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# $Ca^{2+}$ movement from leaky sarcoplasmic reticulum during contraction of rat arterial smooth muscles

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**Abstract**

To examine the $Ca^{2+}$ buffering function of the sarcoplasmic reticulum during arterial contraction, we studied $Ca^{2+}$ movement during stimulation with $K^+$ or norepinephrine in arteries with a leaky sarcoplasmic reticulum. Responses were compared in endothelium-denuded strips of femoral, mesenteric and carotid arteries of the rat. To make the sarcoplasmic reticulum leaky to $Ca^{2+}$, $Ca^{2+}$-induced $Ca^{2+}$ release channels of the sarcoplasmic reticulum were locked open by treatment with ryanodine plus caffeine. After ryanodine treatment, the contractile responses to $K^+$ (3–20 mM) were augmented when compared with control responses in femoral and mesenteric arteries, but were inhibited in the carotid artery. Similar results were obtained when the contractile responses to norepinephrine were determined. The inhibition by ryanodine of the $K^+$- or norepinephrine-contractions seen in the carotid artery was reversed by pretreatment with cyclopiazonic acid (10 μM), an inhibitor of the sarcoplasmic reticulum $Ca^{2+}$-ATPase, but was not by charybdotoxin (100 nM), a blocker of $Ca^{2+}$-activated $K^+$ channels. We conclude that (1) after ryanodine treatment, $Ca^{2+}$ entering from the extracellular space during stimulation with $K^+$ or norepinephrine is first taken up into the leaky sarcoplasmic reticulum and then reaches the myofilaments in femoral and mesenteric arteries, while in the carotid artery, $Ca^{2+}$ leaked from the sarcoplasmic reticulum reaches mainly the plasma membrane from where it is extruded into the extracellular space, and (2) the different movement of $Ca^{2+}$ may be due to the relative location of the sarcoplasmic reticulum in the smooth muscle cell of each artery. 

*Keywords:* Sarcoplasmic reticulum; Superficial buffer barrier hypothesis; $Ca^{2+}$-induced $Ca^{2+}$ release channels; $Ca^{2+}$ movement; $Ca^{2+}$ influx; $Ca^{2+}$ channel, L-type

## 1. Introduction

The cytosolic level of $Ca^{2+}$ ($[Ca^{2+}]_i$) in arterial smooth muscle is determined by an equilibrium among various processes of $Ca^{2+}$ mobilization, such as $Ca^{2+}$ influx across the plasma membrane, $Ca^{2+}$ release from the sarcoplasmic reticulum, $Ca^{2+}$ extrusion across the plasma membrane and $Ca^{2+}$ uptake into the sarcoplasmic reticulum (Van Breemen and Saida, 1989; Chen et al., 1992; Van Breemen et al., 1995). Many studies show that the addition of cyclopiazonic acid or thapsigargin, selective inhibitors of the sarcoplasmic reticulum $Ca^{2+}$-ATPase, to arterial strips causes an elevation of $[Ca^{2+}]_i$ and contraction, suggesting that there is $Ca^{2+}$ influx in the resting state of the arterial smooth muscle and that this influx is buffered by $Ca^{2+}$ uptake into the sarcoplasmic reticulum by sarcoplasmic reticulum $Ca^{2+}$-ATPase (for details, see Nomura et al., 1996, 1997). To maintain this $Ca^{2+}$ buffering function and hence a low $[Ca^{2+}]_i$, it is necessary to extrude sarcoplasmic reticulum $Ca^{2+}$ into the extracellular space. According to the 'superficial buffer barrier' hypothesis proposed by Van Breemen and his colleagues (Van Breemen and Saida, 1989; Chen et al., 1992; Van Breemen et al., 1995; Laporte and Laher, 1997), $Ca^{2+}$ taken up into the sarcoplasmic reticulum is continually extruded from the sarcoplasmic reticulum into the extracellular space. This process is composed of two stages: vectorial release via $Ca^{2+}$-induced $Ca^{2+}$ release channels of the sarcoplasmic reticulum toward the inner surface of the plasma membrane, followed by extrusion via the $Na^+$–$Ca^{2+}$ exchanger and the $Ca^{2+}$-ATPase of the plasma membrane. Ryanodine is a useful tool to evaluate the role of $Ca^{2+}$-induced $Ca^{2+}$ release channels, because this alkaloid locks the channels in an open state (Fleischer et al., 1985; Rousseau et al., 1987; Iino et al., 1988), which results in the establishment of a leaky sarcoplasmic reticulum.

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We have already demonstrated that ryanodine treatment of femoral arterial strips from spontaneously hypertensive rats (SHR) causes a large elevation of $[Ca^{2+}]_i$ and a large contraction (Asano et al., 1996a). In spite of the finding that these responses were inhibited by blockers of L-type $Ca^{2+}$ channels, basal $Ca^{2+}$ influx and net $Ca^{2+}$ entry after ryanodine treatment were not increased, but rather decreased, in this artery. Ryanodine treatment of femoral arterial strips from normotensive Wistar–Kyoto rats (WKY) caused a moderate elevation of $[Ca^{2+}]_i$ but only a very small contraction, and basal $Ca^{2+}$ influx and net $Ca^{2+}$ entry after ryanodine treatment were decreased in this artery (Asano et al., 1996a). These observations suggest that under the open-locked state of $Ca^{2+}$-induced $Ca^{2+}$ release channels, $Ca^{2+}$ entering cells in the resting state of arterial smooth muscle is taken up into a leaky sarcoplasmic reticulum, and that most of the $Ca^{2+}$ that leaks from the sarcoplasmic reticulum reaches the myofilaments, resulting in a large contraction in the SHR artery, and that some $Ca^{2+}$ is likely to be extruded from the cells in this artery. It also suggests that the opposite movement of $Ca^{2+}$ can occur in the WKY artery. Similar results were obtained when the effects of ryanodine were compared in cerebral and peripheral arteries of the dog (Asano et al., 1996b).

Thus, we hypothesized that, after ryanodine treatment, the response of arterial smooth muscle to $Ca^{2+}$ leaking from the sarcoplasmic reticulum is determined by the net balance of contraction, due to the arrival of $Ca^{2+}$ at the myofilaments, and extrusion of $Ca^{2+}$, due to the arrival of $Ca^{2+}$ at the plasma membrane. To further evaluate this hypothesis, the present study was designed to compare $Ca^{2+}$ movement during procedures that increase $Ca^{2+}$ influx (e.g., contraction with elevated extracellular $K^+$ or norepinephrine) under open-lock of the $Ca^{2+}$-induced $Ca^{2+}$ release channels in femoral, mesenteric and carotid arteries of the rat. In this study, we measured mechanical activity, $[Ca^{2+}]_i$ (by using a fluorescent $Ca^{2+}$ indicator fura-PE3), basal $Ca^{2+}$ influx, and net $Ca^{2+}$ entry, and found that $Ca^{2+}$ movement differs in the carotid artery when compared with that in the other arteries studied. A preliminary account of these findings was presented to the 72nd Annual Meeting of the Japanese Pharmacological Society (Asano and Nomura, 1999).

## 2. Materials and methods

### 2.1. Measurement of isometric tension

The preparation of arterial smooth muscle strips and the measurement of isometric tension were done according to methods described in the accompanying paper (Nomura and Asano, 2000). Briefly, endothelium-denuded strips ($0.7–0.8 \times 7–8$ mm) of femoral, mesenteric and carotid arteries of the rat were used. After determination of the maximum contraction induced by 65.9 mM $K^+$ Krebs solution (equimolar substitution of $Na^+$ with $K^+$), the contractile responses to elevated extracellular $K^+$ (3–20 mM addition) or norepinephrine ($10^{-9}$–$10^{-4}$ M) were determined in a cumulative fashion. The sensitivity to $K^+$ or norepinephrine is expressed as $EC_{30}$ (the concentration inducing 30% of the maximum contraction) or $pD_2$ (negative log of the molar concentration inducing 50% of the maximum contraction), respectively. To make the sarcoplasmic reticulum leaky, $Ca^{2+}$-induced $Ca^{2+}$ release channels of the sarcoplasmic reticulum were locked open by treatment of the strips with ryanodine (10 μM) plus caffeine (20 mM), as reported previously (Asano et al., 1996a,b). Since the contraction induced by 20 mM caffeine is a rough index of the amount of $Ca^{2+}$ stored in the sarcoplasmic reticulum, the caffeine-induced contraction was determined. After ryanodine treatment, the $K^+$- and norepinephrine-induced contractions were also determined. In some experiments, the norepinephrine-induced contraction was divided into two components, $Ca^{2+}$ release and

