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Characteristics of Ca^{2+} Stores in Rabbit Cerebral Artery Myocytes

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In a myocyte freshly isolated from rabbit cerebral artery, the characteristics of Ca^{2+} release by histamine or caffeine were studied by microspectrofluorimetry using a Ca^{2+}-binding fluorescent dye, fura-2. Histamine (5 μM) or caffeine (10 mM) induced a phasic rise of cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_c$) which could occur repetitively with extracellular Ca^{2+} but only once or twice in Ca^{2+}-free bathing solution. Also, the treatment with inhibitor of sarcoplasmic reticulum Ca^{2+}-ATPase suppressed the rise of $[Ca^{2+}]_c$ by histamine or caffeine. In Ca^{2+}-free bathing solution, short application of caffeine in advance markedly attenuated the effect of histamine, and *vice versa*. In normal Ca^{2+}-containing solution with ryanodine (2 μM), the caffeine-induced rise of $[Ca^{2+}]_c$ occurred only once and in this condition, the response to histamine was also suppressed. On the other hand, in the presence of ryanodine, histamine could induce repetitive rise of $[Ca^{2+}]_c$ while the amplitude of peak rise became stepwisely decreased and eventually disappeared. These results suggest that two different Ca^{2+}-release mechanisms (caffeine-sensitive and histamine-sensitive) are present in rabbit cerebral artery myocyte and the corresponding pools overlap each other functionally. Increase of $[Ca^{2+}]_c$ by histamine seems to partially activate ryanodine receptors present in caffeine-sensitive pool.

Key Words: Smooth muscle, Ca^{2+} concentration, Ca^{2+} pool, Histamine, Caffeine

INTRODUCTION

The cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_c$) is important in controlling diverse cellular processes including the contraction of smooth muscle (Somlyo & Somlyo, 1994). The processes underlying smooth muscle contraction involve the activation of voltage- and receptor-operated Ca^{2+} influx pathways and the mobilization of Ca^{2+} from sarcoplasmic reticulum (SR), an intracellular store. The relative importance of the Ca^{2+} sources, inside or outside of the smooth muscle, may vary according to the types of muscles as well as to the kinds of stimuli. In smooth muscle, it has been established that upon stimulation with various agonists, such as noradrenaline and histamine, the SR releases Ca^{2+}, via an inositol trisphosphate-sensitive mechanism ($InsP_3$-induced Ca^{2+} release, IICR, Somlyo & Somlyo, 1994). Till now, two types of Ca^{2+} stores have been functionally distinguished in terms of the mechanisms that are involved in Ca^{2+}-release: IICR and Ca^{2+}-induced Ca^{2+} release (CICR) mechanism (Iino, 1990).

CICR is a Ca^{2+} dependent gating of Ca^{2+} permeable channel (ryanodine receptor, RyR) in SR membrane. Ryanodine, a plant alkaloid, has been shown to be bound to the CICR channels when they are open and lock them in a subconductance open state (Meissner, 1986). Experimentally, transient increase of cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_c$) by extracellular application of caffeine has been regarded as an evidence for the presence of CICR in many kinds of cells (Iino, 1990). Caffeine is known to act by shifting the Ca^{2+} sensitivity of RyR to

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lower concentrations (Nagasaki & Kasai, 1984; Endo, 1985).

Physiological studies suggest that different cell types in smooth muscles functionally express different combination of Ca^{2+} pools. A number of studies have been carried out to investigate whether the Ca^{2+} store responsible for one type of Ca^{2+} release mechanism (e.g. CICR) is also susceptible to depletion when another type (e.g. IICR) is activated in the same cell or tissue (Iino, 1990). For example, in rat aorta smooth muscle, the norepinephrine induced Ca^{2+} release via $InsP_3$-receptors is unaffected by prior depletion of the caffeine-sensitive Ca^{2+} pool and vice versa (Kanaide et al, 1987; Matsumoto et al, 1990). In guinea-pig intestinal smooth muscle cells, however, $InsP_3$ mobilizes all of the Ca^{2+} releasable Ca^{2+} pools (Iino et al, 1987), indicating that functional compartmentalization of Ca^{2+} pools varies among smooth muscle tissues.

Different Ca^{2+}-release mechanisms may suggest different physiological roles in $[Ca^{2+}]_c$ regulation. It has been suggested that CICR mechanisms act to amplify small changes in $[Ca^{2+}]_c$. In smooth muscle cells, however, RyRs have low sensitivity to Ca^{2+} and significant CICR appears to occur only at high $[Ca^{2+}]_c$ (>1 μM) in skinned smooth muscle (Iino, 1989). On the other hand, spontaneous discrete Ca^{2+}-release from the SR of smooth muscle has been suggested by spontaneous transient activation of Ca^{2+}-dependent K^+ channels (STOCs, Bolton & Imaizumi, 1996). Since STOCs were abolished by the treatment with ryanodine, localized activation of RyRs may occur in physiological condition.

In a previous report, low concentration of both caffeine (1 mM) and histamine induced oscillations of Ca^{2+}-activated K^+ current in rabbit cerebral arterial myocyte, which indirectly indicated that CICR and IICR pools are present in this cell (Kang et al, 1994). This study endeavoured to elucidate which kind of Ca^{2+}-pool is operational in rabbit cerebral artery. Also, efforts were made to investigate whether RyRs are concomitantly activated during IICR.

