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# $Ca^{2+}$ uptake function of sarcoplasmic reticulum during contraction of rat arterial smooth muscles

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**Abstract**

To determine the $Ca^{2+}$ uptake function of the sarcoplasmic reticulum during contraction, the effects of cyclopiazonic acid or thapsigargin, agents that inhibit sarcoplasmic reticulum $Ca^{2+}$-ATPase, on the contractile responses to $K^+$ or norepinephrine were compared in endothelium-denuded strips of femoral, mesenteric and carotid arteries of the rat. The addition of $K^+$ (3–20 mM) to the strips caused a concentration-dependent contraction, and the sensitivity to $K^+$ was much higher in the carotid artery than in the other arteries. The preincubation of strips with cyclopiazonic acid (10 μM) or thapsigargin (100 nM) caused a leftward shift of the concentration–response curve for $K^+$, and this effect was smaller in the carotid artery than in the other arteries. Inhibition of sarcoplasmic reticulum $Ca^{2+}$ uptake caused the sensitivity to $K^+$ to be similar in the three arteries. Similar results were obtained when the contractile responses to norepinephrine were determined. Cyclopiazonic acid itself induced similar transient contractions in the three arteries. The addition of caffeine (20 mM) caused a transient contraction that was smaller in the carotid artery than in the other arteries. We conclude that (1) the $Ca^{2+}$ influx during stimulation with $K^+$ or norepinephrine is buffered by the sarcoplasmic reticulum in femoral and mesenteric arteries, (2) this function is weak in the carotid artery, probably because the sarcoplasmic reticulum of this artery is almost filled with $Ca^{2+}$ in the resting state, and (3) the $Ca^{2+}$ uptake function of the sarcoplasmic reticulum during contraction is reflected by the contractile sensitivity in these arteries. 

*Keywords:* Sarcoplasmic reticulum; $Ca^{2+}$-ATPase, Sarcoplasmic reticulum; $Ca^{2+}$ uptake function; Superficial buffer barrier hypothesis; $Ca^{2+}$ influx; $Ca^{2+}$ channel, L-type; Arterial contraction

## 1. Introduction

It has been established that the sarcoplasmic reticulum in the peripheral cytoplasm plays an important role in regulating cytosolic $Ca^{2+}$ levels ($[Ca^{2+}]_i$) in smooth muscle cells by serving as a buffer barrier to $Ca^{2+}$ entry. 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), $Ca^{2+}$ that enters the cell through the plasma membrane is taken up into the superficial (peripheral) sarcoplasmic reticulum by the sarcoplasmic reticulum $Ca^{2+}$-ATPase before it reaches the myofilaments. This concept has now been confirmed by many studies using selective sarcoplasmic reticulum $Ca^{2+}$-ATPase inhibitors, such as cyclopiazonic acid (Seidler et al., 1989) or thapsigargin (Thastrup et al., 1990). For instance, the addition of cyclopiazonic acid or thapsigargin to arterial strips causes a contraction and an elevation of $[Ca^{2+}]_i$ (for details, see Nomura et al., 1996, 1997; Asano et al., 1998), suggesting that basal $Ca^{2+}$ entry in the resting state is buffered by sarcoplasmic reticulum $Ca^{2+}$-ATPase.

It also is expected, that during arterial contraction, a fraction of $Ca^{2+}$ that enters cells can be buffered by sarcoplasmic reticulum $Ca^{2+}$-ATPase before it reaches the myofilaments. Several studies have examined the effects of cyclopiazonic acid or thapsigargin on the contractile responses of arterial strips to elevated extracellular $K^+$, but conflicting results have been obtained: augmenting effects of cyclopiazonic acid or thapsigargin were reported in rat aorta (Low et al., 1991; Shima and Blaustein, 1992), rat mesenteric artery (Shima and Blaustein, 1992; Naganobu et al., 1994) and dog mesenteric artery (Low et al., 1991), and inhibitory effects of thapsigargin were reported in rat

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aorta (Shima and Blaustein, 1992). Since the sustained phase of the norepinephrine-induced contraction is due to an influx of extracellular $Ca^{2+}$, the effects of cyclopiazonic acid or thapsigargin on this contraction were also examined. The results from these studies were also conflicting: augmenting effects of cyclopiazonic acid or thapsigargin were reported in rat aorta (Low et al., 1991), rat mesenteric artery (Naganobu and Ito, 1994) and dog mesenteric artery (Low et al., 1991), and no effects of thapsigargin were reported in rat mesenteric artery (Garcha and Hughes, 1995). In a previous study (Asano and Nomura, 1999), we examined the $Ca^{2+}$ buffering function of the sarcoplasmic reticulum during stimulation with methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate (Bay k 8644), an agonist of L-type $Ca^{2+}$ channels, in the rat femoral artery. Bay k 8644 failed to induce a contraction under normal conditions, but induced a strong contraction when sarcoplasmic reticulum $Ca^{2+}$ buffering was inhibited by cyclopiazonic acid, thapsigargin or ryanodine, suggesting that $Ca^{2+}$ influx induced by Bay k 8644 is completely buffered by the sarcoplasmic reticulum.

From a survey of these reports of the diverse effects of cyclopiazonic acid or thapsigargin, we hypothesized that sarcoplasmic reticulum $Ca^{2+}$-ATPase function during contraction is variable in arteries and is reflected by the sensitivity to contractile agonists in each artery. The present study was designed to examine this hypothesis by comparing the effects of cyclopiazonic acid or thapsigargin on $K^+$- and norepinephrine-induced contractions in strips of rat femoral, mesenteric and carotid arteries. We predicted that increased sarcoplasmic reticulum $Ca^{2+}$-ATPase function during stimulation with $K^+$ or norepinephrine causes a fraction of the $Ca^{2+}$ that enters cells to be taken up into the sarcoplasmic reticulum, thereby resulting in a relatively weak contraction in response (i.e., a low sensitivity to the agonist). On the other hand, if sarcoplasmic reticulum $Ca^{2+}$-ATPase function is weak during stimulation with $K^+$ or norepinephrine, a large part of $Ca^{2+}$ that enters cells bypasses the sarcoplasmic reticulum and reaches the myofilaments, resulting in a stronger contraction (i.e., a high sensitivity to the agonist). Moreover, when sarcoplasmic reticulum $Ca^{2+}$-ATPase is inhibited by cyclopiazonic acid or thapsigargin, the sensitivity to the agonist will become similar in these arteries. Since thapsigargin in micromolar concentrations inhibits the function of L-type $Ca^{2+}$ channels (Rossier et al., 1993; Nelson et al., 1994; Buryi et al., 1995; Treiman et al., 1998), an effect which can partly lead to the conflicting results described above, we used 100 (or 30) nM thapsigargin in these studies. This concentration abolishes sarcoplasmic reticulum $Ca^{2+}$-ATPase function without inhibiting L-type $Ca^{2+}$ channels (Nomura et al., 1996, 1997; Asano and Nomura, 1999). A preliminary account of these findings was presented to the 72nd Annual Meeting of the Japanese Pharmacological Society (Nomura and Asano, 1999).

## 2. Materials and methods

### 2.1. Preparation of arterial smooth muscle strips

Male Wistar–Kyoto rats at 12–14 weeks of age (278–319 g) were used. They were obtained from a colony maintained in our laboratory. Femoral arteries (0.6–0.8 mm outside diameter), distal half (0.4–0.7 mm) of the superior mesenteric artery and common carotid arteries (0.9–1.1 mm) were excised from the rat and placed in a Krebs solution of the following composition (in mM): NaCl 115.0, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25.0, $KH_2PO_4$ 1.2 and dextrose 10.0. Arteries were cut into helical strips (0.7–0.8 mm in width) as described previously (Asano et al., 1988, 1993). To avoid possible influences of endothelium-derived factors (e.g., relaxing, hyperpolarizing and contracting factors), the endothelium of the strip was removed by gently rubbing the endothelial surface with a cotton swab. Successful removal of the endothelium was confirmed later by the inability of acetylcholine (1 μM) to induce a relaxation in prostaglandin $F_{2\alpha}$-contracted strips.