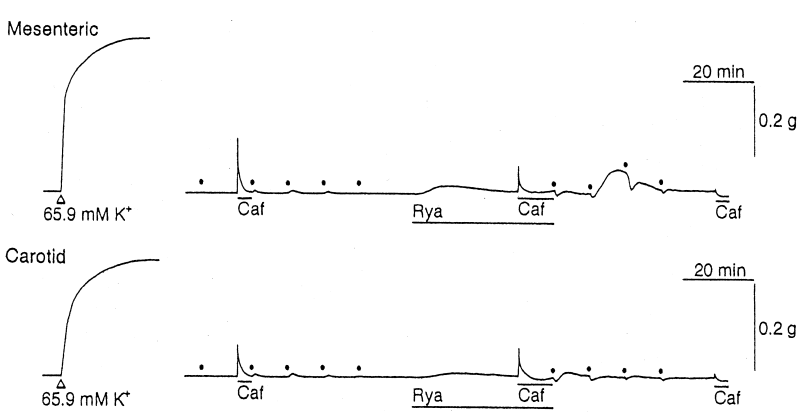


Fig. 1. Typical recordings of ryanodine (Rya; 10 μM) treatment to make a leaky sarcoplasmic reticulum in strips of mesenteric and carotid arteries of the rat. After determination of the maximum contraction induced by 65.9 mM KCl ($K^+$), caffeine (Caf; 20 mM) was added for 4 min. Following washout for 45 min, Rya was added for 30 min. In the presence of Rya, Caf was added for 10 min. After washout for 45 min, Caf was added. Dots (·) denote the washout of the strips with Krebs solution.

$Ca^{2+}$ influx, and the effects of ryanodine treatment on these components were also determined. In these experiments, a nominally $Ca^{2+}$-free solution was used. This solution was prepared by omitting $Ca^{2+}$ from the Krebs solution and by adding 0.1 mM EGTA. In some experiments, a low $Na^+$ (choline-substituted) solution was used. The low $Na^+$ solution was prepared by replacing 115 mM NaCl in Krebs solution with an equimolar amount of choline chloride, so that the $Na^+$ concentration in this solution was reduced from 140 to 25 mM.

### 2.2. Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured as described in the accompanying paper (Nomura and Asano, 2000). Changes in $[Ca^{2+}]_i$ during stimulation with elevated extracellular $K^+$ (10.9, 15.9, and 25.9 mM; equimolar substitution of $Na^+$ with $K^+$) and the effects of ryanodine treatment on this response were determined. For a relative comparison of changes in $[Ca^{2+}]_i$, the ratio of F340 to F380 (F340/F380) obtained in the resting state and in the presence of 65.9 mM $K^+$ (5 min after the addition) was taken as 0 and 100%, respectively.

### 2.3. Measurement of basal $Ca^{2+}$ influx and net $Ca^{2+}$ entry

Basal $^{45}Ca$ influx and net $^{45}Ca$ entry in the resting state were measured by using a cold $La^{3+}$ wash procedure as described previously (Asano et al., 1993, 1996a). Briefly, isolated carotid arteries were opened longitudinally and equilibrated in a Tris-buffered solution of the following composition (in mM): NaCl 154.0, KCl 5.4, $CaCl_2$ 2.5, dextrose 11.0 and Tris 6.0 (pH 7.4). Tris-buffered solutions were maintained at 37°C and aerated with 100% $O_2$. Arteries were then placed in the Tris-buffered solution to which 1 μCi/ml $^{45}Ca$ had been added. After incubation for 5 min (for basal $Ca^{2+}$ influx) or 30 min (for net $Ca^{2+}$ entry), arteries were placed in a series of four test tubes containing 80.8 mM $La^{3+}$-substituted solution (0.5°C) for successive periods of 30 s, 4.5, 15, and 25 min (a total of 45 min) to remove extracellular $^{45}Ca$. Arteries were then transferred to a glass scintillation vial containing 0.1 ml Amersham NCS tissue solubilizer (Amersham International, Buckinghamshire, UK). Solubilized tissues were

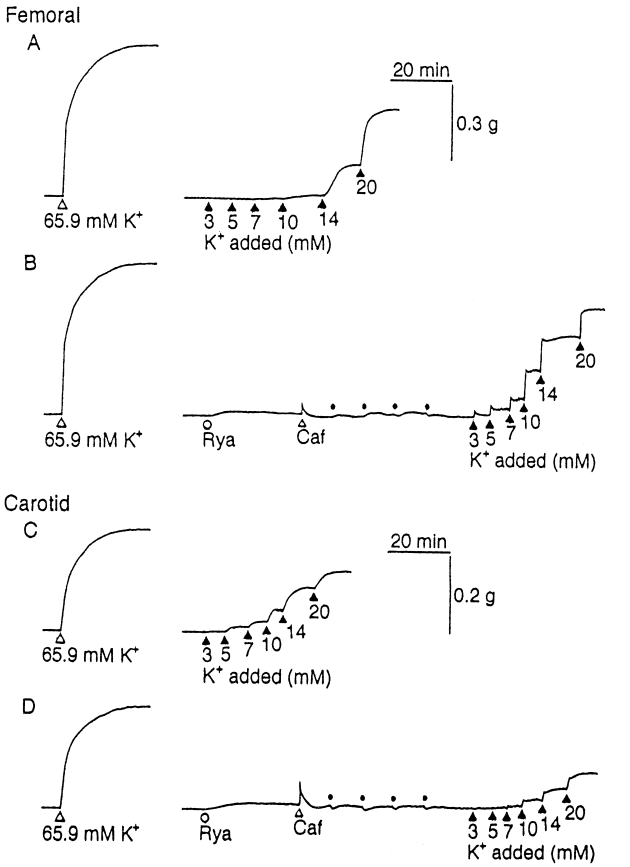


Fig. 2. Typical recordings of the effects of ryanodine (Rya; 10 μM) treatment on the contractile responses to elevated extracellular $K^+$ in strips of femoral (A, B) and carotid (C, D) arteries of the rat. (A, C) Control responses. After determination of the maximum contraction induced by 65.9 mM KCl ($K^+$), the contractile responses to $K^+$ (3–20 mM addition) were determined in a cumulative fashion. (B, D) Effects of Rya treatment on the $K^+$-induced contractions. The Rya treatment to make a leaky sarcoplasmic reticulum was done as described in the legend of Fig. 1.

Table 1
Effects of ryanodine (10 μM) on basal $Ca^{2+}$ influx and net $Ca^{2+}$ entry in rat carotid artery[a]

| $^{45}Ca$ incubation condition[a] | $Ca^{2+}$ taken up by the tissue (nmol/g wet tissue)[a] | |
|---|---|---|
| | Control (−Ryanodine) | +Ryanodine |
| (A) 5.4 mM $K^+$ for 5 min | 68.8 ± 1.1 (14) | 62.0 ± 2.0[b] (14) |
| (B) 5.4 mM $K^+$ for 30 min | 133.4 ± 4.1 (10) | 108.2 ± 7.6[b] (10) |
| (C) 5.4 mM $K^+$ for 5 min after washout | 65.3 ± 2.2 (8) | 54.5 ± 2.0[b] (8) |

Data are expressed as means ± S.E.M. (numbers in parentheses indicate the number of measurements).

[a]Experimental conditions corresponded to those given in the legend of Fig. 1. Arteries were incubated for 5 min (basal $Ca^{2+}$ influx) or 30 min (net $Ca^{2+}$ entry) in the Tris-buffered solution (5.4 mM $K^+$) to which $^{45}Ca$ had been added. (A) Effects of 10 μM ryanodine on basal $Ca^{2+}$ influx. (B) Effects of 10 μM ryanodine on net $Ca^{2+}$ entry. (C) The basal $Ca^{2+}$ influx after treatment with 10 μM ryanodine plus 20 mM caffeine and a washout for 40 min.

[b]Significantly different from the respective Control ($P < 0.05$).