METHODS

Cell isolation and fura-2 loading

Single smooth muscle cells were enzymatically isolated from rabbit middle cerebral artery. 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) containing (in mM) NaCl 140, KCl 5, 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid (HEPES) 10, $MgCl_2$ 1, glucose 10 (pH was adjusted to 7.4 with NaOH). Isolated vessels were transferred to Ca^{2+}-free PSS containing collagenase (1.5 mg/ml, Wako), bovine serum albumin (2 mg/ml; essentially fatty acid free, Sigma) and dithiothreitol (DTT, 1 mg/ml) and incubated at 35°C for 20 min. After collagenase treatment, segments were transferred to fresh Ca^{2+}-free PSS and single myocytes were dispersed by gentle agitation with a fire-polished wide-bored glass pipette.

Isolated single cells were loaded with acetoxymethyl ester form of fura 2 (2 μM diluted from 1 mM stock in dimethyl sulfoxide) in Ca^{2+}-free PSS for 15 minutes at room temperature. After then, the cell suspension was briefly centrifuged (800 r.p.m., 2 min) and washed with Ca^{2+}-free PSS twice. Fura-2 loaded cells were stored at 4°C until used. Experiments were done within 8 hours after the isolation of cells.

$[Ca^{2+}]_c$ measurement

The recording of single cell $[Ca^{2+}]_c$ was performed with a microfluorometric system consisting of an inverted fluorescence microscope (Diaphot 300, Nikon, Japan) with a dry-type fluorescence objective lens ($\times$40, NA 0.85), a photomultiplier tube (type R 1527, Hamamatsu, Japan) and PTI deltascan illuminator (Photon Technology International Inc, USA). One drop of cell suspension was put on a superfusion chamber (100 μl). Cells were allowed to settle down and thereafter superfused at a constant flow of 2 ml/min. Light was provided by a 75 W xenon lamp (Ushino, Japan) in order to control excitation frequency, a chopper wheel alternated the light path to monochromators (340 and 380 nm) with a frequency of 5 or 10 Hz. A short-pass dichroic mirror passed emission light of $<$ 570 nm onto the photomultiplier tube, and intensity at 510 nm was measured. A mechanimcal image mask was placed in the emission path, limiting 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).

Because of the uncertainties involved in calibrating

the fura 2 signals in intact cells, no attempt was made to calibrate $[Ca^{2+}]_c$, the results were instead reported as changes in the 340 nm/380 nm signal ratio ($R_{340/380}$).

Solutions and drugs

PSS contained (in mM) NaCl 140, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1,4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid (HEPES) 10, glucose 10 (pH was adjusted to 7.4 with NaOH). A small chamber (100 μl) was continously perfused (2 ml/min) by PSS and all the experiments were performed at 30°C.

The fluorescence indicator fura 2-AM was purchased from Molecular Probes, Inc (USA) and the all other chemicals and drugs used in this study were purchased from Sigma Chemical Co. (USA).

Statistics

Where applicable, the results of multiple experiments are reported as means±*SEM* (Table 1) but statistical analysis was not used because the resultant analysis was a qualitative comparison between data groups. The figures cited in this article are representative examples selected after confirming the same results.

Table 1. Averaged response of arterial smooth muscle to caffeine and histamine

	mean±*S.E.*	*n*
Resting $R_{340/380}$	1.00±0.042	56
Peak $R_{340/380}$ by caffeine	2.58±0.115	22
Tonic $R_{340/380}$ by caffeine (long)	1.10±0.039	10
Peak $R_{340/380}$ by histamine	2.86±0.111	34
Tonic $R_{340/380}$ by histamine (long)	1.37±0.032	25
$T_{63\%}$ (sec) by caffeine (short)	5.5±0.50	12
$T_{63\%}$ (sec) by caffeine (long)	26.0±5.22	10
$T_{63\%}$ (sec) by histamine (short)	6.6±0.64	8
$T_{63\%}$ (sec) by histamine (long)	7.9±1.21	21

In the case of short application, caffeine or histamine was perfused for three to four seconds. $T_{63\%}$ represent the time after start of decay where 37% of the peak difference was remained. Peak difference = peak $R_{340/380}$ - resting $R_{340/380}$.

RESULTS

Caffeine-, and histamine-induced Ca^{2+} transient

In most cells tested (110~120 cells), caffeine or histamine induced transient increase in $R_{340/380}$, which indicated an increase in intracellular $[Ca^{2+}]$. Fig. 1 shows typical responses to caffeine. 10 mM of caffeine was applied extracellularly for a short (3~4 s, filled bar) or sustained period. The signal ratio of fluorescence excited by the wavelength of 340 nm and 380 nm ($R_{340/380}$) increased abruptly and then diminished by washout or during the application of caffeine. For the sustained application, the decay rate of $R_{340/380}$ was slower than the decay rate observed after a short application of caffeine (Table 1). Under a sustained application of caffeine, $R_{340/380}$ did not return to the control resting level (tonic R340/380). Averaged values of the above results were summarized in Table 1. Frequently, upon washout after the sustained application (>1 min) of caffeine, $R_{340/380}$ declined to a level below initial control value (undershoot by caffeine-washout, Fig. 1B). The result of "undershoot by caffeine-washout" was not ana-