### 2.2. Measurement of isometric tension

Arterial strips (7–8 mm in length) were mounted vertically in water-jacketed muscle baths containing 10 ml Krebs solution. Krebs solutions were maintained at 37°C and aerated with 95% $O_2$ and 5% $CO_2$. The isometric tension was recorded with a force-displacement transducer (TB-612T, Nihon Kohden Kogyo, Tokyo, Japan). The strips were stretched passively to the optimal length for active tension development by imposing a resting tension of 0.6 g for femoral artery, 0.5 g for mesenteric artery and 0.4 g for carotid artery; a 60-min equilibration period preceded each experiment. The optimal resting tensions were determined by a length-passive tension study (Asano et al., 1988, 1993).

After equilibration, the contractile responses of the strips to 65.9 mM $K^+$ Krebs solution (equimolar substitution of $Na^+$ with $K^+$) were repeated (usually twice) until the responses were reproducible. After washout of the strips with Krebs solution, the contractile responses to elevated extracellular $K^+$ (3–20 mM addition) or norepinephrine ($10^{-9}$ to $10^{-4}$ M) were determined in a cumulative fashion. At the end of the $K^+$-induced contraction, the bathing solution was replaced with 65.9 mM $K^+$ to determine the maximum contraction of the strip. The contractile responses to $K^+$ were measured with phenoxybenzamine (2 μM)-treated strips, to eliminate possible α-adrenoceptor responses to endogenously released norepinephrine, and timolol (0.5 μM) was added to the Krebs solution to eliminate possible β-adrenoceptor responses (Asano et al., 1988). The contractile responses to norepinephrine were measured in the presence of timolol. 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 examine sarcoplasmic reticulum $Ca^{2+}$-ATPase function during $K^+$- and norepinephrine-induced contractions, the effects of preincubation with cyclopiazonic acid (10 μM) or thapsigargin (100 nM) on these contractions were determined. Because each inhibitor itself induced a transient contraction, the possible mechanism underlying this contraction was also examined. In some experiments, the norepinephrine-induced contraction was divided into two components, release of intracellular $Ca^{2+}$ and influx of extracellular $Ca^{2+}$, and the effects of thapsigargin on these components were also determined. In some 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.

The transient contraction induced by 20 mM caffeine was determined to estimate the amount of $Ca^{2+}$ stored in the sarcoplasmic reticulum, since higher concentrations of caffeine (> 20 mM) deplete $Ca^{2+}$ stored in the sarcoplasmic reticulum, as shown in other studies (Leijten and Van Breemen, 1986; Naganobu et al., 1994; Nomura et al., 1996, 1997).

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

$[Ca^{2+}]_i$ was measured as described previously (Nomura et al., 1996). Briefly, strips (0.8 × 8 mm) of carotid arteries were loaded with 10 μM acetoxymethyl ester of fura-PE3 (fura-PE3/AM) under protection from light at 37°C. A non-cytotoxic detergent, cremophor EL (0.03%), was added to increase the solubility of fura-PE3/AM. After being loaded for 2.5–3 h, each strip was mounted horizontally in a temperature-controlled perfusion chamber (approximately 1.2 ml volume) attached to a fluorimeter (CAF-100, JASCO, Tokyo, Japan). One end of the strip was connected to a force-displacement transducer for isometric tension recordings with an optimal resting tension of 0.4 g. The strips were perfused at a rate of 2.5 ml/min with an oxygenated Krebs solution at 37°C, and a 50-min equilibration period preceded each experiment. Changes in $[Ca^{2+}]_i$ during the addition of cyclopiazonic acid and the effects of $Ca^{2+}$-free solution and verapamil 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.

### 2.4. 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

The drugs used were cyclopiazonic acid (Sigma, St. Louis, MO, USA), thapsigargin (Sigma), L-norepinephrine

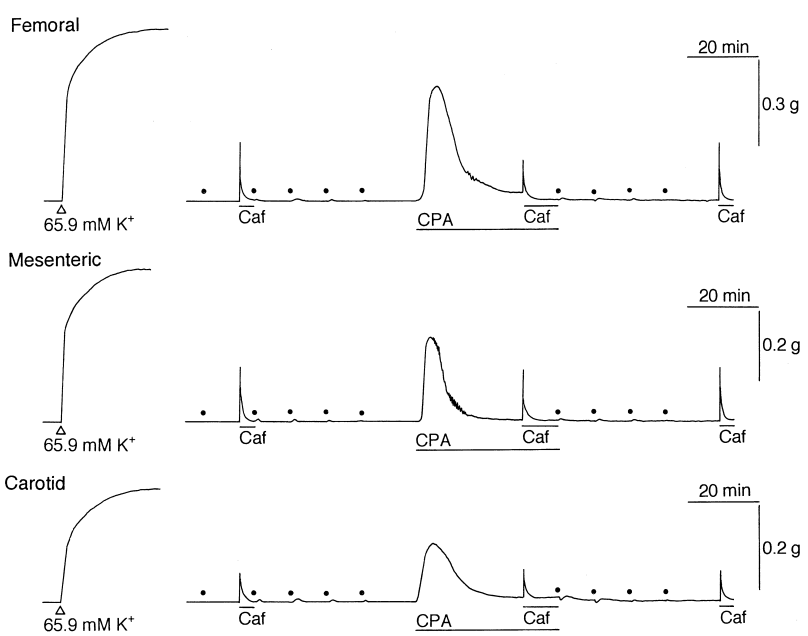


Fig. 1. Typical recordings of the contractions induced by caffeine (Caf; 20 mM) and cyclopiazonic acid (CPA; 10 μM) in strips of femoral, mesenteric and carotid arteries of the rat. After determination of the maximum contraction induced by 65.9 mM KCl ($K^+$), Caf was added for 4 min. Following washout for 45 min, CPA was added for 30 min. In the presence of CPA, Caf was added for 10 min. After washout for 45 min, Caf was added. Dots (·) denote the washout of the strips with Krebs solution.

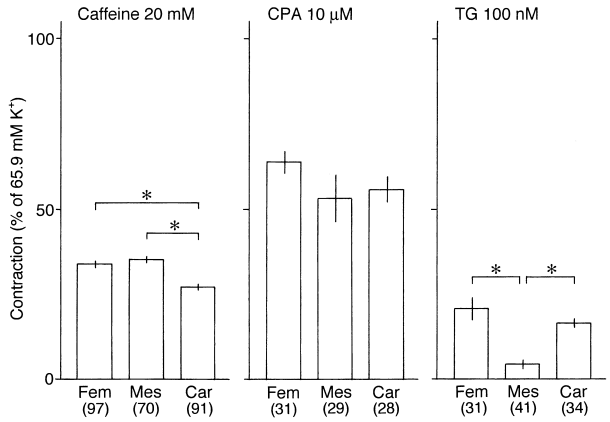


Fig. 2. Comparison of the contractions induced by caffeine (20 mM), cyclopiazonic acid (CPA; 10 μM) and thapsigargin (TG; 100 nM) in strips of femoral (Fem), mesenteric (Mes) and carotid (Car) arteries of the rat. Experimental conditions were the same as in Figs. 1 and 3. Contractions induced by caffeine, CPA and TG are expressed as percentage of the maximum contraction induced by 65.9 mM $K^+$. Data are means of the number of preparations indicated in parentheses (along the bottom of the figure), and S.E.M. are shown by vertical bars. * Significantly different ($P < 0.05$).

bitartrate (Sigma), caffeine (Wako, Osaka, Japan), nifedipine (a kind gift from Bayer Yakuhin, Osaka, Japan), verapamil hydrochloride (a kind gift from Eisai, Tokyo, 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).