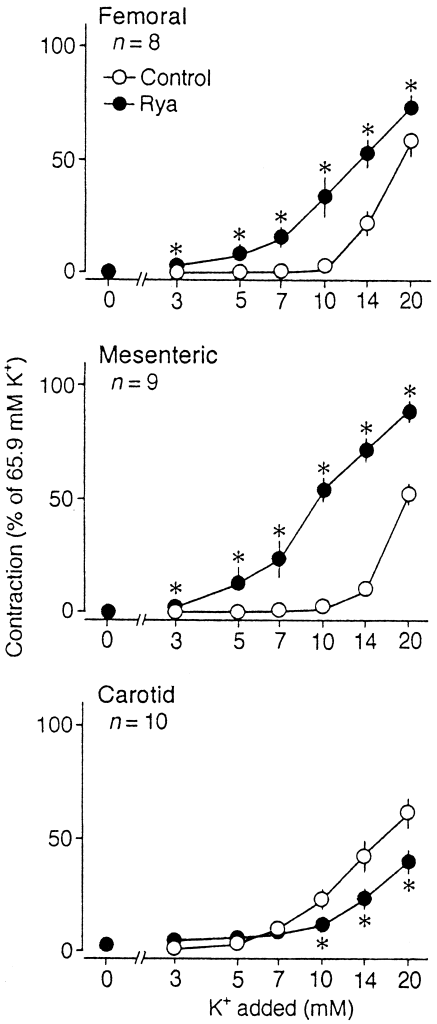


Fig. 3. Effects of ryanodine (Rya; 10 μM) treatment on the concentration–response curve for $K^+$ in strips of femoral, mesenteric and carotid arteries of the rat. Experimental conditions were the same as in Fig. 2. The peak contractions induced by each concentration of $K^+$ are expressed as % of the maximum contraction induced by 65.9 mM $K^+$. At '0' on the abscissa, the Rya-induced contractions remaining before the addition of $K^+$ are expressed as % of the maximum contraction induced by 65.9 mM $K^+$. Data points are means of the number ($n$) of preparations and S.E.M. are shown by vertical bars. * Significantly different from Control ($P < 0.05$).

mixed with 5 ml Amersham ACS II scintillant and counted for radioactivity in an Aloka liquid scintillation counter. The effects of ryanodine on basal $^{45}Ca$ influx and net $^{45}Ca$ entry were also determined. Values for basal $Ca^{2+}$ influx and net $Ca^{2+}$ entry were then calculated and expressed as nmol/g tissue wet weight, as described previously (Asano et al., 1993, 1996a).

### 2.4. Statistical analysis

The results are expressed as means ± S.E.M. ($n$ = number of preparations). The Student's $t$-test for unpaired data was used to determine the significance of differences between means, and a $P$ value of $< 0.05$ was taken as significant.

### 2.5. Drugs and isotope

The drugs used were ryanodine (Wako Pure Chemical Industries, Osaka, Japan), cyclopiazonic acid (Sigma, St. Louis, MO, USA), charybdotoxin (Peptide Institute, Minoh, Japan), caffeine (Wako), L-norepinephrine bitartrate (Sigma), choline chloride (Wako), nifedipine (a kind gift from Bayer Yakuhin, Osaka, Japan), phenoxybenzamine hydrochloride (Nacalai Tesque, Kyoto, Japan), timolol maleate (Sigma), EGTA (Sigma), acetylcholine chloride (Sigma), prostaglandin $F_{2\alpha}$ (Ono Pharmaceutical, Osaka, Japan), fura-PE3/AM (Texas Fluorescence Lab., Austin, TX, USA) and cremophor EL (Nacalai). $^{45}CaCl_2$ (specific activity initially 20 mCi/mg) was obtained from Amersham.

Cyclopiazonic acid (5 mM) and fura-PE3/AM (0.5 mM) were dissolved in 100% dimethyl sulfoxide. Dimethyl sulfoxide at 0.2% (used in the experiments with 10 μM cyclopiazonic acid) neither induced a contraction nor augmented the contractile responses to $K^+$ and norepinephrine. Nifedipine (1 mM) and phenoxybenzamine (2 mM) were dissolved in 99.5% ethanol, with further dilution in distilled water before use. Ethanol at 0.01% (used in the experiments with 100 nM nifedipine) did not inhibit the norepinephrine-induced contractions. Caffeine (20 mM) was dissolved in Krebs solution. Aqueous stock solutions were prepared for other drugs. Concentrations of drugs are expressed as final molar concentrations.

Table 2
Effects of ryanodine (Rya) treatment on the sensitivity to $K^+$ and norepinephrine (NE) of strips of femoral, mesenteric and carotid arteries of the rat[a]

| Condition | Femoral | Mesenteric | Carotid |
|---|---|---|---|
| *(A) $EC_{30}$ for $K^+$ (mM)*[b] | | | |
| Control | 15.8 ± 1.0 (8) | 16.7 ± 0.5 (9) | 11.3 ± 1.1[c] (10) |
| + Rya 10 μM | 10.2 ± 1.1[d] (8) | 7.6 ± 0.5[d] (9) | 17.2 ± 1.2[d,c] (10) |
| *(B) $pD_2$ for NE (− log M)*[e] | | | |
| Control | 6.85 ± 0.13 (9) | 7.17 ± 0.08 (8) | 8.13 ± 0.09[c] (7) |
| + Rya 10 μM | 7.46 ± 0.11[d] (9) | 7.50 ± 0.09[d] (8) | 7.59 ± 0.08[d] (7) |

Data are expressed as means ± S.E.M. (numbers in parentheses indicate the number of preparations used).

[a] Experimental conditions were the same as those described in the legends of Figs. 2–4.

[b] The $EC_{30}$ values for $K^+$ were calculated from the concentration–response curves shown in Fig. 3, and are expressed as mM.

[c] Significantly different from the femoral artery ($P < 0.05$).

[d] Significantly different from the respective Control ($P < 0.05$).

[e] The $pD_2$ values for NE were calculated from the concentration–response curves shown in Fig. 4, and are expressed as a negative log of the molar concentration.

## 3. Results

### 3.1. Leaky sarcoplasmic reticulum induced by ryanodine

As shown in a previous study (Asano et al., 1996a), treatment of the strips of rat femoral artery with ryanodine plus caffeine caused the $Ca^{2+}$-induced $Ca^{2+}$ release channels of the sarcoplasmic reticulum to be locked open, which results in the establishment of a leaky sarcoplasmic reticulum. The sarcoplasmic reticulum of mesenteric and carotid arteries was also made leaky by this procedure (Fig. 1). The addition of 10 μM ryanodine caused a small transient contraction in these arteries. In the presence of ryanodine, the addition of 20 mM caffeine still caused a transient contraction. After washout, however, the caffeine-induced contraction was almost abolished, suggesting that $Ca^{2+}$-induced $Ca^{2+}$ release channels were locked open by this procedure (Fig. 1). The ryanodine-induced contraction was not significantly different among the three arteries: the contraction was $4.0 \pm 1.0\%$ (femoral, $n = 25$), $5.1 \pm 1.0\%$ (mesenteric, $n = 31$), and $4.5 \pm 0.5\%$ (carotid, $n = 81$), respectively, of the maximum contraction induced by 65.9 mM $K^+$.

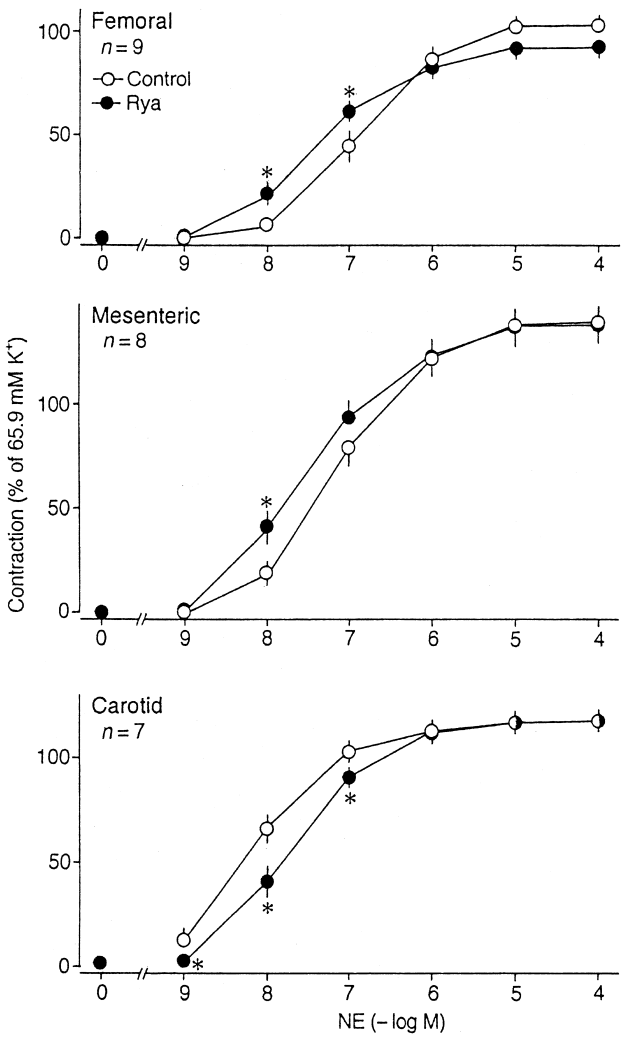


Fig. 4. Effects of ryanodine (Rya; 10 μM) treatment on the concentration–response curve for norepinephrine (NE) in strips of femoral, mesenteric and carotid arteries of the rat. Experimental conditions were the same as in Figs. 2 and 3, except that NE was used as a contractile agonist. The peak contractions induced by each concentration of NE are expressed as % of the maximum contraction induced by 65.9 mM $K^+$. At '0' on the abscissa, the Rya-induced contractions remaining before the addition of NE are expressed as % of the maximum contraction induced by 65.9 mM $K^+$. Data points are means of the number ($n$) of preparations, and S.E.M. are shown by vertical bars. * Significantly different from Control ($P < 0.05$).

