied repetitively (open circles). The dissociation of I_{K(Ca)}⁻ and R_{340/380}-transient was relieved by NMDG replacement. Also, I_{K(Ca)}⁻-transients were increased. The dissociation index (see above) was obtained for each case and plotted in Fig. 5C(a), (b). Fig. 5C(c) shows the responses from the other 3 cells the raw traces of which were not shown here.

As the superficial SR is known to locate just beneath the plasma membrane (Devine et al., 1972), it was plausible that the plasmalemmal Ca²⁺ removal mechanism(s) may affect the refilling procedure; Ca²⁺ released from superficial SR may be preferentially removed to the extracellular space. If this was true, then the blocking of plasmalemmal Ca²⁺ removal mechanism could eliminate the dissociation between I_{K(Ca)}⁻-transient and R_{340/380}-transient. As a number of previous studies had suggested the presence of Na⁺/Ca²⁺ exchanger in vascular
myocytes (Boval et al., 1990; Johaszoa et al., 1994; Karaki et al., 1997), we tried to block the Na+-dependent Ca2+ removal mechanism. In Fig. 6, the extracellular Na+ (140 mM) was replaced by N-methyl-D-glucamine (NMDG), an organic monovalent cation. In this condition, the dissociation between \( I_{K(Ca)} \)-transient and \( R_{340/380} \)-transient was less prominent. Three cells among four cells tested, showed similar responses.

**Discussion**

In this study, we hypothesized that the amplitude of caffeine-induced \( I_{K(Ca)} \)-transient and \( R_{340/380} \)-transient reflect the Ca2+ content of the superficial and total SR, respectively. Although such hypothesis was not strictly proved here, high concentration of caffeine is widely used to induce unequivocal release of Ca2+ from RyR-containing Ca2+ store (Iino, 1990; Ganitkevich & Isenberg, 1996). Also, the disappearance of both signal by CPA supported such hypothesis (Fig. 2). In spite of the narrow cell diameter of cerebral artery smooth muscle (usually less than 5 μm), sharp dissociation of \( I_{K(Ca)} \)-transient from \( R_{340/380} \)-transient by repetitive caffeine application suggests that \( [Ca^{2+}]_{sl} \) is relatively unaffected by the change of deeper cytoplasm. The presence of more than two compartments for \( [Ca^{2+}]_c \) in vascular smooth muscle cells has been also suggested by comparing signals of different Ca2+ binding dyes with different binding affinity (e.g. fura-2 and aequorin, Abe et al., 1995; 1996). In urinary bladder smooth muscle, it was demonstrated that the \( I_{K(Ca)} \) and localized superficial Ca2+-increase (Ca2+-hot spots) was tightly related and both signals were faster than the mean cytoplasmic [Ca2+]) (Imaizumi et al., 1998).