Cyclopiazonic acid (5 mM), thapsigargin (100 μM) 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 cyclopiazonic acid- or norepinephrine-induced contractions. Caffeine (20 mM) was dissolved in Krebs solution. Aqueous stock solutions were prepared for other drugs. Concentrations of the drugs are expressed as final molar concentrations.

## 3. Results

### *3.1. Contractions and elevation of $[Ca^{2+}]_i$ induced by cyclopiazonic acid and thapsigargin*

As shown in our previous studies (Nomura et al., 1996, 1997), the addition of 10 μM cyclopiazonic acid caused a relatively transient contraction in the femoral artery (Fig. 1). In the presence of cyclopiazonic acid (30 min), the addition of 20 mM caffeine caused a transient contraction that was 55.8 ± 7.2% ($n = 8$) of the initial control contraction, suggesting that $Ca^{2+}$ stored in the sarcoplasmic reticulum was reduced during the addition of cyclopiazonic acid (Fig. 1). These experiments were repeated in mesenteric and carotid arteries (Fig. 1). When the control caffeine contraction was compared in the three arteries, this contraction was significantly smaller in the carotid

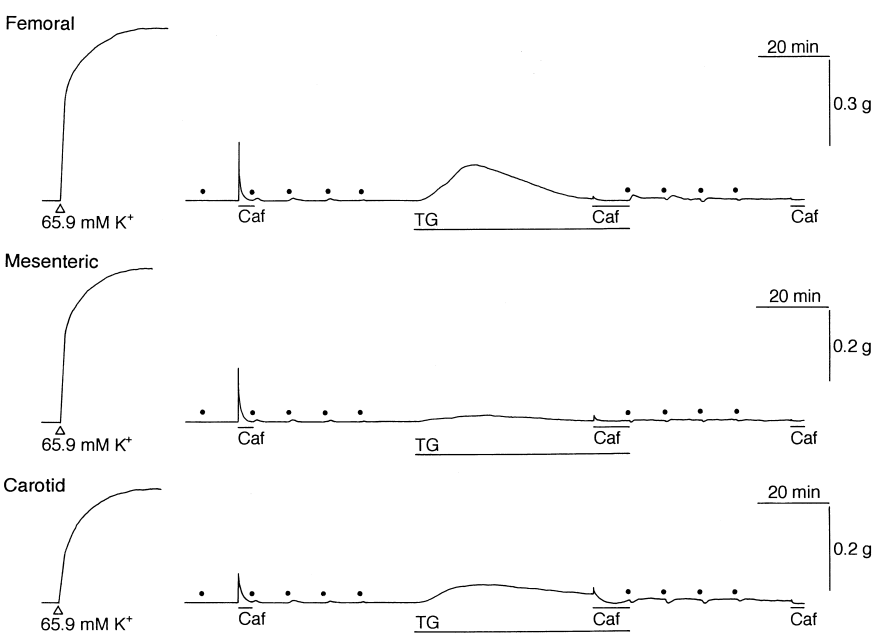


Fig. 3. Typical recordings of the contractions induced by caffeine (Caf; 20 mM) and thapsigargin (TG; 100 nM) in strips of femoral, mesenteric and carotid arteries of the rat. Experimental conditions were the same as in Fig. 1, except that TG was added for 50 min.

artery than in the other arteries (Fig. 2). The cyclopiazonic acid-induced contraction was almost the same in the three arteries (Fig. 2). During the addition of cyclopiazonic acid, the reduction of sarcoplasmic reticulum $Ca^{2+}$ was small in mesenteric and carotid arteries, since the caffeine-induced contraction in the presence of cyclopiazonic acid was 92.9 ± 9.3% (mesenteric, $n = 8$) and 86.3 ± 5.4% (carotid, $n = 8$), respectively, of the control contraction. The caffeine contraction was not further attenuated after 50 min of cyclopiazonic acid treatment. After washout, the caffeine-induced contraction was restored to the control level in the three arteries (Fig. 1).

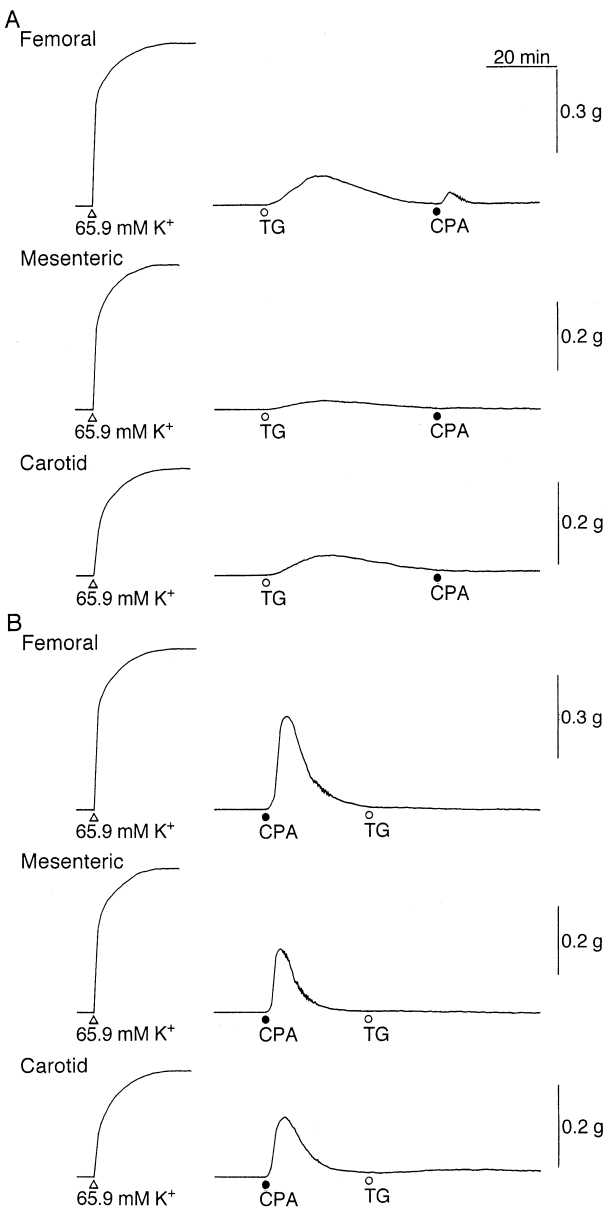


Fig. 4. Typical recordings of the contractions induced by a combination of thapsigargin (TG; 100 nM) and cyclopiazonic acid (CPA; 10 μM). (A) After the 50-min treatment with TG, CPA was added. The CPA-induced contraction was 7.5 ± 3.6% (femoral, $n = 7$), 0.4 ± 0.4% (mesenteric, $n = 4$) and 0.1 ± 0.1% (carotid, $n = 4$), respectively, of the maximum contraction induced by 65.9 mM $K^+$. Each contraction was significantly less than the respective control contraction shown in Fig. 2. (B) After the 30-min treatment with CPA, TG was added. The TG-induced contraction was 0.1 ± 0.1% (femoral, $n = 7$), 0.5 ± 0.5% (mesenteric, $n = 4$) and 3.1 ± 0.6% (carotid, $n = 4$), respectively, of the maximum contraction induced by 65.9 mM $K^+$.