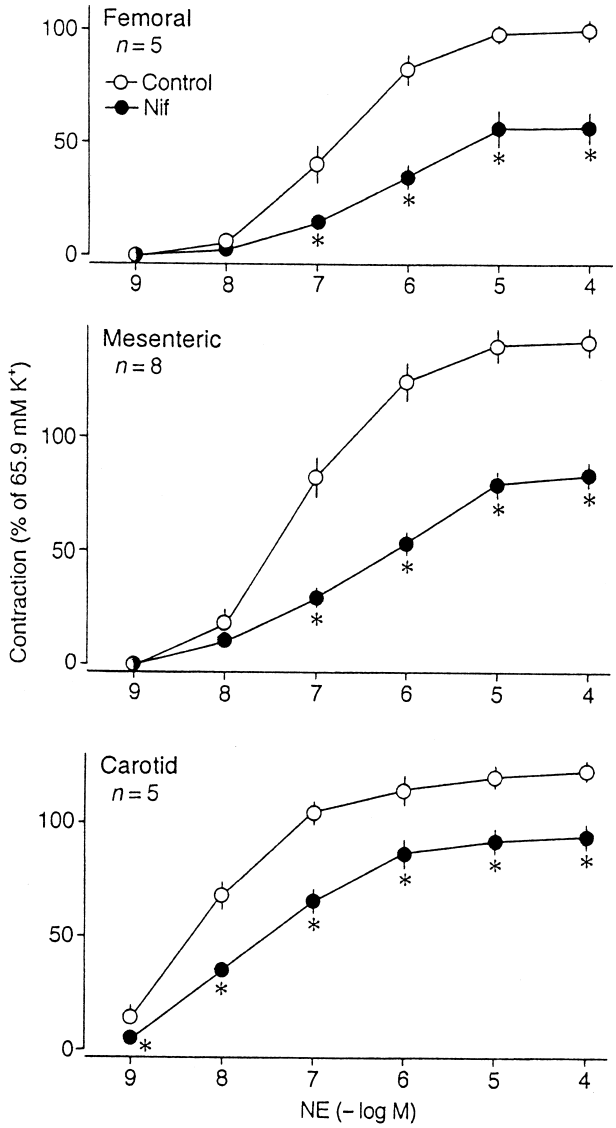


Fig. 5. Effects of nifedipine (Nif; 100 nM) on the concentration–response curve for norepinephrine (NE) in strips of femoral, mesenteric and carotid arteries of the rat. Experimental conditions were the same as in Fig. 4, except that Nif was used instead of ryanodine. The peak contractions induced by each concentration of NE are expressed as % of the maximum contraction induced by 65.9 mM $K^+$. Data points are means of the number ($n$) of preparations, and S.E.M. are shown by vertical bars. * Significantly different from Control ($P < 0.05$).

### 3.2. Effects of ryanodine on basal $Ca^{2+}$ influx and net $Ca^{2+}$ entry

As shown previously (Asano et al., 1996a), ryanodine reduced the basal $Ca^{2+}$ influx and the net $Ca^{2+}$ entry in the femoral artery. Similar results were obtained with the carotid artery (Table 1). The basal $Ca^{2+}$ influx after a 5-min incubation with $^{45}Ca$ was significantly reduced by ryanodine (Table 1A). The net $Ca^{2+}$ entry after a 30-min incubation with $^{45}Ca$ was also reduced by ryanodine (Table 1B). When basal $Ca^{2+}$ influx was measured after treatment of the artery with 10 μM ryanodine plus 20 mM caffeine and a washout for 40 min, this influx was significantly decreased when compared with the basal $Ca^{2+}$ influx determined after treatment with only 20 mM caffeine and a washout for 40 min (Table 1C).

### 3.3. Effects of ryanodine treatment on $K^+$-induced contraction

The addition of $K^+$ (3–20 mM) caused a concentration-dependent contraction in the femoral artery with an $EC_{30}$ value of 15.8 mM (Figs. 2 and 3, Table 2A). The effect of ryanodine treatment on this contraction was then determined. After ryanodine treatment, the concentration–response curve for $K^+$ was shifted to the left with an $EC_{30}$ value of 10.2 mM. The maximum contraction induced by 65.9 mM $K^+$ was not affected by ryanodine treatment (Fig. 2 and Fig. 3, Table 2A).

These experiments were repeated in mesenteric and carotid arteries. Although similar results were obtained in the mesenteric artery (Fig. 3, Table 2A), the results in the carotid artery were different. After ryanodine treatment,

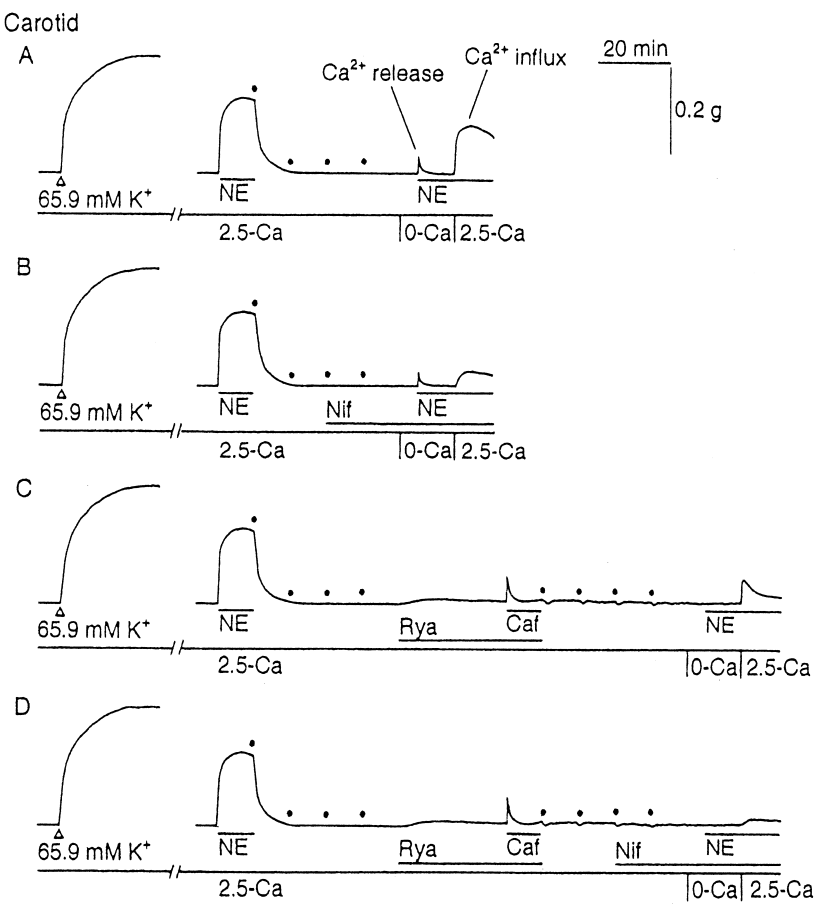


Fig. 6. Typical recordings of the effects of nifedipine (Nif; 100 nM) and ryanodine (Rya; 10 μM) treatment on $Ca^{2+}$ release and $Ca^{2+}$ influx induced by norepinephrine (NE) in strips of rat carotid artery. After determination of the maximum contraction induced by 65.9 mM $K^+$, $7 \times 10^{-9}$ M NE was added to induce 50% of the maximum contraction in this artery. (A) Control response. Following washout with Krebs solution for 40 min, the solution was replaced with a $Ca^{2+}$-free solution (0-Ca). After 5 min, $7 \times 10^{-9}$ M NE was added for 10 min and then 2.5 mM $Ca^{2+}$ was added. The transient contraction induced by NE in a 0-Ca solution was taken as $Ca^{2+}$ release, and the sustained contraction upon the addition of 2.5 mM $Ca^{2+}$ was taken as $Ca^{2+}$ influx. (B) The effect of Nif (100 nM). NE and 2.5 mM $Ca^{2+}$ were added in the presence of Nif. Nif was added 20 min before replacement of the solution. (C) The effect of Rya treatment. The Rya treatment was done as described in the legend of Fig. 1, and then NE and 2.5 mM $Ca^{2+}$ were added as in (A). (D) The effect of a combination of Rya treatment and Nif (100 nM). The Rya treatment was done as in Fig. 1, and the effect of Nif was determined as in (B). Dots (·) denote the washout of the strips with Krebs solution.

the concentration–response curve for $K^+$ in the carotid artery was shifted to the right (Figs. 2 and 3, Table 2A). The maximum contraction induced by 65.9 mM $K^+$ was not inhibited after ryanodine treatment. Thus, after ryanodine treatment, the $K^+$ contraction was augmented in femoral and mesenteric arteries but was inhibited in the carotid artery.

