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Ca^{2+} buffering action of sarcoplasmic reticulum on Bay k 8644-induced Ca^{2+} influx in rat femoral arterial smooth muscle

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Abstract

We examined the Ca^{2+} buffering action of sarcoplasmic reticulum during the stimulation of arterial smooth muscle with Bay k 8644 [methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate]. The effects of Bay k 8644 on tension and cellular Ca^{2+} level were first determined in endothelium-denuded strips of rat femoral artery. The Ca^{2+} buffering action was examined by using cyclopiazonic acid and thapsigargin to inhibit Ca^{2+}-ATPase of sarcoplasmic reticulum and ryanodine to deplete Ca^{2+} stored in sarcoplasmic reticulum. The addition of Bay k 8644 (0.3–300 nM) to the resting strips almost failed to cause a contraction. When the strips were preincubated with 10 μM cyclopiazonic acid, Bay k 8644 induced a concentration-dependent contraction that is antagonized by nifedipine. The maximum contraction induced by Bay k 8644 in the presence of cyclopiazonic acid was comparable to the maximum contraction induced by 65.9 mM K^+-depolarization and the ED_{50} value for Bay k 8644 was around 5 nM. Similar results were obtained when the strips were preincubated with 30 nM thapsigargin or 10 μM ryanodine. Bay k 8644 also induced a strong contraction when the extracellular K^+ concentration was elevated. During the stimulation with 100 nM Bay k 8644, the Ca^{2+} influx was increased. We conclude that in rat femoral arterial smooth muscle, (1) the Ca^{2+} influx induced by Bay k 8644 is completely buffered by Ca^{2+} uptake into the sarcoplasmic reticulum, and (2) this sarcoplasmic reticulum can buffer a large amount of Ca^{2+} that induces a maximum contraction.

Keywords: 'Superficial buffer barrier' hypothesis; Sarcoplasmic reticulum; Ca^{2+}-ATPase; Ca^{2+} level, cellular; Ca^{2+} influx; Ca^{2+} channel, L-type; Arterial contraction

1. Introduction

L-type Ca^{2+} channels are heteromultimetric proteins that play important roles in the cardiovascular system (Triggle, 1990; McDonald et al., 1994; Hockerman et al., 1997). These channels are activated at relatively high voltages, conduct large, relatively long-lasting currents and are sensitive to dihydropyridines. The dihydropyridines act on the specific receptors of L-type Ca^{2+} channels as either antagonists favoring the inactivated states or agonists favoring the open state. Nifedipine is a typical dihydropyridine receptor antagonist that inhibits Ca^{2+} influx via L-type Ca^{2+} channels and thus used as a valuable tool to investigate the function of these channels in a variety of tissues. Due to the potent vasodilator properties, nifedipine and related dihydropyridines have already gained wide use in the treatment of hypertension. Bay k 8644 [methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate] is a typical dihydropyridine receptor agonist that increases the Ca^{2+} influx through the increase in the open probability of the channel. This agonist is therefore expected to possess potent vasoconstrictor properties that are antagonized by nifedipine. However, the first report by Schramm et al. (1983) showed that Bay k 8644 did not induce a contraction in rabbit aortic strips unless the K^+ concentration in the bathing solution was increased to 15 mM. A need of such preactivation has been confirmed later in many studies, while some studies clearly showed the Bay k 8644-induced strong contraction without the preactivation (for details, see Asano et al., 1987; Wanstall and O'Donnell, 1989). Thus, the inability of Bay k 8644 to induce a contraction may indicate two

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opposite possibilities: (1) Ca^{2+} influx induced by Bay k 8644 is small, or (2) the influx is large but the entered Ca^{2+} is completely buffered by Ca^{2+} uptake into the sarcoplasmic reticulum.

By measuring the $^{45}Ca^{2+}$ influx, Hwang and Van Breemen (1985) noted a marked disparity between Bay k 8644-induced tension and net Ca^{2+} content; under the resting condition (5 mM K^+), Bay k 8644 did not induce tension but did induce net $^{45}Ca^{2+}$ gain in the caffeine-releasable sarcoplasmic reticulum. These authors concluded that the superficial sarcoplasmic reticulum accumulates Ca^{2+} entering through the L-type Ca^{2+} channels before it reaches the myofilaments. To examine the Ca^{2+} buffering action of sarcoplasmic reticulum, several valuable tools are now available; cyclopiazonic acid and thapsigargin to inhibit Ca^{2+}-ATPase of sarcoplasmic reticulum and ryanodine to deplete Ca^{2+} stored in sarcoplasmic reticulum. However, such examinations have never been evaluated. We therefore determined the Ca^{2+} buffering action of sarcoplasmic reticulum during the stimulation with Bay k 8644 in rat femoral artery. The prediction is that if sarcoplasmic reticulum of the artery is a buffer barrier to the Ca^{2+} influx induced by Bay k 8644, the functional elimination of sarcoplasmic reticulum Ca^{2+} buffering by each of the agents will augment the contractions to Bay k 8644. Here, we show that under the inhibition of sarcoplasmic reticulum Ca^{2+} buffering, Bay k 8644 induced a strong contraction that was comparable to the maximum contraction induced by K^+-depolarization. A preliminary account of these findings was presented to the 71st Annual Meeting of the Japanese Pharmacological Society (Nomura and Asano, 1998).