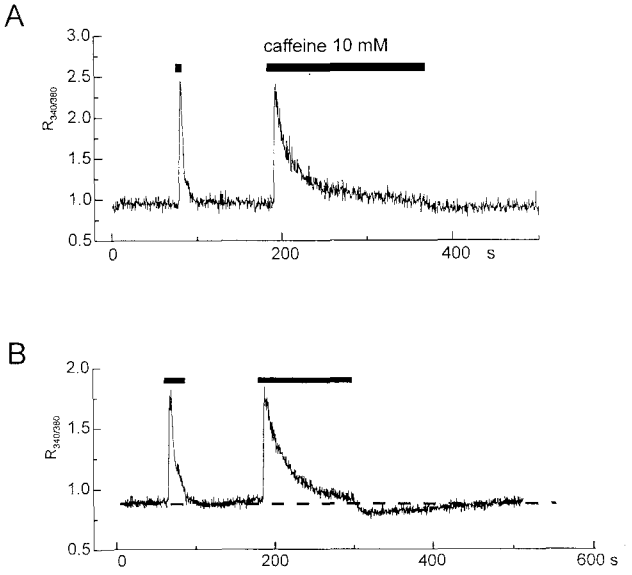

Fig. 1. Effects of caffeine on $[Ca^{2+}]_c$ in rabbit cerebral arterial myocytes. A & B, The change in fluorescence ratio ($R_{340/380}$) was drawn against the recording time. Caffeine (10 mM) was superfused during time indicated by filled bar. Compare the different decay rates of $R_{340/380}$ in the absence and presence of caffeine. In case of B, the myocyte showed a clear undershoot by caffeine-washout (see dotted line).

lysed since the extent of undershoot was irregular and was not prominent in many cases.

In Fig. 2, bath-applied histamine (5 μM) also induced a sharp rise in $R_{340/380}$ which decayed rapidly to a level higher than the resting concentration (tonic increase of $R_{340/380}$ by long exposure to histamine, see Table 1). Histamine-induced tonic increase of $R_{340/380}$ was variable in the amplitude or in the shape depending on individual myocytes (Fig. 2A & B). In 6 out of 25 cells tested for sustained application (>2 min) of histamine, oscillations of $R_{340/380}$ were superimposed upon the tonic increase of $R_{340/380}$ (Fig. 3A & Fig. 4B).

As shown in Fig. 3, effects of caffeine and histamine were compared in the same cell. Typical responses to caffeine and to histamine were sequentially induced in the same myocyte (40 out of 55 cells tested). In some myocytes, however, only caffeine-induced increase was observed (14 out of 55 cells).

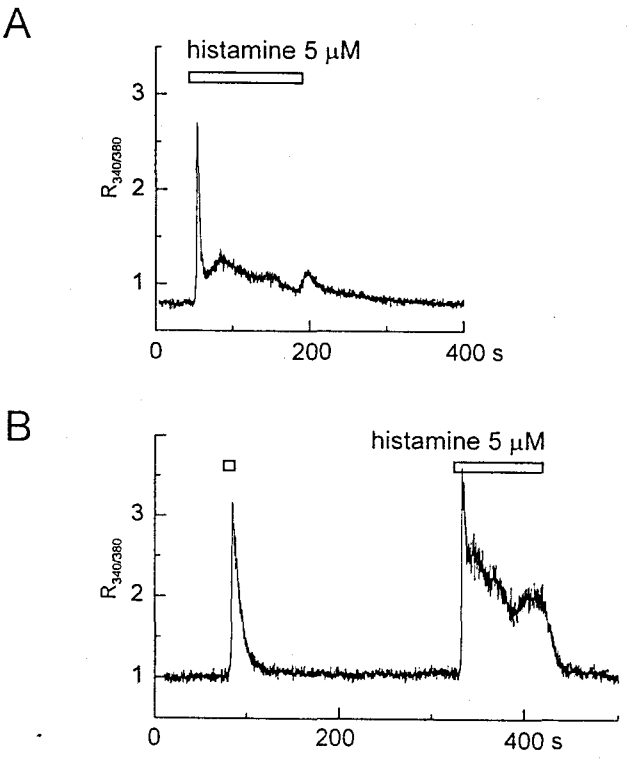

Fig. 2. Effects of histamine on $[Ca^{2+}]_c$ in cerebral arterial myocytes. Responses to 5 μM histamine are composed of fast transient increase and sustained tonic increase of $R_{340/380}$. Histamine was superfused during time indicated by open bar. The level of tonic increase was variable depending on the myocyte (compare the responses of A and B from different myocyte).

Only histamine-induced increase was observed in one myocyte. As mentioned above, a representative case by 5 μM histamine (Fig. 3A) showed oscillations superimposed upon tonic increase of $R_{340/380}$ (Fig. 3A).