### 3.4. Effects of ryanodine treatment on norepinephrine-induced contraction

Since the sustained phase of norepinephrine-induced contraction is due to the influx of extracellular $Ca^{2+}$, the effect of ryanodine treatment on this contraction was also determined. Under control conditions, the addition of norepinephrine ($10^{-9}$–$10^{-4}$ M) caused a concentration-dependent contraction in the femoral artery with a $pD_2$ value of 6.85 (Fig. 4, Table 2B). After ryanodine treatment, the concentration–response curve for norepinephrine was shifted to the left with a $pD_2$ value of 7.46 (Fig. 4, Table 2B).

These experiments were repeated in mesenteric and carotid arteries (Fig. 4, Table 2B). Although similar results were obtained in the mesenteric artery (Fig. 4, Table 2B), the results in the carotid artery were different. After ryanodine treatment, the concentration–response curve for norepinephrine in the carotid artery was shifted to the right (Fig. 4, Table 2B). Thus, after ryanodine treatment, the sustained contractions induced by lower concentrations of norepinephrine were augmented in femoral and mesenteric arteries but were inhibited in the carotid artery.

### 3.5. Effects of nifedipine on norepinephrine-induced contraction

To determine the contribution of $Ca^{2+}$ influx via L-type $Ca^{2+}$ channels to the norepinephrine-induced sustained contraction, the effects of 100 nM nifedipine were determined in the three arteries (Fig. 5). Nifedipine at 100 nM, which blocked completely the $K^+$ contraction, shifted the concentration–response curve for norepinephrine to the right, together with a reduction of the maximum contraction in the three arteries. Although the extent of the rightward shift was not significantly different among the three arteries, the extent of the reduction of the maximum contraction was smaller in the carotid artery.

### 3.6. Effects of ryanodine treatment on norepinephrine-induced $Ca^{2+}$ release and influx

In a $Ca^{2+}$-free solution, norepinephrine at $7 \times 10^{-9}$ M (the concentration that induced 50% of the maximum

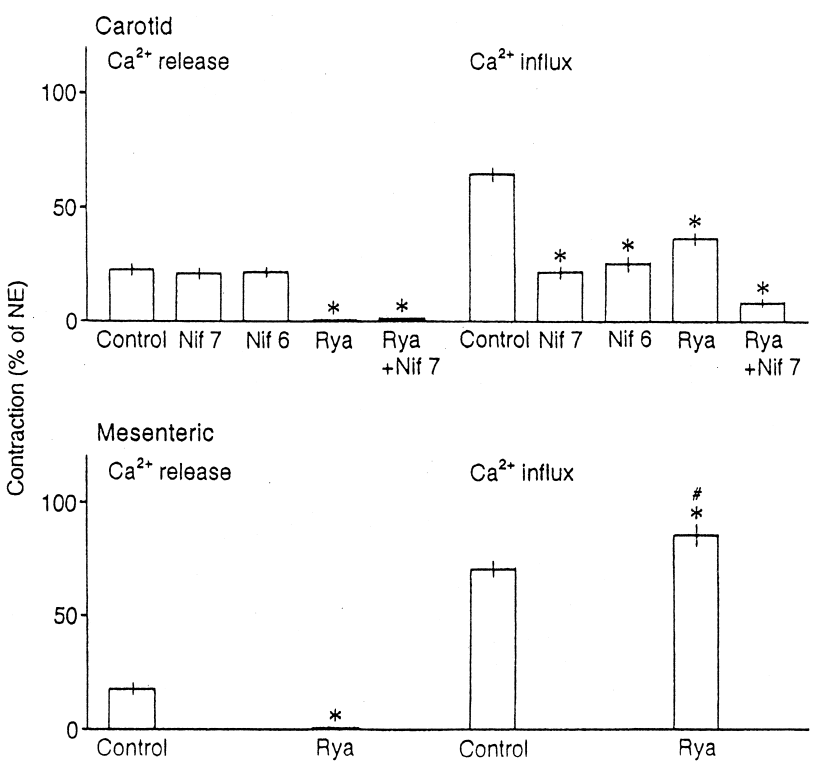


Fig. 7. Effects of nifedipine (Nif), ryanodine (Rya; 10 μM) treatment and a combination of the two agents on $Ca^{2+}$ release and $Ca^{2+}$ influx induced by norepinephrine (NE) in strips of carotid and mesenteric arteries of the rat. Experimental conditions were the same as in Fig. 6. To induce 50% of the maximum contraction, $7 \times 10^{-9}$ M NE (carotid) or $7 \times 10^{-8}$ M NE (mesenteric) was used. The contractions due to $Ca^{2+}$ release and $Ca^{2+}$ influx shown in Fig. 6 are expressed as % of the control NE contraction determined prior to these contractions. Concentrations of Nif are expressed as a negative log of the molar concentration. Data are means of six preparations, and S.E.M. are shown by vertical bars. * Significantly different from the respective Control ($P < 0.05$). # Significantly different from the carotid artery ($P < 0.05$).

contraction as shown in Table 2) induced a transient contraction due to the release of intracellular $Ca^{2+}$ in the carotid artery (Figs. 6A and 7). Under these conditions, the addition of 2.5 mM $Ca^{2+}$ caused a sustained contraction due to the influx of extracellular $Ca^{2+}$ (Figs. 6A and 7). When 100 nM nifedipine was added during this procedure, the sustained contraction, but not the transient one, was inhibited (Figs. 6B and 7). No further inhibition was observed when the higher concentration of nifedipine (1 μM) was used (Fig. 7). After ryanodine treatment, the transient contraction was abolished, and the sustained contraction was inhibited (Figs. 6C and 7). When the effects of a combination of ryanodine treatment and nifedipine were determined, the sustained contraction was inhibited more strongly than it was by each agent alone (Figs. 6D and 7), suggesting that the mechanism of action of ryanodine on the norepinephrine-induced $Ca^{2+}$ influx differs from that of nifedipine.

After ryanodine treatment of the mesenteric artery, the transient contraction was also abolished, but the sustained contraction was augmented (Fig. 7).

### *3.7. Effects of charybdotoxin and cyclopiazonic acid on the inhibitory action of ryanodine on $K^+$-induced contraction*

The possible mechanism underlying the inhibitory action of ryanodine on the $K^+$ contraction in the carotid artery was examined. Since $Ca^{2+}$-induced $Ca^{2+}$ release channels of the sarcoplasmic reticulum are involved in the vectorial release of $Ca^{2+}$ from the superficial sarcoplasmic reticulum, resulting in the activation of $Ca^{2+}$-activated $K^+$ channels ($Ca^{2+}$ sparks; for review, see Bolton and Imaizumi, 1996; Laporte and Laher, 1997), the possible involvement of this process in the inhibitory action of ryanodine was examined (Fig 8A). When $Ca^{2+}$-activated $K^+$ channels were blocked by 100 nM charybdotoxin after ryanodine treatment, there was no change in the effect of ryanodine (Fig. 8D).