2. Materials and methods

2.1. Preparation of arterial smooth muscle strips and measurement of isometric tension

Male Wistar–Kyoto rats at 12 to 14 weeks of age (270–315 g) were used. They were inbred in our laboratory. Rats were decapitated, and femoral arteries (0.6–0.8 mm o.d.) were excised 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; Nomura et al., 1996). To avoid the possible influences of the 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 relaxation in prostaglandin $F_{2\alpha}$-contracted strips.

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). Strips were stretched passively to optimal length by imposing a resting tension of 0.6 g and a 60-min equilibration period preceded each experiment. The optimal resting tension was determined by a length-passive tension study (Asano et al., 1988). All experiments were done under protection from light.

After the equilibration, contractile responses of the strips to the Krebs solution containing 65.9 mM KCl (K^+) (equimolar substitution of Na^+ with K^+) were repeated (usually twice) until the responses were reproducible. After washout of the strips with a Krebs solution, contractile responses to Bay k 8644 were determined in a cumulative fashion. Bay k 8644 in concentrations ranging from 0.3 to 300 nM was used, since this agonist at 1 μM exhibited a Ca^{2+} channel antagonistic effect (Asano et al., 1986, 1987). To examine the Ca^{2+} buffering action of sarcoplasmic reticulum during the responses to Bay k 8644, effects of preincubation with cyclopiazonic acid, thapsigargin or ryanodine on these responses were determined. In some experiments, these agents were added after the addition of Bay k 8644. Effects of elevation of extracellular K^+ concentration on the responses to Bay k 8644 were also determined.

Since the contractions to Bay k 8644 were markedly augmented by cyclopiazonic acid, thapsigargin or ryanodine, the effects of nifedipine on the strong contraction to Bay k 8644 in the presence of 10 μM cyclopiazonic acid were determined. Four strips from the same animal were prepared and subjected to different treatments; three strips were preincubated with nifedipine (10, 30 or 100 nM), while the fourth strip was the control without nifedipine. Nifedipine was added for 20 min before the determination of the strong contraction. The data were subjected to a Schild plot analysis according to the method of Arunlakshana and Schild (1959) and a pA_2 value for nifedipine and a slope of the line were determined from the regression analysis (Asano et al., 1986, 1987).

Since the contraction induced by caffeine is a rough index of the amount of Ca^{2+} stored in sarcoplasmic reticulum, the caffeine-induced contractions in the absence and presence of Bay k 8644 were compared.

2.2. Measurement of basal Ca^{2+} influx and net Ca^{2+} entry

Basal $^{45}Ca^{2+}$ influx and net $^{45}Ca^{2+}$ entry were measured by using a cold La^{3+} wash procedure as described previously (Asano et al., 1993, 1996a). In brief, isolated arteries were opened longitudinally and equilibrated in the

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 into the Tris-buffered solution to which 1 μCi/ml $^{45}Ca^{2+}$ had been added. After the incubation for 5 min (for basal Ca^{2+} influx) or 30 min (for net Ca^{2+} entry), arteries were placed into 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 min, 15 min and 25 min (a total of 45 min) to remove extracellular $^{45}Ca^{2+}$. Arteries were then transferred to a glass scintillation vial containing 0.1 ml Amersham NCS tissue solubilizer (Amersham International, Buckinghamshire, UK). Solubilized tissues were mixed with 5 ml Amersham ACS II scintillant and counted for radioactivity in an Aloka liquid scintillation counter. Changes in basal $^{45}Ca^{2+}$ influx or net $^{45}Ca^{2+}$ entry during the stimulation with Bay k 8644 were determined. Values for basal Ca^{2+} influx and net Ca^{2+} entry were then calculated and expressed as μmol/kg tissue wet weight, as described previously (Asano et al., 1993, 1996a).