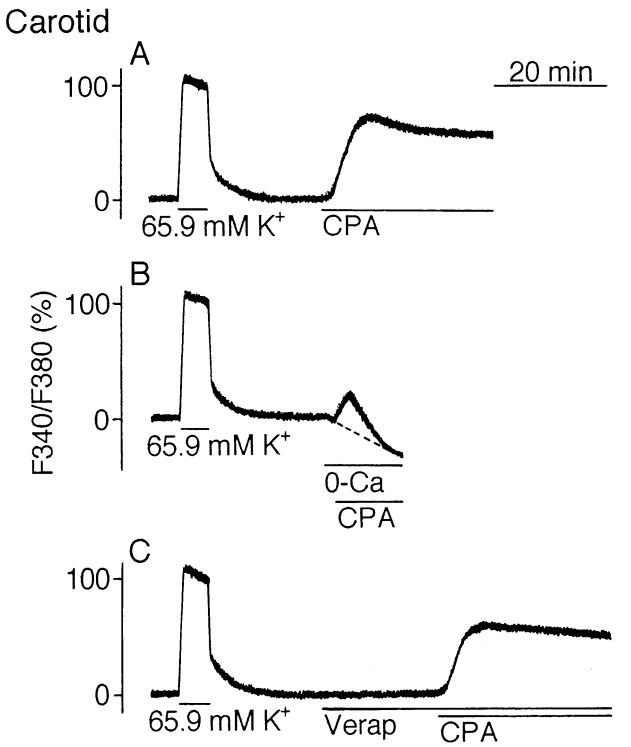


Fig. 5. Typical recordings of the cyclopiazonic acid (CPA; 10 μM)-induced elevation of $[Ca^{2+}]_i$ (indicated by F340/F380) and the effects of $Ca^{2+}$-free solution (0-Ca) and verapamil (Verap; 3 μM) on this elevation in strips of rat carotid artery. (A) Control response of the CPA-induced elevation of $[Ca^{2+}]_i$. After loading with fura-PE3, the strips were exposed to 65.9 mM $K^+$ for 5 min. Following washout with Krebs solution for 20 min, CPA was added. (B) The effect of $Ca^{2+}$-free solution. As shown in Table 1, CPA was added after the 2-min exposure to a $Ca^{2+}$-free solution. Broken line indicates the decrease in resting $[Ca^{2+}]_i$ induced by $Ca^{2+}$-free solution itself. (C) The effect of verapamil. Verapamil was added 20 min before the addition of CPA.

Similar experiments with thapsigargin were also performed in the three arteries (Fig. 3). The addition of 100 nM thapsigargin also caused a relatively transient contraction in the three arteries. In the presence of thapsigargin (50 min), the addition of 20 mM caffeine caused a very small contraction, suggesting that the sarcoplasmic reticulum $Ca^{2+}$ was almost depleted during the addition of thapsigargin. After washout, the caffeine-induced contraction was not restored (Fig. 3). The thapsigargin-induced contraction was significantly larger in femoral and carotid arteries than in the mesenteric artery (Fig. 2).

To determine whether cyclopiazonic acid and thapsigargin caused a contraction by acting at sarcoplasmic reticulum $Ca^{2+}$-ATPase, the contractile effect of cyclopiazonic acid (10 μM) in the presence of thapsigargin (100 nM) was determined in the three arteries (Fig. 4). As shown in

Table 1
Effects of $Ca^{2+}$-free solution and nifedipine on cyclopiazonic acid (CPA)-, thapsigargin (TG)- and caffeine-induced contractions in strips of mesenteric and carotid arteries of the rat

| Condition | Contraction (% of 65.9 mM $K^+$)[a] | |
|---|---|---|
| | Mesenteric | Carotid |
| (A) CPA 10 μM (Control) | 53.6±7.2 (29) | 55.9±3.8 (28) |
| $Ca^{2+}$-free solution[b] | 0.3±0.2[c] (4) | 0.3±0.2[c] (5) |
| +Nifedipine 100 nM[d] | 6.4±3.3[c] (6) | 36.6±2.6[c,e] (9) |
| +Nifedipine 1 μM[d] | 5.7±2.2[c] (6) | 38.0±3.6[c,e] (6) |
| (B) TG 100 nM (Control) | 4.4±1.4 (41) | 16.5±1.0[e] (34) |
| $Ca^{2+}$-free solution[b] | N.D.[f] | 0.3±0.2[c] (4) |
| +Nifedipine 100 nM[d] | N.D.[f] | 11.3±0.9[c] (6) |
| (C) Caffeine 20 mM (Control) | 34.6±3.0 (7) | 26.5±2.3[e] (8) |
| $Ca^{2+}$-free solution[b] | 18.2±1.4[c] (6) | 19.0±2.0[c] (8) |
| +Nifedipine 100 nM[d] | 29.2±2.3 (7) | 22.6±1.9[e] (8) |

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

[a] Contractions induced by CPA (10 μM), TG (100 nM) and caffeine (20 mM) are expressed as % of the maximum contraction induced by 65.9 mM $K^+$.

[b] After the 2-min exposure to a $Ca^{2+}$-free solution, CPA, TG or caffeine was added.

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

[d] Nifedipine was added 20 min before the addition of CPA, TG or caffeine.

[e] Significantly different from the mesenteric artery ($P < 0.05$).

[f] N.D., not determined.

Fig. 4A, after the 50-min treatment with thapsigargin, the addition of cyclopiazonic acid almost failed to cause a

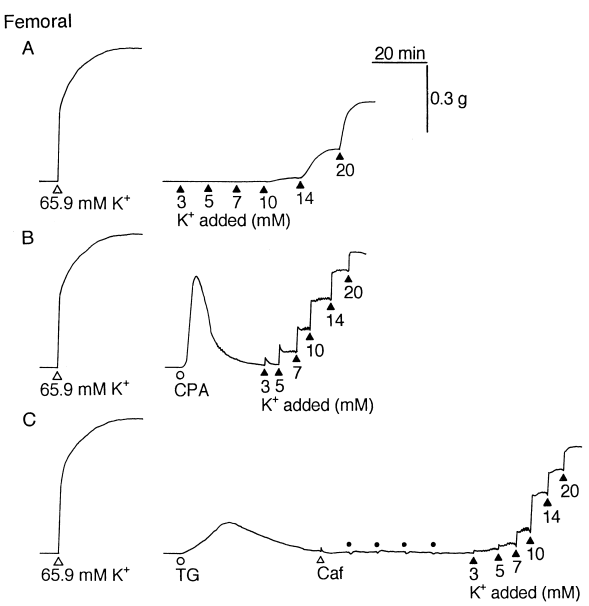


Fig. 6. Typical recordings of the effects of cyclopiazonic acid (CPA; 10 μM) and thapsigargin (TG; 100 nM) on the contractile responses to elevated extracellular $K^+$ in strips of rat femoral artery. (A) Control response. After determination of the maximum contraction induced by 65.9 mM KCl ($K^+$), the contractile responses to $K^+$ (3–20 mM addition) were determined in a cumulative fashion. Effects of preincubation with CPA (10 μM) and treatment with TG (100 nM) plus caffeine (Caf; 20 mM) on the $K^+$-induced contractions are shown in (B) and (C), respectively. (B) CPA was added 35 min before the addition of $K^+$. (C) The TG treatment was done as in Fig. 3.