Changes in activity of potassium channels represent a major mechanism that regulates vascular tone. Activation of potassium channels induces hyperpolarization of the cell membrane, closure of voltage-operated Ca2+ channel. The membrane potential of cerebral vascular muscle in vivo is not exactly known. In experimental condition, changes in membrane potential of only a few millivolts are associated with substantial changes in vascular tone (Knot and Nelson, 1998). In various smooth muscle cells including cerebral vascular myocyte, \( I_{K(Ca)} \) is one of the major outward currents elicited by both membrane depolarization and cytosolic Ca2+ increase. The spontaneous Ca2+-release from peripheral SR which activates STOCs is sensitive to both caffeine and ryanodine (Bolton and Imaizumi, 1996) and, therefore, considered to be mediated by opening of ryanodine receptor Ca2+-releasing channels (Bolton and Imaizumi, 1996). \( I_{K(Ca)} \) is greatly reduced by low concentrations of tetraethylammonium (TEA), charybdotoxin or iberiotoxin, suggesting that the current is mainly due to the activation of large conductance Ca2+-dependent K+ channels (BKCa, Bolton and Beech, 1992). In cerebral arteries, application of BKCa blockers produces contraction in vitro and in vivo, which indicates the contribution of STOCs to the regulation of basal tone of cerebral arteries; offsetting membrane potential to the negative direction and reducing Ca2+-influx through voltage-dependent Ca2+ channels (Nelson et al., 1995). Similarly, inhibition of Ca2+ store function by CPA enhanced both electrical excitability and phasic contractions in ileal smooth muscle. In rat portal vein thapsigargin increases the amplitude of the spontaneous contractions (Mikkelsen et al., 1992).
The present study focused on the relationship between fluorescence ratio and \(I_{K(Ca)}\). The results demonstrate the dissociated regulation of the \([Ca^{2+}]_{si}\) and \([Ca^{2+}]_{c}\) in rabbit cerebral arterial myocyte as has been reported in coronary arterial smooth muscle (Ganitkevich and Isenberg). The decrease of the \(I_{K(Ca)}\) transient upon successive application of caffeine can be due to an exhaustion of stored \(Ca^{2+}\) in superficial SR after a large \(Ca^{2+}\) release. Alternatively, \(Ca^{2+}\) release might be preferably suppressed in superficial SR after a large release by negative feedback mechanism, e.g. protein kinase C (Kitamura et al., 1992). Although the latter possibility was not excluded experimentally, it is more likely that the \(Ca^{2+}\) released from superficial SR would have more chance to be removed from cytosol because of the anatomical contiguity with plasma membrane where many \(Ca^{2+}\) removal mechanisms locate (e.g., plasmalemmal \(Ca^{2+}\)-ATPase (PMCA) and \(Na^{+}/Ca^{2+}\) exchanger). In fact, a digitized three-dimensional image study in smooth muscle revealed that \(Na^{+}/Ca^{2+}\) exchanger and \(Na^{+}/K^{+}\) pump are clustered in the region of plasma membrane close to portions of the SR specialized for \(Ca^{2+}\) storage (Moore et al., 1993). By the hypothesis of 'superficial buffer barrier', the subsarcolemmal gap space between superficial SR and plasma membrane forms a functional \(Ca^{2+}\) extrusion compartment; the high \([Ca^{2+}]_{si}\) was thought to stimulate \(Ca^{2+}\) extrusion via PMCA and \(Na^{+}/Ca^{2+}\) exchange (van Breemen et al., 1995). In our experimental results, the less dissociation after removing extracellular \(Na^{+}\) suggests that \(Na^{+}/Ca^{2+}\) exchanger also participates the regulation of \([Ca^{2+}]\) of rabbit cerebral vascular myocyte.

In our study, we could also find that the more negative membrane potential facilitates the recovery of \(I_{K(Ca)}\)-transient, i.e. refilling state of superficial SR. We interpret this result that the retarded refilling of superficial SR was responsible for the dissociation of \(I_{K(Ca)}\)-transient and \(R_{340/380}\)-transient observed here. This result also implies that one of the sources of \(Ca^{2+}\) which refills the subsarcolemmal SR depends upon the electrical gradient across plasma membrane. We could not, however, identify the passive \(Ca^{2+}\) influx pathway for the recovery of \(I_{K(Ca)}\) transient in this report. So called CRAC channels (\(Ca^{2+}\)-release activated \(Ca^{2+}\) channels, Hoth and Penner, 1992) might be responsible for the \(Ca^{2+}\) entry after SR depletion. The presence of \(Ca^{2+}\) store-dependent \(Ca^{2+}\) influx has been also suggested in smooth muscle cells (Putney, 1990; Wayman et al., 1998).

In summary, our study demonstrated the dissociated regulation of superficial vs. deep cytoplasmic \([Ca^{2+}]\) in cerebral arterial myocytes. Further elucidation of regulatory mechanisms for \([Ca^{2+}]_{si}\) (e.g. PMCA, \(Na^{+}/Ca^{2+}\) exchanger) might be helpful to understand the physiological roles of compartmentalized \(Ca^{2+}\) regulation in smooth muscle.