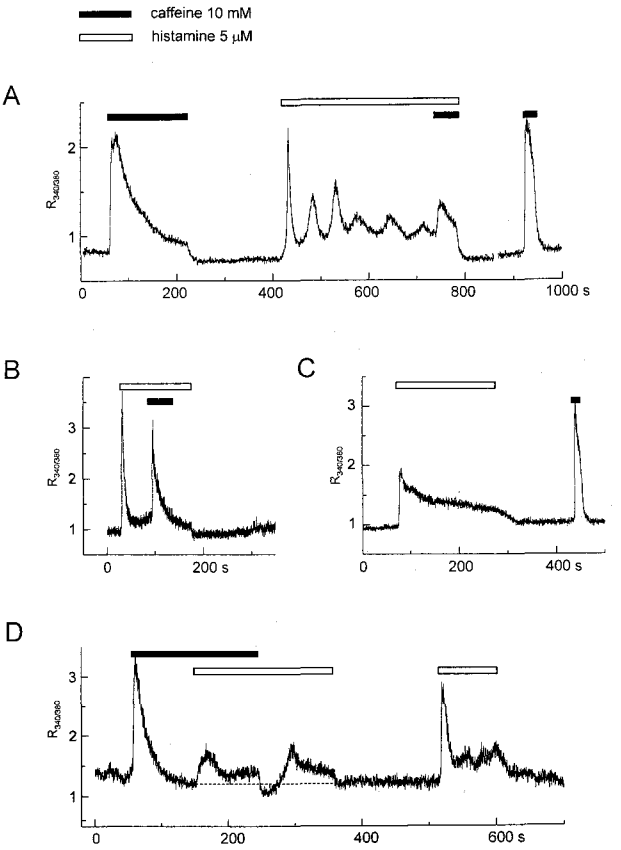

Fig. 3. Effects of caffeine and histamine on $[Ca^{2+}]_c$ compared in the same myocyte. Caffeine (10 mM) and/or histamine (5 μM) were applied separately or simultaneously to the same myocyte. Each panel represents the result from different myocyte with different pattern of response. In panel A, prolonged application of histamine induced $[Ca^{2+}]_c$ oscillations of decreasing amplitude. Peak amplitudes of the responses to histamine and caffeine were similar, while in the presence of histamine, additional application of caffeine could induce small additional increase. In panel B, however, additional application of caffeine could still induce large increase of $[Ca^{2+}]_c$. In panel C, relative amplitude of the response to caffeine was larger than the response to histamine. In panel D, the addition of histamine in the presence of caffeine induced small additional increase of $[Ca^{2+}]_c$ and in this state, withdrawal of caffeine only induced a prominent undershoot and subsequent rebound increase of $R_{340/380}$ (see dotted line). Usual response to histamine only was confirmed by second application. Small amplitudes of $[Ca^{2+}]_c$ oscillations are also observable in panel D.

The relative amplitudes of $R_{340/380}$ increase by caffeine or histamine were variable depending on individual myocytes (compare Fig. 3A and Fig. 3C).

In five cells, a test was performed to determine whether additional increase of $R_{340/380}$ could be induced by an application of caffeine when histamine is present. As shown in Fig. 3B, additional application of caffeine induced additional increase in $R_{340/380}$ which was far smaller than the maximal increase induced by caffeine alone. In two out of five cells tested, however, additional increase by caffeine was quite comparable to the effect of histamine. When the order of application was changed, an additional application of histamine further increased $R_{340/380}$ only in one out of four cells tested. The amplitude of additional increase by histamine was also much smaller than the response to histamine alone (Fig. 3D). In this myocyte under the presence of histamine, washout of caffeine induced a decrease of $R_{340/380}$ below the control level (undershoot by caffeine-washout, see dotted line in Fig. 3D).

Contribution of extracellular Ca^{2+}

The tonic increase of $R_{340/380}$ was largely dependent upon the presence of external Ca^{2+}. Both the caffeine- and histamine-induced tonic increases were abolished when the normal bath solution was changed to Ca^{2+}-free PSS containing 1 mM EGTA (Fig. 4A & B). In the absence of extracellular Ca^{2+}, repetitive application of caffeine or histamine did not induce a corresponding increase in $R_{340/380}$ (Fig. 4A & C). When returned to the normal bath solution, however, caffeine- or histamine-induced increase of $R_{340/380}$ was recovered slowly (Fig. 4A & C). Similar results were obtained in three other cells.

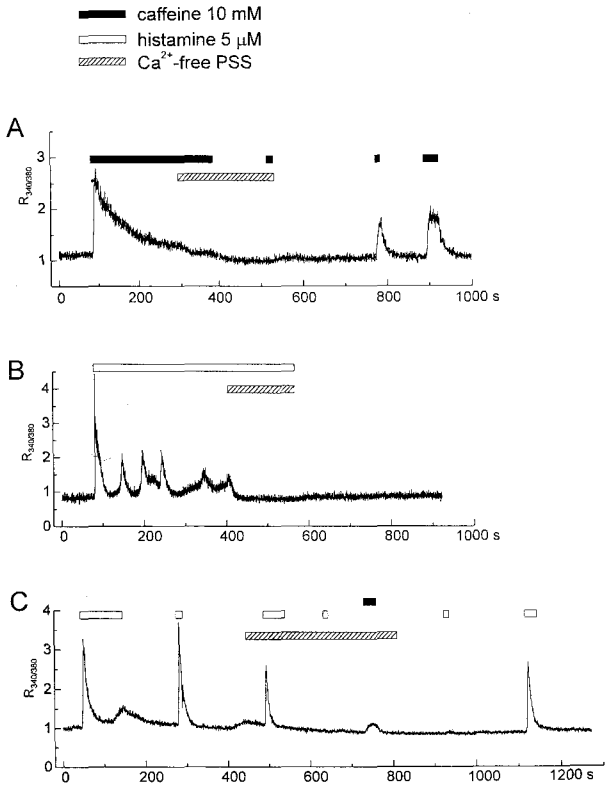

Fig. 4. Effects of caffeine and histamine in Ca^{2+}-free solution on $[Ca^{2+}]_c$. A, Ca^{2+}-free (EGTA 1 mM) PSS was perfused (hatched bar) during the prolonged stimulation with caffeine (filled bar). In the continued absence of Ca^{2+}, response to caffeine was abolished. B, Histamine-induced tonic increase and oscillations of $[Ca^{2+}]_c$ were abolished by eliminating extracellular Ca^{2+}. C, Only a single transient increase of $[Ca^{2+}]_c$ was induced in the absence of extracellular Ca^{2+} and in this condition, 10 mM caffeine could induce a small additional increase of $[Ca^{2+}]_c$.