According to the 'superficial buffer barrier' hypothesis, $Ca^{2+}$ taken up into the sarcoplasmic reticulum is continually extruded from the sarcoplasmic reticulum lumen to the extracellular space via the $Na^+$–$Ca^{2+}$ exchanger and the $Ca^{2+}$-ATPase of the plasma membrane (Van Breemen and Saida, 1989; Chen et al., 1992; Van Breemen et al., 1995; Laporte and Laher, 1997). The possible involvement of this process was then examined. Since no specific inhibitors are available for the $Na^+$–$Ca^{2+}$ exchanger or the $Ca^{2+}$-ATPase of the plasma membrane, we used an indirect method to inhibit this sarcoplasmic reticulum–plasma membrane $Ca^{2+}$ extrusion system by using cyclopiazonic acid, a sarcoplasmic reticulum $Ca^{2+}$-ATPase inhibitor (Fig. 8B). We reasoned that if sarcoplasmic reticulum $Ca^{2+}$ uptake is inhibited, $Ca^{2+}$ extrusion into the extracellular space would be secondarily inhibited. After ryanodine treatment, the addition of 10 μM cyclopiazonic acid caused a transient contraction and the concentration–response

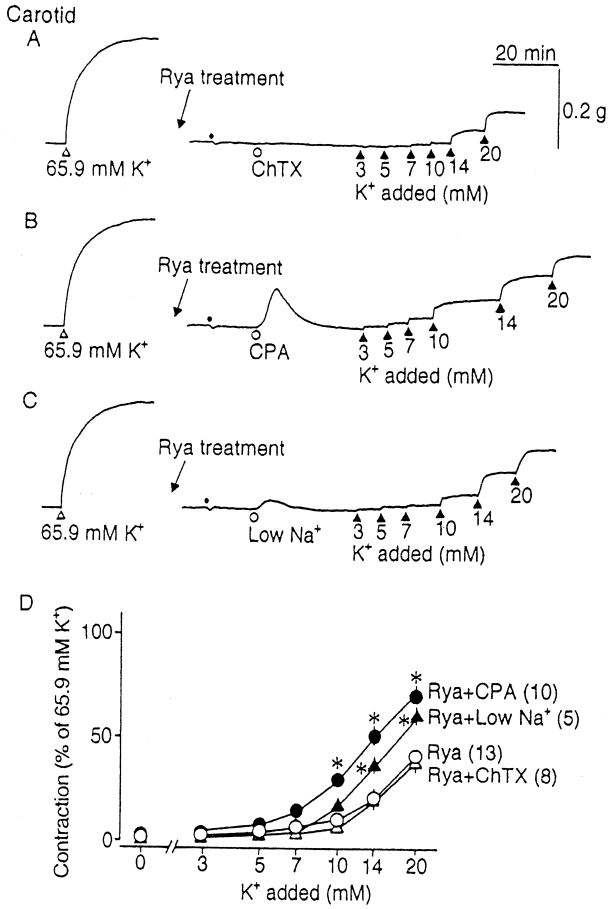


Fig. 8. Effects of charybdotoxin (ChTX; 100 nM), cyclopiazonic acid (CPA; 10 μM), and low $Na^+$ solution on the ryanodine (Rya)-induced inhibition of the $K^+$ contraction in strips of rat carotid artery. (A, B, C) Typical recordings of the effects of ChTX (A), CPA (B) or low $Na^+$ solution (C) on the Rya action. After Rya treatment as described in the legend of Fig. 1, ChTX (A), CPA (B) or low $Na^+$ solution (C) was incubated for 30 min and then the contractile responses to $K^+$ (3–20 mM addition) were determined. (D) Summary of the effects of ChTX, CPA or low $Na^+$ solution on the Rya action. The peak contractions induced by each concentration of $K^+$ are expressed as % of the maximum contraction induced by 65.9 mM $K^+$. At '0' on the abscissa, the contractions remaining before the addition of $K^+$ are expressed as % of the maximum contraction induced by 65.9 mM $K^+$. Data points are means of the number of preparations indicated in parentheses, and S.E.M. are shown by vertical bars. * Significantly different from ryanodine treatment (Rya) ($P < 0.05$).

curve for $K^+$ was shifted to the left (Fig. 8D). The $EC_{30}$ value calculated from this curve ($10.7 \pm 0.7$ mM, $n = 10$) was not significantly different from the control $EC_{30}$ value ($11.3 \pm 1.1$ mM, $n = 10$) determined in the absence of any blocker, suggesting that cyclopiazonic acid abolished the action of ryanodine. As shown in Fig. 8B, the addition of cyclopiazonic acid to the ryanodine-treated strips caused a moderate contraction, suggesting that when $Ca^{2+}$-induced $Ca^{2+}$ release channels are locked open, $Ca^{2+}$ entering under resting conditions continues to be accumulated in the leaky sarcoplasmic reticulum by sarcoplasmic reticulum $Ca^{2+}$-ATPase.

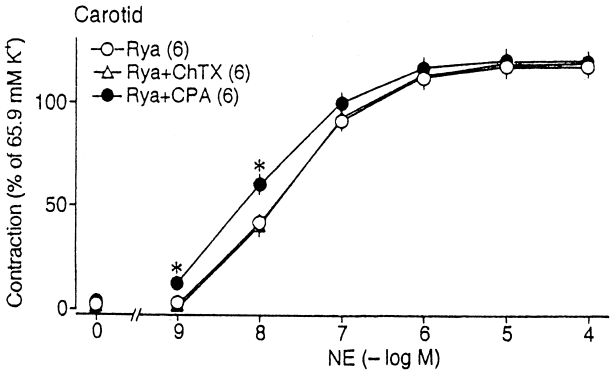


Fig. 9. Effects of charybdotoxin (ChTX; 100 nM) and cyclopiazonic acid (CPA; 10 μM) on the ryanodine (Rya)-induced inhibition of the norepinephrine (NE) contraction in strips of rat carotid artery. Experimental conditions were similar to those described in the legends of Figs. 7 and 8, except that NE was used as a contractile agonist. The peak contractions induced by each concentration of NE are expressed as % of the maximum contraction induced by 65.9 mM $K^+$. At '0' on the abscissa, the contractions remaining before the addition of NE are expressed as % of the maximum contraction induced by 65.9 mM $K^+$. Data points are means of six preparations, and S.E.M. are shown by vertical bars. * Significantly different from ryanodine treatment (Rya) ($P < 0.05$).

The possible role of the $Na^+$–$Ca^{2+}$ exchanger of the plasma membrane in the inhibitory action of ryanodine on the $K^+$ contraction was also determined in the carotid artery (Fig. 8C,D). When $Na^+$–$Ca^{2+}$ exchange was inhibited by lowering extracellular $Na^+$ to 25 mM after ryanodine treatment, the concentration–response curve for $K^+$ was shifted to the left (Fig. 8D). The $EC_{30}$ value calculated from this curve was $12.3 \pm 1.0$ mM ($n = 5$). As shown in Fig. 8C, the low $Na^+$ solution itself induced a small transient contraction in the ryanodine-treated strips, suggesting that when $Ca^{2+}$-induced $Ca^{2+}$ release channels are locked open, some $Ca^{2+}$ that leaked from the sarcoplasmic reticulum was extruded from the plasma membrane via the $Na^+$–$Ca^{2+}$ exchanger.

Similar experiments were performed with the femoral artery. After ryanodine treatment, the addition of 10 μM cyclopiazonic acid caused a transient contraction and the concentration–response curve for $K^+$ was further shifted to the left. The $EC_{30}$ value calculated from this curve ($7.5 \pm 0.6$ mM, $n = 8$) was significantly smaller than the $EC_{30}$ value ($10.2 \pm 1.1$ mM, $n = 8$) determined after ryanodine treatment but was similar to the $EC_{30}$ value ($7.2 \pm 1.1$ mM, $n = 8$) determined under the inhibition by cyclopiazonic acid of sarcoplasmic reticulum $Ca^{2+}$-ATPase shown in the accompanying paper (Nomura and Asano, 2000). These observations suggest that some $Ca^{2+}$ that leaks from the sarcoplasmic reticulum is also extruded from the plasma membrane in the femoral artery. The cyclopiazonic acid-induced leftward shift of the concentration–response curve for $K^+$ in ryanodine-treated strips was calculated as 6.5 mM (from 17.2 to 10.7 mM) for the carotid artery and 2.7 mM (from 10.2 to 7.5 mM) for the femoral artery. This suggests a preferential movement of $Ca^{2+}$ from $Ca^{2+}$-induced $Ca^{2+}$ release channels toward the plasma membrane in the carotid artery when compared with the femoral artery.