**Acknowledgement**

This paper was supported by NON DIRECTED RESEARCH FUND, Korea Research Foundation, 1996.

**References**

Abe, F., Karaki, H. and Endoh, M. (1996) Effects of cyclopiazonic acid and ryanodine on cytosolic
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Abe, F., Mitsui, M., Karaki, H. and Endoh, M. (1995) Calcium compartments in vascular smooth muscle cells as detected by aequorin signal. *Br. J. Pharmacol.* **118**: 1711-1716.

Bolton, T.B. and Imaizumi, Y. (1996) Spontaneous transient outward currents in smooth muscle cells. *Cell Calcium* **20**: 141-152.

Boval, S., Goldman, W.F., Yauan, X.J. and Blaustein, M.P. (1990) Influence of Na\(^+\) gradient on Ca\(^{2+}\) transients and contraction in vascular smooth muscle. *Am. J. Physiol.* **259**: H409-H423.

Devine, C.E., Somlyo, A.V. and Somlyo, A.P. (1972) Sarcoplasmic reticulum and excitation-contraction coupling in mammalian smooth muscles. *J. Cell. Biol.* **52**: 690-718.

Gabella, G. (1981) Structure of smooth muscles. In: Smooth Muscle: an Assessment of Current Knowledge, ed. by A.F. Bulbring, A.W. Jones and T. Tomita., Edward Arnold (Publishers) Ltd, London, pp. 1-46.

Ganitkevich, V.Ya. (1998) Use of Indo-1FF for measurement of rapid micromolar cytoplasmic free Ca\(^{2+}\) increments in a single smooth muscle cells. *Cell Calcium* **23**: 313-322.

Ganitkevich, V.Ya. and Isenberg, G. (1996) Dissociation of subsarcolemmal from global cytosolic \([\text{Ca}^{2+}]\) in myocytes from guinea-pig coronary artery. *J. Physiol. (Lond)* **490**: 305-318.

Hamill, O.P., Marty, A., Neher, E., Sakmann, B. and Sigworth, F.J. (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.* **391**: 85-100.

Hoth, M. and Penner, R. (1992) Depletion of intracellular calcium stores activates a calcium current in mast cells. *Nature* **355**: 353-355.

Iino, M. (1990) Calcium release mechanisms in smooth muscle. *Jap. J. Pharmacol.* **54**: 345-354.

Imaizumi, Y., Torii, Y., Ohi, Y., Nagano, N., Atsuki, K., Yamamura, H., Muraki, K., Watanabe, M. and Bolton, T.B. (1998) Ca\(^{2+}\) images and K\(^+\) current during depolarization in smooth muscle cells of the guinea-pig vas deferens and urinary bladder. *J. Physiol. (Lond)* **510**: 705-719

Isenberg, G. and Klöckner, U. (1982) Calcium tolerant ventricular myocytes delivered by pre-incubation in a "KB-medium". *Pflügers Arch.* **395**: 358-360.

Juhaszova, M., Ambesi, A., Lindenmayer, G.E., Bloch, R.J. and Blaustein, M.P. (1994) Na\(^+\)-Ca\(^{2+}\) exchanger in arteries: identification by immunoblotting and immunofluorescence microscopy. *Am. J. Physiol.* **266**: C234-C242.

Kang, T.M., So, I., Kim, K.W. (1995) Caffeine- and histamine-induced oscillations of K\(_{\text{Ca}}\) current in single smooth muscle cells of rabbit cerebral artery. *Pflügers Arch.* **431**: 91-100.

Karaki, H., Ozaki, H., Hori, M., Mitsui-Saito, M., Amano, K-I., Harada, K-I., Miyamoto, S., Nakazawa, H., Won, K-J. and Sato, K. (1997) Calcium movements, distribution, and functions in smooth muscle. *Pharmacol. Rev.* **49**(2): 157-230.