2.3. Statistical analysis

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

2.4. Drugs and isotope

The drugs used were Bay k 8644 (Bayer, Wuppertal, Germany), cyclopiazonic acid (Sigma, St. Louis, MO, USA), thapsigargin (Sigma), ryanodine (Wako, Osaka, Japan), caffeine (Wako), nifedipine (Bayer Yakuhin, Osaka, Japan), acetylcholine chloride (Sigma) and prostaglandin $F_{2\alpha}$ (Ono Pharmaceutical, Osaka, Japan). $^{45}CaCl_2$ (specific activity initially 14.6–28.5 mCi/mg) was obtained from Amersham International (Buckinghamshire, UK).

Bay k 8644 (1 mM) and nifedipine (1 mM) were dissolved in 99.5% ethanol, with further dilution in distilled water before use. Cyclopiazonic acid (5 mM) and

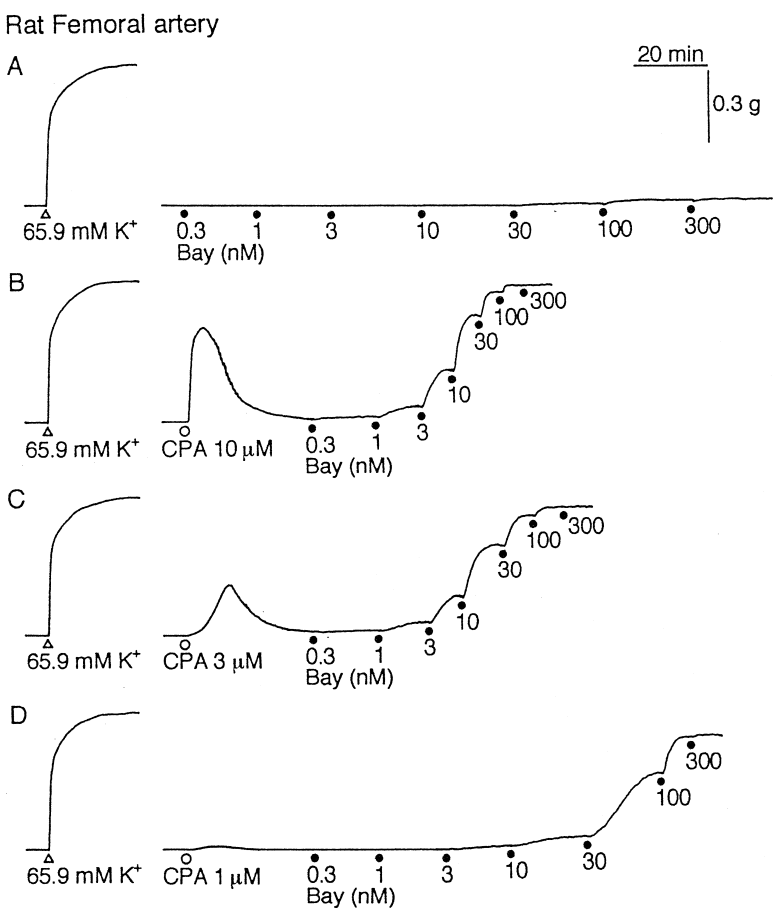

Fig. 1. Typical recordings of the effects of cyclopiazonic acid (CPA) on the responses to Bay k 8644 in strips of rat femoral artery. After determination of the maximum contraction induced by 65.9 mM KCl (K^+), the contractile responses to Bay k 8644 (0.3–300 nM) were determined in a cumulative fashion (A). Effects of the preincubation of the strips with 10, 3 and 1 μM CPA on the responses to Bay k 8644 are shown in B, C and D, respectively. CPA was added 35 min before the addition of Bay k 8644.

Table 1
Effects of cyclopiazonic acid (CPA), thapsigargin (TG), ryanodine and low concentrations of K^+ on the contractile responses to Bay k 8644 in strips of rat femoral artery