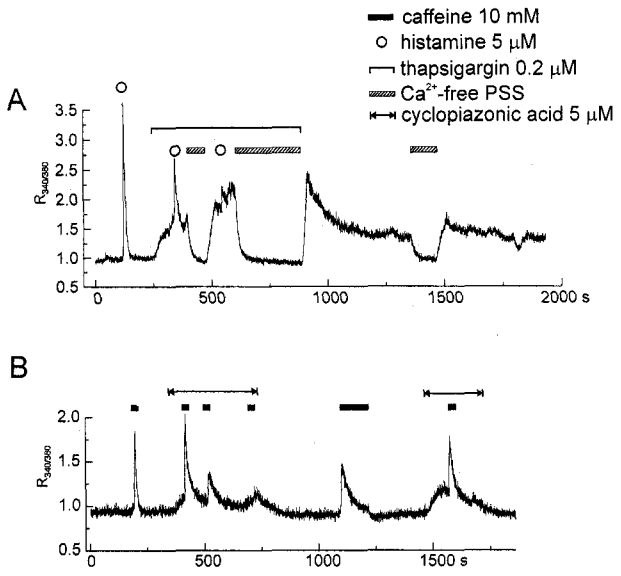

Fig. 5. Effects of the SERCA inhibitor on caffeine- or histamine-induced increase of $[Ca^{2+}]_c$. A, Application of 0.2 μM thapsigargin slowly increased $R_{340/380}$ and suppressed the response to histamine. Thapsigargin-induced tonic increase of $[Ca^{2+}]_c$ was sharply decreased by removal of extracellular Ca^{2+}. B, Response to 10 mM caffeine was gradually suppressed by prolonged application of cyclopiazonic acid (CPA) in a reversible manner. Note the slowed decay rate of $R_{340/380}$ in the presence of CPA.

Effects of SERCA inhibitors

Both caffeine- and histamine-induced Ca^{2+} transients were dependent upon the refilling function of SR. It has been reported that the Ca^{2+} ATPase in SR membrane (SERCA) is pharmacologically inhibited by cyclopiazonic acid (CPA) or by thapsigargin. In Fig 5A, after the effect of histamine was observed, 0.2 μM thapsigargin was applied, which induced a slow increase in $R_{340/380}$.

As has been reported elsewhere, thapsigargin-induced $R_{340/380}$ increase was also sensitively abolished when external Ca^{2+} was reversed (Iwasawa et al, 1997). Increased $R_{340/380}$ was reversed to the partial extent by washout of thapsigargin. Histamine-induced transient increase of $R_{340/380}$ stopped when the full effect of thapsigargin had been attained (third open circle in Fig. 5A). A similar relationship was observed between CPA and caffeine (Fig. 5B). Unlike the irreversible effect of thapsigargin, the tonic increase of $R_{340/380}$ by CPA was reversed by washout. Also, the inhibitory effect of CPA on caffeine-induced $R_{340/380}$ transient was reversible (Fig. 5B). Similar results were observed in three other cells.

Effects of RyR inhibitor

Caffeine is known to activate the Ca^{2+}-induced Ca^{2+} release channel (ryanodine receptor, RyR) by lowering the threshold of RyR to $[Ca^{2+}]_c$ (Endo, 1985). Ryanodine, a plant alkaloid, binds with open-state RyRs and irreversibly locks them in subconductance open state (Meissner, 1986). On the other hand, tetracaine, a local anesthetic drug, directly inhibits RyRs in a reversible manner (Pacaud & Loirand, 1995; Guibert et al, 1996). Fig. 6A demonstrates that pretreatment with 0.3 mM tetracaine completely suppressed the transient increase of $R_{340/380}$ induced by caffeine. In contrast, the pretreatment with ryanodine could not suppress the initial activation of $R_{340/380}$ transient by caffeine. The second activation of $R_{340/380}$ transient, however, was suppressed completely and irreversibly even after the washout of ryanodine (Fig. 6B). Identical results were obtained in five other cells. In this state, separate application of histamine did (Fig. 6B) or did not (Fig. 6C) induce $R_{340/380}$ increase of small amplitude.

These results (Fig. 6B & C) indicate that pre-applied ryanodine is readily bound to RyRs activated by caffeine and finally depletes SR which is sensitive

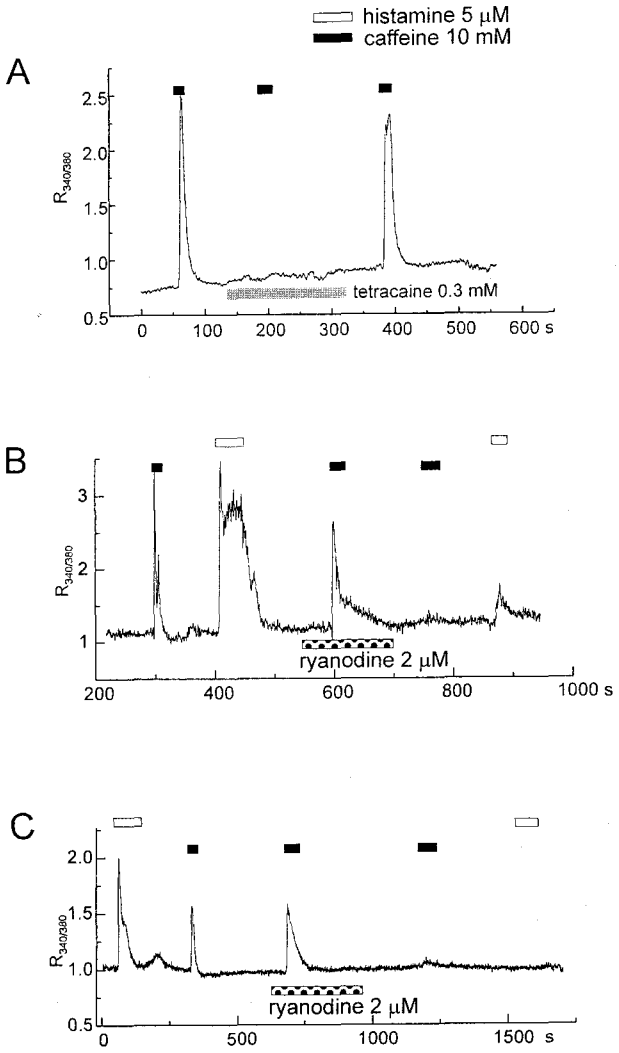

Fig. 6. Effects of tetracaine and ryanodine on caffeine-induced increase of $[Ca^{2+}]_c$. A, The pretreatment with 0.3 mM tetracaine (gray bar) completely suppressed the response to caffeine. B & C, responses to caffeine were evoked repetitively in the absence, presence and washout of ryanodine (2 μM, dotted bar). Caffeine-induced increase of $[Ca^{2+}]_c$ was irreversibly abolished by ryanodine. The response to histamine was partly remained (panel B) or not (panel C).