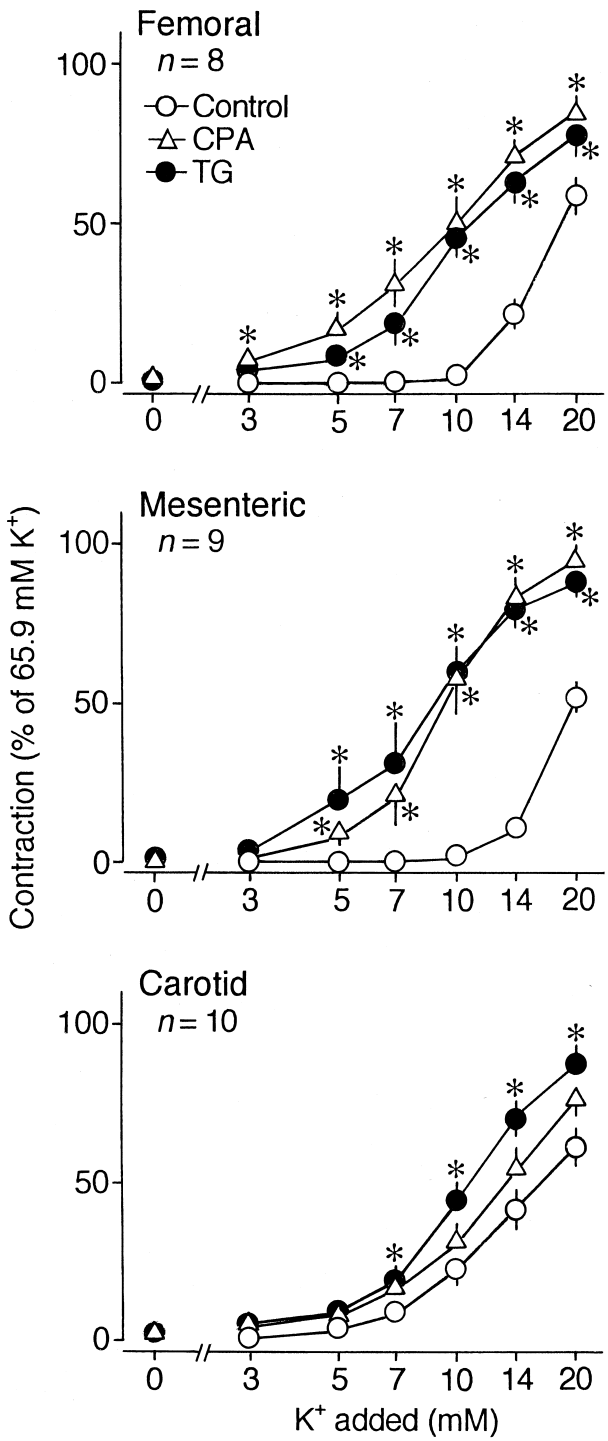


Fig. 7. Effects of cyclopiazonic acid (CPA; 10 μM) and thapsigargin (TG; 100 nM) on the concentration–response curve for $K^+$ in strips of femoral, mesenteric and carotid arteries of the rat. Experimental conditions were the same as in Fig. 6. The peak contractions induced by each concentration of $K^+$ are expressed as percentage of the maximum contraction induced by 65.9 mM $K^+$. At '0' on the abscissa, the CPA- and TG-induced contractions remaining before the addition of $K^+$ are expressed as percentage 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 the respective Control ($P < 0.05$).

contraction. Moreover, after the 30-min treatment with cyclopiazonic acid, the addition of thapsigargin failed to cause a contraction (Fig. 4B). These results strongly suggest that the two inhibitors cause a contraction by acting at the same site: the sarcoplasmic reticulum $Ca^{2+}$-ATPase.

As shown previously (Nomura et al., 1996), the cyclopiazonic acid-induced elevation of $[Ca^{2+}]_i$ was relatively sustained in the femoral artery in spite of the transient contraction. The same result was obtained in the carotid artery (Fig. 5A). The addition of 10 μM cyclopiazonic acid caused an initial peak followed by a sustained elevation of $[Ca^{2+}]_i$ in the carotid artery, where the cyclopiazonic acid-induced contraction was transient, as shown in Fig. 1. The peak elevation of $[Ca^{2+}]_i$ and at 30 min after the addition of cyclopiazonic acid was 72.4 ± 6.6% ($n = 4$) and 55.9 ± 5.4% ($n = 4$), respectively, of the response to 65.9 mM $K^+$. These values are similar to those for the femoral artery (Nomura et al., 1996).

### 3.2. Effects of $Ca^{2+}$-free solution and nifedipine on cyclopiazonic acid- and thapsigargin-induced contractions

As shown previously (Nomura et al., 1996), the cyclopiazonic acid- and thapsigargin-induced contractions in the femoral artery were abolished by $Ca^{2+}$-free solution and were strongly inhibited by 100 nM nifedipine, suggesting that the contractions were due to an influx of extracellular $Ca^{2+}$ via L-type $Ca^{2+}$ channels. These experiments were performed in mesenteric and carotid arteries (Table 1). The cyclopiazonic acid-induced contractions in these arteries also were abolished by a 2-min exposure to a $Ca^{2+}$-free solution. Although the contractions were inhibited by 100 nM nifedipine, the extent of the inhibition was different in the two arteries: 90% inhibition in the mesenteric artery but only 30% inhibition in the carotid artery (Table 1A). A similar inhibition was obtained for the thapsigargin-induced contraction in the carotid artery (Table 1B). The caffeine-induced contractions in the two arteries were not abolished (but were reduced) by $Ca^{2+}$-free solution and were not inhibited by nifedipine (Table 1C).

Table 2
Effects of cyclopiazonic acid (CPA) and thapsigargin (TG) 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) |
| +CPA 10 μM | 7.2 ± 1.1[d] (8) | 8.1 ± 0.8[d] (9) | 9.9 ± 0.8 (10) |
| +TG 100 nM | 8.2 ± 0.5[d] (8) | 7.1 ± 0.8[d] (9) | 8.3 ± 0.4[d] (10) |
| *(B) $pD_2$ for NE (− log M)*[e] | | | |
| Control | 6.82 ± 0.15 (8) | 7.17 ± 0.08 (8) | 8.15 ± 0.09[c] (6) |
| +CPA 10 μM | 7.76 ± 0.17[d] (8) | 7.63 ± 0.10[d] (8) | 8.11 ± 0.10 (6) |
| +TG 100 nM | 7.88 ± 0.11[d] (8) | 7.82 ± 0.11[d] (8) | 8.17 ± 0.10 (6) |

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

[a] Experimental conditions were the same as in Figs. 6–8.

[b] The $EC_{30}$ values for $K^+$ were calculated from the concentration–response curves shown in Fig. 7, 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. 8, and are expressed as a negative log of the molar concentration.