Kargacin, G.J. (1991) Calcium signaling in restricted diffusion spaces. *Biophys J* **67**: 262-272.

Kim, S.J., Kim, J.K., So, I., Suh, S.H., Lee, S.J. and Kim, K.W. (1998) Characteristics of Ca\(^{2+}\) stores in rabbit cerebral artery myocytes. *Kor. J. Physiol. Pharmacol.* **2**: 313-322.

Knot, H.J. and Nelson, N.T. (1998) Regulation of arterial diameter and wall \([\text{Ca}^{2+}]\) in cerebral arteries of rat by membrane potential and intravascular pressure. *J. Physiol. (Lond)* **508**: 199-209.

Knot, H.J., Standen, N.B. and Nelson, M.T. (1998) Ryanodine receptors regulate arterial diameter and wall \([\text{Ca}^{2+}]\) in cerebral arteries of rat via Ca\(^{2+}\)-dependent K\(^+\) channels. *J. Physiol. Lond.* **508**: 211-221.

Mikkelsen, E.O., Poulsen, S.H. and Christensen, S.B. (1992) Comparison of the effects of thapsigargin and Bay K 8644 on spontaneous mechanical activity in rat portal vein and contractile responses of rat cardiac muscle. *Pharmacol. Toxicol.* **70**: 152-156.

Moore, E.D.W., Etter, E.F., Philipson, K.D., Carrington, W.A., Fogarty, K.E., Lifshitz, L.M. and Fay,
F.S. (1993) Coupling of the Na\(^+\)/Ca\(^{2+}\) exchanger, Na\(^+\)/K\(^+\) pump and sarcoplasmic reticulum in smooth muscle. Nature 365: 657–660.

Nelson, M.T., Cheng, H., Rubart, M., Santana, L.F., Bonev, A.D., Knot, H.J. and Lederer, W.J. (1995) Relaxation of arterial smooth muscle by calcium sparks. Science 270, 633–637.

Putney, J.W.JR. (1990) Capacitative calcium entry revisited. Cell Calcium 11: 611–624.

Seidler, N.W., Jona, I., Vegh, M. and Martonosi, A. (1989) Cyclopiazonic acid is a specific inhibitor of the Ca\(^{2+}\)-ATPase of sarcoplasmic reticulum. J. Biol. Chem. 264: 17817–17823.

Stehno-Bittel, L., and M. Sturek. Spontaneous sarcoplasmic reticulum calcium release and extrusion from bovine, not porcine, coronary artery smooth muscle. J. Physiol. Lond. 451: 49–78, 1992.

Uyama, Y., Imaizumi, Y. and Watanabe, M. (1993) Cyclopiazonic acid, an inhibitor of Ca\(^{2+}\)-ATPase in sarcoplasmic reticulum, increases excitability in ileal smooth muscle. Br. J. Pharmacol. 110: 567–572.

Van Breeman, C. and Saida, K. (1989) Cellular mechanisms regulating [Ca\(^{2+}\)]\(_i\), of smooth muscle. Annu. Rev. Physiol. 51: 315–329.

Van Breemen, C., Chen, Q. and Laher, I. (1995) Superficial buffer barrier function of smooth muscle sarcoplasmic reticulum. T.I.P.S. 98–105.

Wayman, C.P., Gibson, A. and McFadzean, I. (1998) Depletion of either ryanodine- or IP\(_3\)-sensitive calcium stores activates capacitative calcium entry in mouse anococcygeus smooth muscle cells. Pflügers Arch. 435: 231–239.

Xu, L., Lai, F.A., Cohn, A., Etter, E., Guerrero, A., Fay, F.S. and Meissner, G. (1994) Evidence for a Ca\(^{2+}\)-gated ryanodine-sensitive Ca\(^{2+}\) release channel in visceral smooth muscle. Proc. Natl. Acad. Sci. USA 91: 3294–3298.

(Received November 9, 1998 : Accepted December 9, 1998)