to histamine as well as caffeine. Based on this property of ryanodine, a test was conducted on whether the CICR via RyRs contributed to the histamine-induced increase of $[Ca^{2+}]$; i.e. whether histamine-induced transient increases of $R_{340/380}$ are easily affected by the treatment with ryanodine. Fig. 7 shows a representative result. Histamine-induced $R_{340/380}$ transients were not as easily abolished by the pretreatment with ryanodine as the caffeine-induced response. In this myocyte, five times of transient increases with

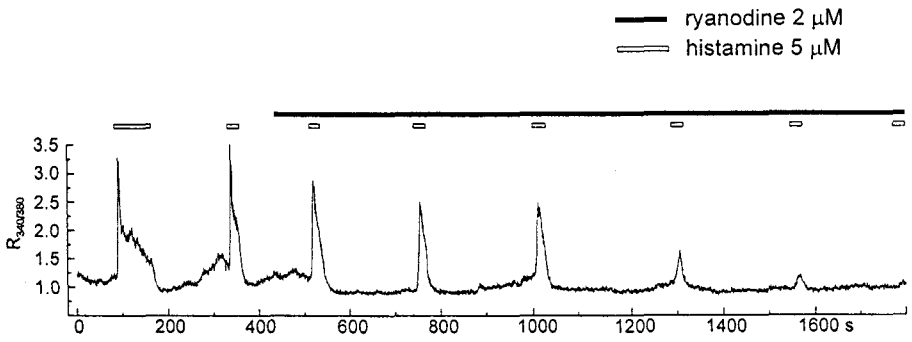

Fig. 7. Effects of ryanodine on histamine-induced increase of $[Ca^{2+}]_c$. Histamine (5 μM) was repetitively applied (open bar) in the continued presence of 2 μM ryanodine (filled bar).

decreasing amplitudes could be repetitively induced by histamine and finally abolished. Similar results were observed in three other cells.

DISCUSSION

Relationship between two Ca^{2+} pools

The results of this study showed that both caffeine and histamine induce transient rise in $[Ca^{2+}]_c$, which indirectly indicates the presence of both CICR and IICR pools in the smooth muscle of rabbit cerebral artery. In various smooth muscles, agonists can induce contraction independent of membrane depolarization, which has been termed pharmaco-mechanical coupling (Somlyo & Somlyo, 1994). Those agonists (noradrenaline, acetylcholine, histamine etc.) commonly induce rapid formation of $InsP_3$ which has been shown to provoke Ca^{2+} release by acting on its own receptors in SR membrane (Karaki et al, 1997). The role of CICR in smooth muscle is, however, not so clear. In cardiac myocyte, membrane depolarization induces Ca^{2+} influx via L-type Ca^{2+} channels and an elevation of the cytoplasmic Ca^{2+} concentration can provoke the opening RyRs (CICR), which is important for the excitation-contraction coupling (Berridge, 1997). In smooth muscle cells, however, the recruitment of CICR during membrane depolarization has not been clearly proven by experiments adopting whole-cell patch clamp technique and microspectrofluorimetry (Grgoire, 1993; Kamishima & McCarron, 1996; Kim et al, 1997). Such controversial reports may be due to a low sensitivity of smooth muscle RyRs to Ca^{2+} (Iino, 1989) or to an experimental limitation (e.g. Ca^{2+} indicator dye itself buffers effective increase of free Ca^{2+}). As for another physiological role of RyRs in smooth muscle, it could be speculated that the increase in $[Ca^{2+}]_c$ by IICR could activate CICR mechanism in the same myocyte. CICR may contribute to the total increase of $[Ca^{2+}]_c$ when stimulated by noradrenaline or histamine. This was examined indirectly from the effect of ryanodine pretreatment on histamine-induced $[Ca^{2+}]_c$ transient (Fig. 7). The data obtained from this examination suggest that a small population of RyRs is activated by histamine-induced $[Ca^{2+}]_c$ transient and locked at sub-conductance state. The partial openings of RyRs slowly deplete the SR and finally suppress Ca^{2+} release by histamine. This data will be discussed later.

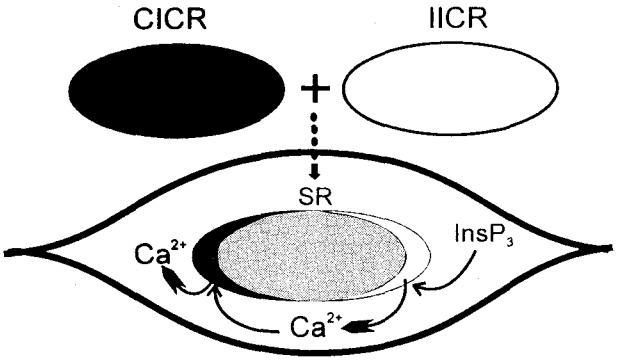

Fig. 8. Schematic illustration of suggestive relationship between two Ca^{2+}-pools in rabbit cerebral arterial myocyte. Filled area, hollow area, gray area represent SRs containing ryanodine receptors, $InsP_3$ receptors and both, respectively. CICR: Ca^{2+}-induce Ca^{2+} release, IICR: $InsP_3$-induced Ca^{2+} release, SR: sarcoplasmic reticulum