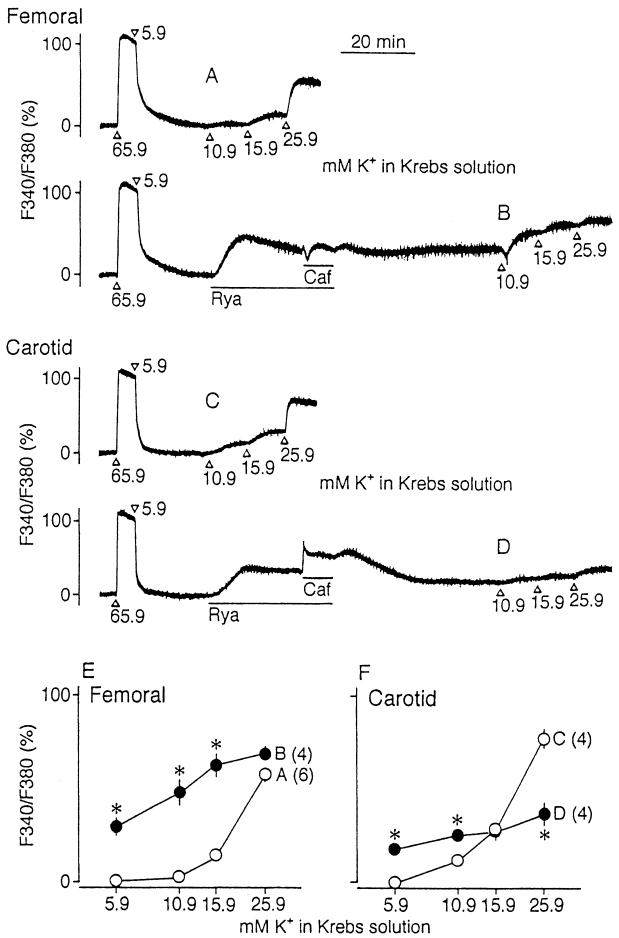


Fig. 10. Effects of ryanodine (Rya; 10 μM) treatment on the $K^+$-induced elevation of $[Ca^{2+}]_i$ (indicated by F340/F380) in strips of femoral (A, B, E) and carotid (C, D, F) arteries. Experimental conditions were the same as described in the legends of Figs. 2 and 3. (A, C) Control responses. After loading with fura-PE3, the strips were exposed to 65.9 mM $K^+$ for 5 min. Following washout with a normal Krebs solution (5.9 mM $K^+$) for 20 min, Krebs solution containing 10.9, 15.9, and 25.9 mM $K^+$ (equimolar substitution of $Na^+$ with $K^+$) was added in a cumulative fashion as indicated. (B, D) Effects of Rya treatment. The Rya treatment was done as described in the legend of Fig. 1, and then the responses to $K^+$ were determined as indicated. (E, F) Summary of the effects of Rya treatment on the $K^+$-induced elevation of $[Ca^{2+}]_i$. The peak values of the elevation of $[Ca^{2+}]_i$ induced by each concentration of $K^+$ are expressed as % of the maximum response to 65.9 mM $K^+$. At '5.9' on the abscissa of B and D responses, the Rya-induced elevation of $[Ca^{2+}]_i$ remaining before the addition of the 10.9-mM $K^+$ Krebs solution is expressed as % of the maximum response to 65.9 mM $K^+$. The Krebs solutions containing 5.9, 10.9, 15.9 and 25.9 mM $K^+$ in this figure corresponded to the addition of 0, 5, 10 and 20 mM $K^+$, respectively, shown in Figs. 2 and 3. Data points are means of the number of preparations indicated in parentheses, and S.E.M. are shown by vertical bars. * Significantly different from Control ($P < 0.05$).

### 3.8. Effects of charybdotoxin and cyclopiazonic acid on the inhibitory action of ryanodine on norepinephrine-induced contraction in the carotid artery

When $Ca^{2+}$-activated $K^+$ channels were blocked by 100 nM charybdotoxin after ryanodine treatment, there were no effects on the ryanodine-induced inhibition of the norepinephrine contraction (Fig. 9). However, when sarcoplasmic reticulum $Ca^{2+}$ uptake was inhibited by 10 μM cyclopiazonic acid after ryanodine treatment, the concentration–response curve for norepinephrine was shifted to the left (Fig. 9). Thus, these observations were quite similar to those for the $K^+$ contraction shown in Fig. 8.

### 3.9. Effects of ryanodine treatment on $K^+$-induced elevation of $[Ca^{2+}]_i$

The elevation of extracellular $K^+$ caused a concentration-dependent elevation of $[Ca^{2+}]_i$ in femoral and carotid arteries (Fig. 10). The effects of ryanodine treatment on this elevation were then determined. After ryanodine treatment, the resting $[Ca^{2+}]_i$ was moderately elevated in the two arteries, and the concentration–response curve for $K^+$ was shifted to the left in the femoral artery (Fig. 10E), while the $K^+$-induced elevation was inhibited in the carotid artery (Fig. 10D,F). Thus, with a leaky sarcoplasmic reticulum, the $K^+$-induced elevation of $[Ca^{2+}]_i$ was augmented in the femoral artery but was inhibited in the carotid artery.

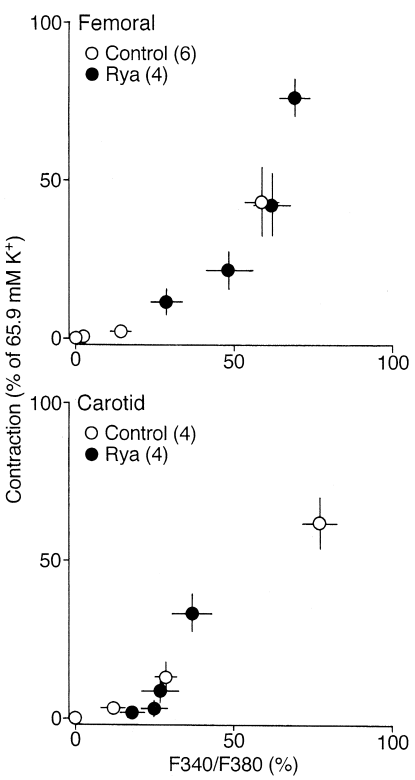


Fig. 11. Effects of ryanodine (Rya; 10 μM) treatment on the $K^+$-induced $[Ca^{2+}]_i$–tension relationship in strips of femoral and carotid arteries. The $[Ca^{2+}]_i$–tension relationship was obtained by the simultaneous measurement of $[Ca^{2+}]_i$ and tension induced by cumulative addition of the Krebs solution containing 10.9, 15.9, and 25.9 mM $K^+$ (equimolar substitution of $Na^+$ with $K^+$) shown in Fig. 10. The peak values of the elevation of $[Ca^{2+}]_i$ (indicated by F340/F380) induced by each concentration of $K^+$ are expressed as % of the maximum response to 65.9 mM $K^+$. The contractions induced by each concentration of $K^+$ are expressed as % of the maximum response to 65.9 mM $K^+$. Data points are means of the number of preparations indicated in parentheses, and S.E.M. are shown by vertical bars.

### 3.10. Effects of ryanodine treatment on $K^+$-induced $[Ca^{2+}]_i$–tension relationship

The effects of ryanodine treatment on the $Ca^{2+}$ sensitivity of the contractile apparatus were evaluated by examining the $[Ca^{2+}]_i$–tension relationship of the data shown in Fig. 10. As shown in Fig. 11, the $[Ca^{2+}]_i$–tension relationship, based on data obtained by simultaneous measurement of $[Ca^{2+}]_i$ and tension induced by the cumulative addition of $K^+$ in the same preparation, indicated that there was a positive correlation between the two parameters. There was no significant difference in the $[Ca^{2+}]_i$–tension relationship of the control $K^+$ response between the femoral and carotid arteries. This $[Ca^{2+}]_i$–tension relationship was not significantly altered by ryanodine treatment in the two arteries.

## 4. Discussion

Ryanodine binds to $Ca^{2+}$-induced $Ca^{2+}$ release channels of the sarcoplasmic reticulum, keeping them in an open, semi-conducting state. After treatment with ryanodine plus caffeine, the sarcoplasmic reticulum becomes empty and unable to accumulate $Ca^{2+}$, resulting in the establishment of a leaky sarcoplasmic reticulum (for details, see Iino et al., 1988; Asano et al., 1996a,b). Under these conditions, stimulation with $K^+$ or norepinephrine causes an influx of extracellular $Ca^{2+}$, and a fraction of the $Ca^{2+}$ that has entered is initially taken up into the leaky sarcoplasmic reticulum before it reaches the deeper myoplasm (myofilaments), where it can move to several regions of the cytoplasm. When $Ca^{2+}$ moves to myofilaments, the contraction should be augmented. In contrast, when $Ca^{2+}$ moves to the plasma membrane, the extrusion of $Ca^{2+}$ from the cell by the $Na^+$–$Ca^{2+}$ exchanger and the $Ca^{2+}$-ATPase of the plasma membrane and/or the inhibition of $Ca^{2+}$ influx through the activation of $Ca^{2+}$-activated $K^+$ channels (i.e., $Ca^{2+}$ sparks) should occur, resulting in an inhibition of the contraction.