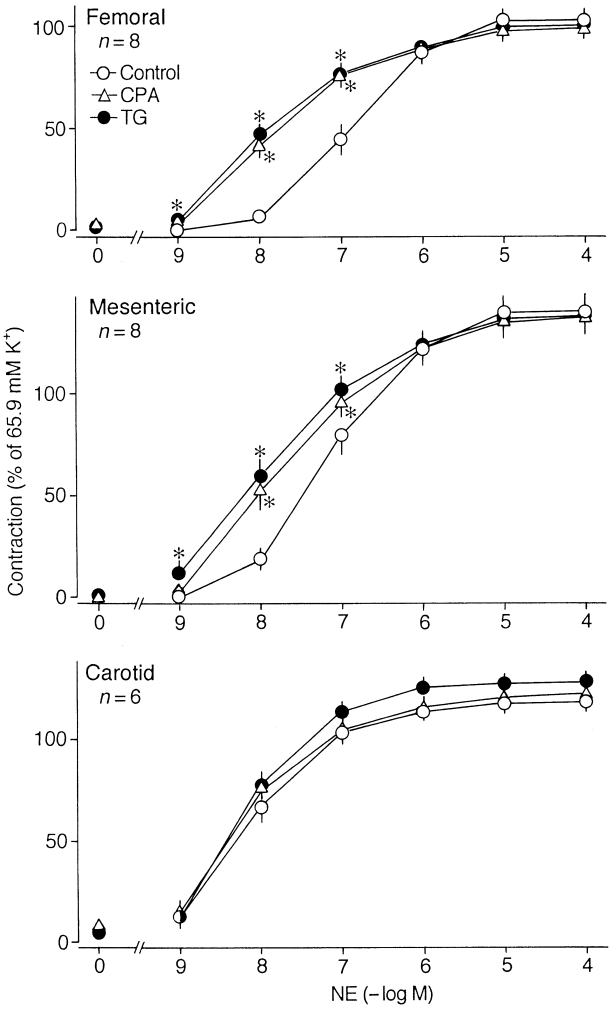


Fig. 8. Effects of cyclopiazonic acid (CPA; 10 μM) and thapsigargin (TG; 100 nM) on the concentration–response curve for norepinephrine (NE) in strips of femoral, mesenteric and carotid arteries from the rat. Experimental conditions were the same as in Figs. 6 and 7, except that NE was used as a contractile agonist. The peak contractions induced by each concentration of NE are expressed as percentage of the maximum contraction induced by 65.9 mM $K^+$. At '0' on the abscissa, the CPA- and TG-induced contractions remaining before the addition of NE are expressed as percentage 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 the respective Control ($P < 0.05$).

### 3.3. Effects of $Ca^{2+}$-free solution and verapamil on cyclopiazonic acid-induced elevation of $[Ca^{2+}]_i$

As also shown previously (Nomura et al., 1996), the cyclopiazonic acid-induced elevation of $[Ca^{2+}]_i$ in the

femoral artery was inhibited by $Ca^{2+}$-free solution and verapamil, but the inhibition of the $[Ca^{2+}]_i$ was smaller than that of the contraction. These experiments were performed in the carotid artery (Fig. 5). In a $Ca^{2+}$-free solution, cyclopiazonic acid induced a small transient elevation of $[Ca^{2+}]_i$ (Fig. 5B). This pattern of the elevation was similar to that in the femoral artery (Nomura et al., 1996). Although in the presence of 3 μM verapamil the cyclopiazonic acid-induced elevation of $[Ca^{2+}]_i$ tended to be decreased ($64.6 \pm 7.0\%$ of the response to 65.9 mM $K^+$, $n = 4$), this verapamil effect was not statistically significant (Fig. 5C). This effect was different from the effect in the femoral artery (Nomura et al., 1996). The decrease in the resting $[Ca^{2+}]_i$ produced by verapamil itself was smaller in the carotid artery ($1.9 \pm 1.1\%$ decrease of the response to 65.9 mM $K^+$, $n = 4$) than in the femoral artery ($11.9 \pm 1.5\%$ decrease, $n = 24$, Nomura et al., 1996).

### 3.4. Effects of cyclopiazonic acid and thapsigargin on $K^+$-induced contraction

The addition of $K^+$ (3–20 mM) caused a concentration-dependent contraction at relatively high concentrations in the femoral artery with an $EC_{30}$ value of 15.8 mM (Figs. 6A and 7, Table 2A). The effect of 10 μM cyclopiazonic acid on this contraction was then determined (Figs. 6B and 7, Table 2A). After the cyclopiazonic acid-induced contraction had declined and reached a plateau (35 min), addition of $K^+$ caused a strong contraction with an $EC_{30}$ value of 7.2 mM. This was the maximum augmenting effect of cyclopiazonic acid, because a similar response to $K^+$ ($EC_{30}$: $7.7 \pm 0.5$ mM, $n = 5$) was also observed in the presence of 3 μM cyclopiazonic acid. Similar results were obtained when 100 or 30 nM thapsigargin was used (Figs. 6C and 7, Table 2A). The maximum contraction induced by 65.9 mM $K^+$ was not affected by cyclopiazonic acid or thapsigargin. Thus, the concentration–response curve for $K^+$ in the femoral artery was shifted to the left by cyclopiazonic acid or thapsigargin (Fig. 7).

These experiments were repeated in mesenteric and carotid arteries (Fig. 7, Table 2A). Although similar results were obtained in the mesenteric artery, the results in the carotid artery were different. The control $EC_{30}$ value for $K^+$ in the carotid artery (11.3 mM) was significantly smaller than that in the other arteries (Table 2A). Although the concentration–response curve for $K^+$ in the carotid artery was shifted to the left by cyclopiazonic acid or thapsigargin, the extent of the leftward shift was much smaller in the carotid artery than in the other arteries (Fig. 7, Table 2A). Thus, the sensitivity to $K^+$ for inducing a contraction was significantly different in the three arteries under normal conditions, and became similar after sarcoplasmic reticulum $Ca^{2+}$-ATPase inhibition (Table 2A).

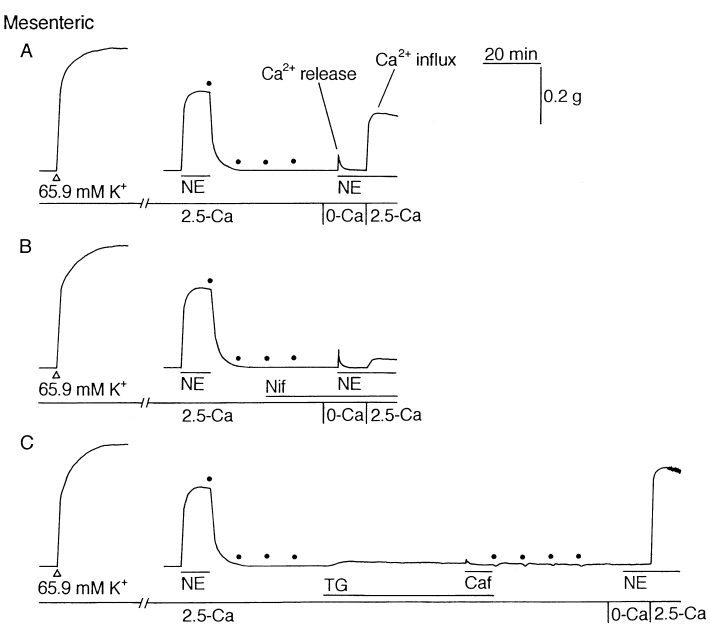


Fig. 9. Typical recordings of the effects of nifedipine (Nif; 100 nM) and thapsigargin (TG; 100 nM) on $Ca^{2+}$ release and $Ca^{2+}$ influx induced by norepinephrine (NE) in strips of rat mesenteric artery. After determination of the maximum contraction induced by 65.9 mM $K^+$, $7 \times 10^{-8}$ 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, $7 \times 10^{-8}$ 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 the replacement of the solution. (C) The effect of TG (100 nM). The TG treatment was done as in Fig. 3, and then NE and 2.5 mM $Ca^{2+}$ were added as in (A). Dots (·) denote the washout of the strips with Krebs solution.