The data also indicate that above two Ca^{2+} pools

are functionally not separable; i.e. histamine and caffeine release Ca^{2+} from the same store (Fig. 8). Prior treatment with one agonist for Ca^{2+} release markedly inhibited the effect of the other agonist and vice versa (Fig. 3). The degree of overlap, however, seems to be different from cell to cell as there were such cells that showed a persistent increase in $R_{340/380}$ by histamine even in the presence of caffeine (Fig. 3). The histamine-induced oscillation or transient increase of intracellular Ca^{2+} is known to involve the production of $InsP_3$ and the mobilization of Ca^{2+} from an $InsP_3$- sensitive store (Bielkiewics-Vollrath et al, 1987). However, caffeine is known to have many nonspecific effects other than RyRs activation, e.g. inhibition of $InsP_3$ formation (Prestwich & Bolton, 1995), direct inhibition or $InsP_3$ receptor function (Sanchez-Bueno et al, 1994). Therefore, the interpretation of the inhibitory effect of caffeine on the response to another agonist like histamine has to be made with caution. To avoid any confusion, the "functional removal" of CICR pool by using ryanodine pretreatment or by eliminating external Ca^{2+} during agonist stimulation is often used (Iino, 1990). Experiments similar to this study still indicated that most of the releasable pool is depleted by functional removal of either CICR pool or IICR pool (Fig. 4 & 6).

These results suggest that both $InsP_3$ receptor and RyR may be expressed anatomically in the same SR membrane. In that case, it is probable that the Ca^{2+} released via $InsP_3$ receptor channel activates the RyR nearby and induces CICR. In other kinds of cells, the propagating activation of Ca^{2+} release channels resulted in a characteristic Ca^{2+}-wave or Ca^{2+}-oscillation (Wakui et al, 1990). In this arterial myocyte, however, such a positive-feedback mechanism does not seem to operate because the self-propagating activation of RyRs would have limited the response to histamine once or twice in the presence of ryanodine, which is not the case here(Fig. 7). In contrast, the response to caffeine was readily blocked by ryanodine (Fig. 6). Therefore, only a small population of RyR was activated by the IICR-induced $[Ca^{2+}]_c$ increase even though those two receptors are co-expressed in the same SR. Direct investigation on the spatial distribution of each Ca^{2+} release channel is necessary to prove the above speculation.

Regulation of refilling Ca^{2+} stores

The dependence of sustained $[Ca^{2+}]_c$ increase on the presence of extracellular Ca^{2+} suggests that there is a continuous exchange of Ca^{2+} between extracellular space and SR. Part of the released Ca^{2+} would have been removed to the extracellular space as well as having been sequestered into SR. This is quite possible because the repetitive application of caffeine without sufficient resting interval can not induce the same amplitude of maximal increase in $R_{340/380}$ (data not shown here). To refill the depleted intracellular stores, Ca^{2+} has to be provided partially from the extracellular space. Refilling could be prevented by SERCA inhibitors, CPA and thapsigargin, which implies that Ca^{2+} enters into the cytoplasmic space before accumulating in the SR.

$R_{340/380}$ decay in the presence of 10 mM caffeine was always considerably slower than the one observed after a short application of caffeine (Fig. 1 & Table 1). The fact that $[Ca^{2+}]_c$ recovers more quickly following a brief exposure to caffeine has been noted by previous experiments (Ganitkevich & Isenberg, 1992; Baro & Eisner, 1995). They have suggested that, when caffeine is removed, the caffeine-sensitive store can re-accumulate Ca^{2+} as well as other removal mechanisms. However, in the continued presence of caffeine, calcium must either be pumped out of the cell or be sequestered into caffeine-insenstive stores. Consequently, the difference in the decay rate between short and long application of caffeine may reflect the kinetics of Ca^{2+}-removal mechanisms of caffeine- sensitive SR. Unlike the effect of caffeine, either a short- or long-application of histamine induced fastly decaying transient increase of $[Ca^{2+}]_c$ (Fig. 2 & Table 1). The fast decay of $R_{340/380}$ may reflect transient formation and release of $InsP_3$ during histamine application. If that is the case, even in the presence of histamine, released Ca^{2+} could be sequestered into histamine-sensitive SR by SERCA as well as be removed across the cell membrane.

In the rat mesenteric arterial myocyte, the decay rates were compared to explain dissect the contribution of Ca^{2+} removal mechanisms and in that experiment, caffeine-sensitive stores had contributed about 80% of the maximal rate of removal (Baro & Eisner, 1995). In the present study, however, we did not carry out further dissection of the contribution from each removal mechanism because it was hard to selectively block the respective Ca^{2+} removal mechanisms. In fact, while the treatment with CPA seemed to slow the decay rate of $R_{340/380}$ induced by a short application of caffeine (Fig. 5B), the degree

of deceleration was lower than the one induced by a long application of caffeine (Fig. 5). A persistent application of caffeine might have directly inhibited the Ca^{2+} removal mechanism(s) in smooth muscle cell. Otherwise, CPA might have not blocked the SERCA completely in this myocyte.