It is likely that in femoral and mesenteric arteries with a leaky sarcoplasmic reticulum (i.e., after ryanodine treatment), most $Ca^{2+}$ that is leaked from the sarcoplasmic reticulum during stimulation with $K^+$ or norepinephrine moves mainly to the myofilaments. This assumption comes

from observations that after ryanodine treatment, the $K^+$ contraction, the $K^+$-induced elevation of $[Ca^{2+}]_i$, the norepinephrine contraction and the norepinephrine-induced $Ca^{2+}$ influx were all augmented. This $Ca^{2+}$ movement is in accordance with several studies showing an augmenting effect of ryanodine on $K^+$ or norepinephrine contractions in rat small mesenteric artery (Julou-Schaeffer and Freslon, 1988; Shima and Blaustein, 1992; Gustafsson and Nilsson, 1993; Naganobu and Ito, 1994) and dog mesenteric artery (Low et al., 1993). As shown in Figs. 5–7, the norepinephrine-induced tonic contraction was in part due to $Ca^{2+}$ influx via L-type $Ca^{2+}$ channels in the three arteries studied.

In the carotid artery with a leaky sarcoplasmic reticulum, most $Ca^{2+}$ that is leaked from the sarcoplasmic reticulum during stimulation with $K^+$ or norepinephrine moves mainly to the plasma membrane. This assumption comes from observations that after ryanodine treatment, the four responses described above were all inhibited. Thus, with a leaky sarcoplasmic reticulum, $Ca^{2+}$ movement in the carotid artery was different from that in the other arteries. It is likely that $Ca^{2+}$ leaking from the sarcoplasmic reticulum to the plasma membrane in the carotid artery is extruded from the cell by the $Na^+$–$Ca^{2+}$ exchanger and the $Ca^{2+}$-ATPase of the plasma membrane rather than that there is inhibition of $Ca^{2+}$ influx through the activation of $Ca^{2+}$-activated $K^+$ channels (i.e., $Ca^{2+}$ sparks). As shown in Fig. 8, the inhibitory action of ryanodine in the carotid artery was abolished by cyclopiazonic acid, an indirect inhibitor of the sarcoplasmic reticulum–plasma membrane $Ca^{2+}$ extrusion system (as explained in Fig. 8), and also by the inhibition of $Na^+$–$Ca^{2+}$ exchange, but was not affected by charybdotoxin, a blocker of $Ca^{2+}$-activated $K^+$ channels. Furthermore, as shown in Figs. 6 and 7, the mechanism of action of ryanodine was different from that of nifedipine, a blocker of L-type $Ca^{2+}$ channels, because each effect was additive when the effect of a combination of ryanodine and nifedipine was determined.

To our knowledge, this is a first report suggesting that the inhibitory effects of ryanodine on $K^+$ and norepinephrine contractions in the carotid artery are mainly due to the extrusion of $Ca^{2+}$ from the sarcoplasmic reticulum to the extracellular space. Naganobu et al. (1994) have previously shown the inhibition of the depolarization-dependent contraction (40 mM $K^+$ and 0.1 mM $Ca^{2+}$) after ryanodine treatment in rat small mesenteric artery, but the mechanism of action of ryanodine was different from that in the present study. These authors concluded that the $Ca^{2+}$-induced $Ca^{2+}$ release mechanism is involved in the depolarization-dependent contraction and that after ryanodine treatment, the $Ca^{2+}$-induced $Ca^{2+}$ release mechanism was lost so that the contraction was strongly inhibited.

Since it is likely that with a leaky sarcoplasmic reticulum, $Ca^{2+}$ leaked from the sarcoplasmic reticulum moves to both the myofilaments and the plasma membrane, then the final response to $K^+$ or norepinephrine could be determined by the net balance of the augmentation and the inhibition of the contraction. If one assumes that the augmentation and the inhibition are equal, the $K^+$ or norepinephrine contraction seems to be unaffected as a consequence. Such a situation may explain the results showing that the $K^+$ or norepinephrine contraction was not affected by ryanodine in rat aorta (Julou-Schaeffer and Freslon, 1988; Low et al., 1993) and rat small mesenteric artery (Garcha and Hughes, 1995, 1997). In the present study, $Ca^{2+}$ movement from the leaky sarcoplasmic reticulum to the plasma membrane also occurred in femoral and mesenteric arteries. When the effects of cyclopiazonic acid on the $K^+$ contraction were determined after ryanodine treatment in the femoral artery, as in the carotid artery shown in Fig. 8, the concentration–response curve for $K^+$ was further shifted to the left when compared with the concentration–response curve determined after ryanodine treatment. This suggests that when the sarcoplasmic reticulum of the femoral artery is made leaky, $Ca^{2+}$ from the sarcoplasmic reticulum moves to both the myofilaments and the plasma membrane, and the amount of $Ca^{2+}$ moved to the former was larger than to the latter. This assumption is also supported by the finding of our previous study that during the addition of ryanodine to the femoral artery, $^{45}Ca$ extrusion from the cell was secondarily increased (Asano and Nomura, 1997). Although we could not show the movement of $Ca^{2+}$ from the leaky sarcoplasmic reticulum to the myofilaments in the carotid artery, we consider that a small amount of $Ca^{2+}$ moves in this direction.

With a leaky sarcoplasmic reticulum, $Ca^{2+}$ from the sarcoplasmic reticulum appears to cycle across the sarcoplasmic reticulum membrane during stimulation with $K^+$ or norepinephrine: a fraction of $Ca^{2+}$ is taken up again into the leaky sarcoplasmic reticulum by sarcoplasmic reticulum $Ca^{2+}$-ATPase. This assumption is based on the finding that after ryanodine treatment, cyclopiazonic acid still induced a strong contraction in the three arteries (e.g., carotid artery shown in Fig. 8B). This also suggests that $Ca^{2+}$ entering under resting conditions is still taken up into a leaky sarcoplasmic reticulum.

A possible explanation for the different $Ca^{2+}$ movement seen in the carotid artery is the relative location of the sarcoplasmic reticulum in the cells. If one assumes that the sarcoplasmic reticulum of the carotid artery is located close to the plasma membrane (compared with the other arteries), then it is possible to explain why $Ca^{2+}$ leaking from the sarcoplasmic reticulum is effectively extruded into the extracellular space in the carotid artery. In fact, ultrastructural studies have shown that a portion of the sarcoplasmic reticulum in vascular smooth muscle cells is located less than 25 nm from the plasma membrane (Devine et al., 1972; Forbes et al., 1979). This region of close apposition is now considered to play an important role in the regulation of $[Ca^{2+}]_i$, because a number of $Ca^{2+}$

transport proteins, such as the $Na^+$–$Ca^{2+}$ exchanger and the $Na^+$–$K^+$-ATPase, are localized in this region (for details, see Laporte and Laher, 1997). Moreover, functional linkage of the sarcoplasmic reticulum with the $Ca^{2+}$ extrusion system of plasma membrane has been reported (Sturek et al., 1992; Stehno-Bittel and Sturek, 1992; Nazer and Van Breemen, 1998; Rembold and Chen, 1998).

Ryanodine caused a sustained elevation of $[Ca^{2+}]_i$ (Fig. 10D) but only a small transient contraction (Fig. 1) in both femoral and carotid arteries. This may suggest the existence of a mechanism of desensitization of the contractile apparatus, especially in the carotid artery, where the subsequent addition of $K^+$ after ryanodine treatment caused a decreased contraction. In smooth muscle, it is known for example that high $[Ca^{2+}]_i$ maintained for a long time stimulates the CaM kinase II which then phosphorylates the myosin light chain kinase (Ikebe and Reardon, 1990; Tansey et al., 1992). However, this seems to be unlikely, because ryanodine treatment did not alter the $[Ca^{2+}]_i$–tension relationship in either artery, as shown in Fig. 11.

In conclusion, our results suggest that (1) with a leaky sarcoplasmic reticulum, $Ca^{2+}$ entering from the extracellular space during contraction is first taken up into the leaky sarcoplasmic reticulum and then moves to either the myofilaments or the inner surface of the plasma membrane, (2) the final response is determined by the net balance of these $Ca^{2+}$ movements, and (3) the direction of $Ca^{2+}$ movement depends on the relative location of the sarcoplasmic reticulum in the smooth muscle cell of each artery. The results of the present study, taken together with the results in the accompanying paper (Nomura and Asano, 2000), strongly suggest that the sarcoplasmic reticulum of the rat carotid artery has the following unique characteristics when compared with the sarcoplasmic reticulum of femoral and mesenteric arteries: (1) the capacity of the sarcoplasmic reticulum to store $Ca^{2+}$ is small, (2) the sarcoplasmic reticulum is almost filled to capacity with $Ca^{2+}$ in the resting state, so that the $Ca^{2+}$ uptake function of the sarcoplasmic reticulum during contraction is weak, and (3) when the sarcoplasmic reticulum is made leaky by ryanodine, $Ca^{2+}$ leaked from the sarcoplasmic reticulum during contraction is extruded into the extracellular space.

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