### 3.5. Effects of cyclopiazonic acid and thapsigargin on norepinephrine-induced contraction

Since the sustained phase of the norepinephrine-induced contraction is due to an influx of extracellular $Ca^{2+}$, the effects of cyclopiazonic acid and thapsigargin on this contraction were determined. The addition of norepinephrine ($10^{-9}$ to $10^{-4}$ M) caused a concentration-dependent contraction in the femoral artery with a $pD_2$ value of 6.82 (Fig. 8, Table 2B). The effect of 10 μM cyclopiazonic acid on this contraction was then determined (Fig. 8, Table 2B). After the cyclopiazonic acid-induced contraction had reached a plateau (35 min), addition of norepinephrine caused a contraction with a $pD_2$ value of 7.76. Similar data were obtained when 100 nM thapsigargin was used (Fig. 8, Table 2B). Thus, the concentration–response curve for norepinephrine in the femoral artery was also shifted to the left by cyclopiazonic acid or thapsigargin (Fig. 8).

These experiments were repeated in mesenteric and carotid arteries (Fig. 8, Table 2B). Although similar results were obtained for the mesenteric artery, results for the carotid artery were different. The control $pD_2$ value for norepinephrine in the carotid artery (8.15) was significantly larger than that in the other arteries (Table 2B). In the carotid artery, cyclopiazonic acid and thapsigargin failed to augment the norepinephrine-induced contractions (Fig. 8, Table 2B).

Again, the norepinephrine sensitivity was significantly different in the three arteries under normal conditions, and became similar after sarcoplasmic reticulum $Ca^{2+}$-ATPase inhibition (Table 2B).

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

When extracellular $Ca^{2+}$ was removed by using a $Ca^{2+}$-free solution (5 min), norepinephrine at $7 \times 10^{-8}$ M (the concentration that produced 50% of the maximum contraction as shown in Table 2) induced a transient contraction due to the release of intracellular $Ca^{2+}$ in the mesenteric artery (Figs. 9A and 10). Under the same conditions, 65.9 mM $K^+$ failed to induce a contraction (data not shown). After the norepinephrine-induced transient contraction had ceased (10 min), the addition of 2.5 mM $Ca^{2+}$ caused a sustained contraction due to an influx of extracellular $Ca^{2+}$ (Figs. 9A and 10). When 100 nM nifedipine was added during this procedure, the sustained contraction, but not the transient one, was inhibited (Figs. 9B and 10). After thapsigargin treatment, the transient contraction was abolished, and the sustained contraction was strongly augmented (Figs. 9C and 10). This augmented contraction was also inhibited by 100 nM nifedipine (Fig. 10).

Although similar results were obtained for the carotid artery, the extent of the augmentation by thapsigargin of the sustained contraction was smaller in the carotid artery than in the mesenteric artery (Fig. 10).

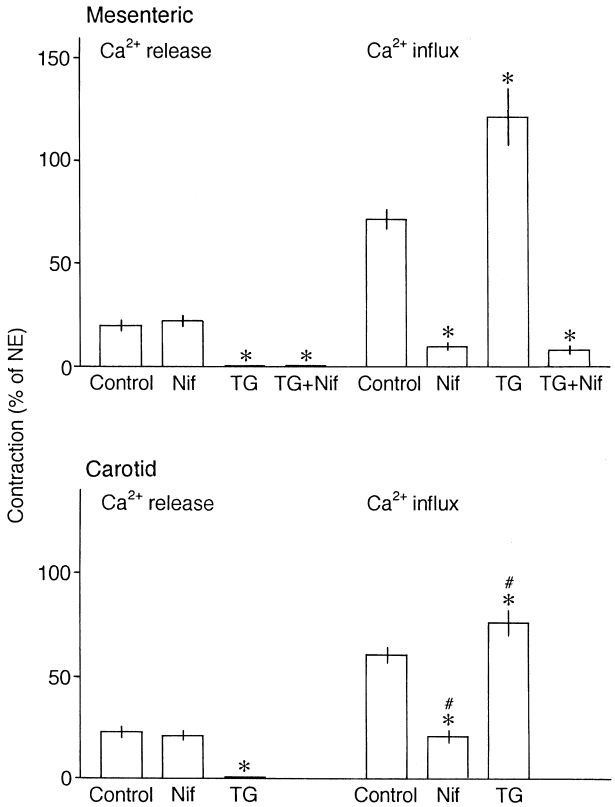


Fig. 10. Effects of nifedipine (Nif; 100 nM) and thapsigargin (TG; 100 nM) on $Ca^{2+}$ release and $Ca^{2+}$ influx induced by norepinephrine (NE) in strips of mesenteric and carotid arteries of the rat. Experimental conditions were the same as in Fig. 9. To induce 50% of the maximum contraction, $7 \times 10^{-8}$ M NE (mesenteric) or $7 \times 10^{-9}$ M NE (carotid) was used. The contractions due to $Ca^{2+}$ release and $Ca^{2+}$ influx shown in Fig. 9 are expressed as percentage of the control NE contraction determined prior to these contractions. 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 mesenteric artery ($P < 0.05$).

## 4. Discussion

The present study suggested that during arterial contraction evoked by $K^+$ or norepinephrine, the sarcoplasmic reticulum serves as a buffer barrier to $Ca^{2+}$ entry by taking up $Ca^{2+}$ into the sarcoplasmic reticulum by sarcoplasmic reticulum $Ca^{2+}$-ATPase, and that this function is quite different in femoral, mesenteric and carotid arteries. We conclude that sarcoplasmic reticulum $Ca^{2+}$-ATPase function during contraction is an important factor in determining the sensitivity of the artery to contractile agonists.

According to the superficial buffer barrier hypothesis (Van Breemen and Saida, 1989; Chen et al., 1992; Van Breemen et al., 1995; Laporte and Laher, 1997), the sarcoplasmic reticulum serves as a buffer barrier to $Ca^{2+}$ entry, based on mechanisms of $Ca^{2+}$ uptake into the

sarcoplasmic reticulum and unloading (extrusion) of $Ca^{2+}$ into the extracellular space. This hypothesis was derived from the observation that the magnitude of vascular smooth muscle contraction depends on the rate, rather than on the net amount, of $Ca^{2+}$ influx (Van Breemen, 1977), and was confirmed later by a number of studies using cyclopiazonic acid and thapsigargin (for details, see Nomura et al., 1996, 1997; Asano et al., 1998). As described in the Introduction, the effects of cyclopiazonic acid or thapsigargin on the contractile responses to $K^+$ or norepinephrine are complicated and not always consistent among different arteries. A strong $Ca^{2+}$ buffering function of the sarcoplasmic reticulum on Bay k 8644-induced $Ca^{2+}$ influx was demonstrated in the rat femoral artery (Asano and Nomura, 1999). In the present study, we hypothesized that sarcoplasmic reticulum $Ca^{2+}$-ATPase function differs in arteries and that this function is reflected by the sensitivity of the artery to a contractile agonist. The prediction was that if sarcoplasmic reticulum $Ca^{2+}$-ATPase function is increased during stimulation with an agonist, a larger fraction of $Ca^{2+}$ that enters cells can be taken up into the sarcoplasmic reticulum and so lead to a relatively low sensitivity to the agonist (a weak contraction with the agonist). In contrast, if sarcoplasmic reticulum $Ca^{2+}$-ATPase function is weak, a large part of $Ca^{2+}$ that enters cells bypasses the sarcoplasmic reticulum and reaches the myofilaments, which results in a high sensitivity to the agonist (a strong contraction with the agonist). Therefore, with the sarcoplasmic reticulum $Ca^{2+}$-ATPase inhibition evoked by cyclopiazonic acid or thapsigargin, the extent of the augmentation of the contraction differs in the arteries. This is clearly shown in the present study. Thus, the extent of the leftward shift of the concentration–response curve for the agonist by cyclopiazonic acid or thapsigargin is a rough index of sarcoplasmic reticulum $Ca^{2+}$-ATPase function during contraction.