Tonic increase of $[Ca^{2+}]c$ under agonist stimulation

In virtually all systems examined in other reports, the initial response to stimulation of receptors that stimulate hydrolysis of phosphatidylinositol 4,5-bisphosphate resulted in two phases: (i) a phasic $[Ca^{2+}]_c$ increase due to the release from intracellular store and (ii) a tonic increase of $[Ca^{2+}]_c$ mostly due to extracellular Ca^{2+} entry (Karaki et al, 1997). There have been many controversies about the mechanisms responsible for the sustained Ca^{2+} entry phase. Two major candidates are 1) receptor-operated nonselective cation channels (ROC) which have Ca^{2+} permeability and 2) store-depletion operated channels (SOC) which have high Ca^{2+} permeability (Berridge, 1997; Karaki et al, 1997). In non-excitable cells, Ca^{2+} release activated Ca^{2+} current (I_{CRAC}) has been reported already (Hoth & Penner, 1992). In the present experiment, tonic $[Ca^{2+}]_c$ increase was observed both by histamine and by caffeine (Table 1). Since the histamine-induced tonic increase was partially inhibited by nicardipine (data not shown here), part of the influx was due to the voltage-operated L-type Ca^{2+} channel. The nature of remaining influx has not been determined yet. Tonic increase of $[Ca^{2+}]_c$ by SERCA inhibitors has been generally considered as a sign of the presence of SOC or CRAC channels (Karaki et al, 1997). In this arterial myocyte, thapsigargin or CPA could also induce tonic increase of $R_{340/380}$ while the extent of increase was different from cell to cell. Further investigation has to be made about the responsible mechanisms.

In conclusion, this study found two different Ca^{2+}-release mechanisms, presumably IICR and CICR, in rabbit cerebral arterial smooth muscle. Both mechanisms seem to be expressed in almost the same Ca^{2+} pool, at least functionally, and the increase of $[Ca^{2+}]_c$ by the activation of IICR seems to concomitantly activate a small population of RyRs.

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REFERENCES

Bar I, Eisner DA. Factors controlling changes in intracellular Ca^{2+} concentration produced by noradrenaline in rat mesenteric artery smooth muscle cells. *J Physiol (Lond)* 482: 247−258, 1995

Berridge MJ. Elementary and global aspects of calcium signalling. *J Physiol (Lond)* 499: 291−306, 1997

Bielkiewicz-Vollrath B, Carpenter J_R, Schultz R, Cook DA. Early production of 1,4,5-inositol trisphosphate and 1,3,4,5-inositol tetrakisphosphate by histamine and carbachol in ileal smooth muscle. *Mole Pharmacol* 31: 513−522, 1987

Endo M. Calcium release from sarcoplasmic reticulum. *Physiol Rev* 57: 71−108, 1985

Ganitkevich VYa, Isenberg G. Caffeine-induced release and reuptake of Ca^{2+} by Ca^{2+} stores in myocytes from guinea-pig urinary bladder. *J Physiol (Lond)* 458: 99−117, 1992

Grgoire G, Loirand G, Pacaud P. Ca^{2+} and Sr^{2+} entry induced Ca^{2+} release from the intracellular Ca^{2+} store in smooth muscle cells of rat portal vein. *J Physiol (Lond)* 472: 483−500, 1993

Guibert C, Pacaud P, Loirand G, Marthan R, Savineau J-P. Effects of extracellular ATP on cytosolic Ca^{2+} concentration in rat pulmonary artery myocyte. *Am J Physiol* 271: L450−L458, 1996

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

Iino M. Calcium-induced calcium release mechanism in guinea-pig taenia caeci. *J Gen Physiol* 94: 363−383, 1989

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

Iino M, Kobayashi T, Endo M. Use of ryanodine for functional removal of the calcium store in smooth muscle cells of the guinea-pig. *Biochem Biophys Res Commun* 152: 417−422, 1988

Iwasawa K, Nakajima T, Hazama H, Goto A, Shin WS, Toyo-oka T, Omata M. Effects of extracellular pH on receptor-mediated Ca^{2+} influx in A7r5 rat smooth muscle cells: involvement of two different types of channel. *J Physiol (Lond)* 503: 237−251, 1997

Kamishima T, McCarron JG. Depolarization-evoked increases in cytosolic calcium concentration in isolated smooth muscle cells of rat portal vein. *J Physiol (Lond)* 492: 61−74, 1996

Kang TM, So I, Kim KW. Caffeine- and hitamine-induced oscillations of $K_{(Ca)}$ current in single smooth muscle cells of rabbit cerebral artery. *Pflügers Arch* 431: 91−100, 1995

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

Kim SJ, Ahn SC, Kim JK, Kim YC, So I, Kim KW. Changes in intracellular Ca^{2+} concentration induced by L-type Ca^{2+} channel current in guinea pig gastric myocytes. *Am J Physiol* 273: C1947−C1956, 1997

Matsumoto T, Kanaide H, Shoagkiuchi Y, Nakamura M. Characteristics of the histamine-sensitive calcium store in vascular smooth muscle. *J Biol Chem* 265: 5610−5616, 1990

Meissner G. Ryanodine activation and inhibition of the Ca^{2+} release channel of sarcoplasmic reticulum. *J Biol Chem* 261: 6300−6306, 1986

Nagasaki K, Kasai M. Channel selectivity and gating specificity of calcium-induced calcium release channel in isolated sarcoplasmic reticulum. *J Biochem* 96: 1769−1775, 1984

Pacaud P, Loirand G. Release of Ca^{2+} by noradrenaline and ATP from the same Ca^{2+} store sensitive to both InsP3 and Ca^{2+} in rat portal vein myocytes. *J Physiol (Lond)* 484: 549−555, 1995

Sanchez-Bueno A, Marrero I, Cobbold PH. Caffeine inhibits agonists-induced cytoplasmic Ca^{2+} oscillations in single rat hepatocytes. *Biochem Biophys Res Comm* 198(2): 728−733, 1994

Somlyo AP, Somlyo AV. Signal transduction and regulation in smooth muscle. *Nature* 372: 231−236, 1994

Wakui M, Osipshuk YV, Petersen OH. Receptor-activated cytoplasmic Ca^{2+} spiking mediated by inositol trisphosphate is due to Ca^{2+}-induced Ca^{2+} release. *Cell* 63: 1025−1032, 1990