The elevation of extracellular $K^+$ induces a contraction that is due to an influx of extracellular $Ca^{2+}$ via L-type $Ca^{2+}$ channels. Under normal conditions, the sensitivity to $K^+$ was higher in the carotid artery than in the other arteries. After sarcoplasmic reticulum $Ca^{2+}$-ATPase inhibition, the extent of the leftward shift of the concentration–response curve for $K^+$ was smaller in the carotid artery than in the other arteries. Thus, sarcoplasmic reticulum $Ca^{2+}$-ATPase function during $K^+$ stimulation was weak in the carotid artery when compared with that in the other arteries. Following inhibition, the sensitivity to $K^+$ was the same in the three arteries. From these observations, we conclude that, during $K^+$ stimulation, sarcoplasmic reticulum $Ca^{2+}$-ATPase function is an important factor in the sensitivity to $K^+$.

This conclusion is also based on the results for norepinephrine-induced contractions. Agonist stimulation of α-adrenoceptors frequently induces a biphasic contraction; the initial phase is due to the release of $Ca^{2+}$ from sarcoplasmic reticulum, while the sustained phase is due to the influx of extracellular $Ca^{2+}$. When the effects of thapsigargin on the sustained phase of the norepinephrine contraction were compared in mesenteric and carotid arteries, the augmentation of the response by thapsigargin was smaller in the carotid artery. Again, sarcoplasmic reticulum $Ca^{2+}$-ATPase function during norepinephrine stimulation was weak in the carotid artery.

A possible explanation for the weak sarcoplasmic reticulum $Ca^{2+}$-ATPase function seen in the carotid artery is that the sarcoplasmic reticulum of this artery is small and is almost filled to capacity with $Ca^{2+}$ in the resting state. This assumption comes from the following observations: (1) sarcoplasmic reticulum $Ca^{2+}$-ATPase function during stimulation with $K^+$ or norepinephrine is weak, as described above, (2) sarcoplasmic reticulum $Ca^{2+}$-ATPase function in the resting state is enough to buffer basal $Ca^{2+}$ entry, as assessed by the cyclopiazonic acid- and thapsigargin-induced contractions, and (3) the capacity of sarcoplasmic reticulum to store $Ca^{2+}$ is small, as assessed by the caffeine-induced contraction. The present study also examined sarcoplasmic reticulum $Ca^{2+}$-ATPase function in the resting state of the three arteries by measuring the contractile effects of cyclopiazonic acid and thapsigargin. As reported previously (Nomura et al., 1996, 1997; Asano et al., 1998), the contractile effects of cyclopiazonic acid and thapsigargin reflect the buffering function of sarcoplasmic reticulum $Ca^{2+}$ ATPase against basal $Ca^{2+}$ entry. Moreover, as shown in Fig. 4, cyclopiazonic acid and thapsigargin caused a contraction by acting at the same site. As shown in Figs. 1–3, the contractile effect of cyclopiazonic acid or thapsigargin was not weak in the carotid artery when compared with that in the other arteries. Moreover, the cyclopiazonic acid-induced elevation of $[Ca^{2+}]_i$ in the carotid artery was similar to that in the femoral artery (Nomura et al., 1996). These results suggest that sarcoplasmic reticulum $Ca^{2+}$ ATPase function in the resting state of the carotid artery is not plentiful but is sufficient to buffer basal $Ca^{2+}$ entry. As estimated from the contractile responses to caffeine, the amount of $Ca^{2+}$ stored in the sarcoplasmic reticulum was smaller in the carotid artery than in the other arteries. This suggests that the capacity of sarcoplasmic reticulum to store $Ca^{2+}$ was relatively small in the carotid artery.

According to the superficial buffer barrier hypothesis (Van Breemen and Saida, 1989; Chen et al., 1992; Van Breemen et al., 1995; Laporte and Laher, 1997), sarcoplasmic reticulum $Ca^{2+}$-ATPase function depends on the state of $Ca^{2+}$ loading of the sarcoplasmic reticulum; an empty sarcoplasmic reticulum will buffer $Ca^{2+}$ entry whereas an overloaded sarcoplasmic reticulum cannot. Therefore, it is likely that in femoral and mesenteric arteries, sarcoplasmic reticulum $Ca^{2+}$ uptake does not reach the maximum level in the resting state, so that a fraction of $Ca^{2+}$ that enters cells during stimulation with $K^+$ or norepinephrine is still taken up into the sarcoplasmic reticulum, resulting in a relatively weak contrac-

tion with the agonist. When sarcoplasmic reticulum $Ca^{2+}$-ATPase is inhibited by cyclopiazonic acid or thapsigargin, $Ca^{2+}$ that enters cells can bypass the sarcoplasmic reticulum and reach the myofilaments, resulting in increased contraction, as proposed by us earlier (Asano and Nomura, 1999) on the basis of the $Ca^{2+}$ buffering function of the sarcoplasmic reticulum on Bay k 8644-induced $Ca^{2+}$ influx in the femoral artery. However, in the carotid artery, the sarcoplasmic reticulum $Ca^{2+}$ uptake has already reached a near maximum level in the resting state, so that this sarcoplasmic reticulum cannot buffer the $Ca^{2+}$ that has entered, resulting in a strong contraction with the agonist, and cyclopiazonic acid and thapsigargin failed to cause a further augmentation of the contraction. In addition, the spontaneous $Ca^{2+}$ leak from the sarcoplasmic reticulum is lower in the carotid artery, based on the lower reduction of the caffeine-induced contraction in a $Ca^{2+}$-free solution in the carotid artery, than in the mesenteric artery (Table 1). Altogether, we consider that, in the resting state of carotid artery, the sarcoplasmic reticulum is filled to capacity with $Ca^{2+}$.

We have previously demonstrated the existence of a relatively large compartment of cytosolic $Ca^{2+}$ that does not contribute to contraction during the addition of cyclopiazonic acid or thapsigargin to the femoral artery (Nomura et al., 1996). This $Ca^{2+}$ compartment was also observed in the carotid artery of the present study. Cyclopiazonic acid induced a sustained elevation of $[Ca^{2+}]_i$, but the contraction was relatively transient, suggesting the cytosolic localization of $Ca^{2+}$. This assumption was also supported by the finding that $Ca^{2+}$-free solution or nifedipine (or verapamil) inhibited the cyclopiazonic acid-induced contraction more strongly than the cyclopiazonic acid-induced elevation of $[Ca^{2+}]_i$. These contraction-independent cytosolic $Ca^{2+}$ compartments probably reflect the $Ca^{2+}$ gradient localized between the sarcoplasmic reticulum and plasma membrane during the addition of cyclopiazonic acid, as already demonstrated in the femoral artery (Nomura et al., 1996). When the source of the cyclopiazonic acid-induced elevation of $[Ca^{2+}]_i$ was analyzed from the data shown in Fig. 5, a large part of the elevated $[Ca^{2+}]_i$ was due to $Ca^{2+}$ influx from the extracellular space; approximately 10% of the $Ca^{2+}$ entered the cell via L-type $Ca^{2+}$ channels and the rest entered the cell via other pathways (e.g., leak pathway). These percentages in the carotid artery were quite different from those in the femoral artery (Nomura et al., 1996).

Altogether, our results suggest that (1) the $Ca^{2+}$ influx during stimulation with $K^+$ or norepinephrine is buffered by sarcoplasmic reticulum $Ca^{2+}$-ATPase in femoral and mesenteric arteries, (2) this function is weak in the carotid artery, probably because the sarcoplasmic reticulum of this artery is almost filled to capacity with $Ca^{2+}$ in the resting state, and (3) sarcoplasmic reticulum $Ca^{2+}$-ATPase function during contraction is reflected by the contractile sensitivity of these arteries.

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