Condition[a]	*n*	Preincubated agents[b]		Bay k 8644[c]	
		Peak (%)	Remained (%)	Maximum (%)	EC_{50} (nM)
(A) Control	11			2.8 ± 0.5	NC[d]
(B) CPA 10 μM	17	65.8 ± 1.8	3.1 ± 2.0	96.2 ± 1.7	4.9 ± 0.6
CPA 3 μM	10	37.0 ± 6.9[e]	3.5 ± 1.6	91.7 ± 1.6	6.5 ± 1.4
CPA 1 μM	6	1.3 ± 0.8[e]	0[e]	80.1 ± 2.5[e]	23.8 ± 2.6[e]
(C) TG 30 nM	5	19.3 ± 6.9	1.5 ± 0.5	95.4 ± 2.2	3.4 ± 0.7
TG 10 nM	5	2.7 ± 0.6[f]	0.4 ± 0.2[f]	95.3 ± 1.8	3.9 ± 0.7
TG 3 nM	5	0.7 ± 0.3[f]	0[f]	85.0 ± 2.2[f]	10.2 ± 1.9[f]
(D) Ryanodine 10 μM	5	3.4 ± 1.1	0.4 ± 0.3	80.0 ± 3.6	13.8 ± 1.5
(E) K^+ 11 mM	6	2.4 ± 0.6	1.8 ± 0.5	94.5 ± 3.3	5.7 ± 0.6
K^+ 7 mM	6	0[g]	0[g]	88.4 ± 3.4	24.0 ± 6.8[g]

[a] Experimental conditions were the same as in Figs. 1, 2 and 3.
[b] The peak contraction induced by CPA, TG, ryanodine or K^+ and the remained contraction before the addition of Bay k 8644 is expressed as % of the maximum contraction induced by 65.9 mM K^+.
[c] The maximum contraction induced by Bay k 8644 is expressed as % of the maximum contraction induced by 65.9 mM K^+. The EC_{50} value for Bay k 8644 is expressed as nM. Data are expressed as means ± S.E.M., and *n* indicates the number of preparations used.
[d] NC, not calculated.
[e] Significantly different from the respective value of CPA 10 μM ($P < 0.05$).
[f] Significantly different from the respective value of TG 30 nM ($P < 0.05$).
[g] Significantly different from the respective value of K^+ 11 mM ($P < 0.05$).

thapsigargin (30 μM) were dissolved in 100% dimethyl sulfoxide, with further dilution in the same solution before use. Caffeine (20 mM) was dissolved in the Krebs solution. Aqueous stock solutions were prepared for other drugs. Concentrations of drugs are expressed as final molar concentrations.

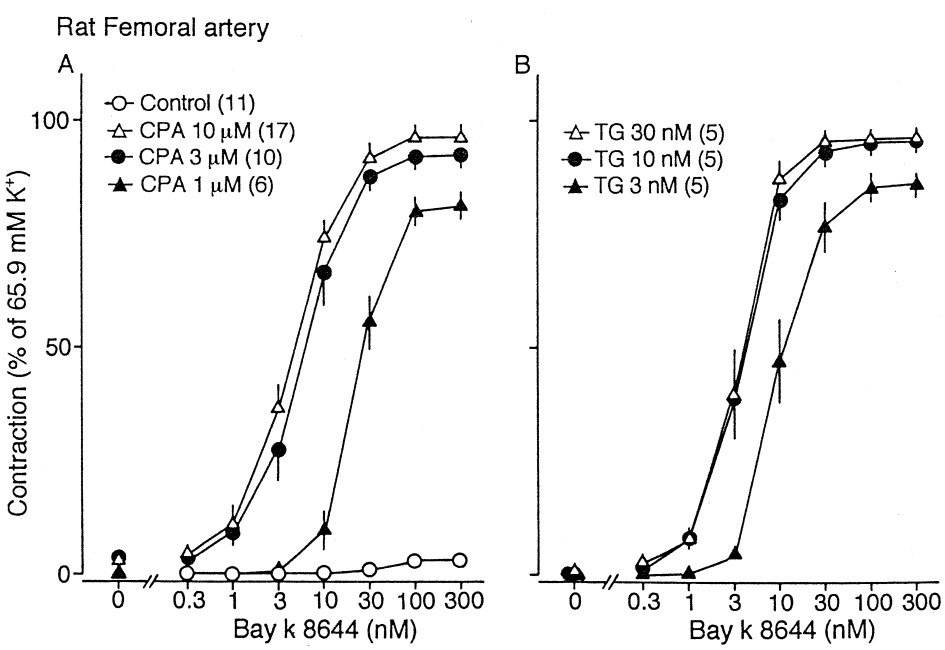

Fig. 2. Effects of cyclopiazonic acid (CPA, A) and thapsigargin (TG, B) on the concentration–response curve for Bay k 8644 in strips of rat femoral artery. Experimental conditions were the same as in Fig. 1. The peak contractions induced by each concentration of Bay k 8644 are expressed as % of the maximum contraction induced by 65.9 mM K^+. At '0' on the abscissa of A and B panels, the CPA-induced contractions (A) and TG-induced contractions (B) remained before the addition of Bay k 8644 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.

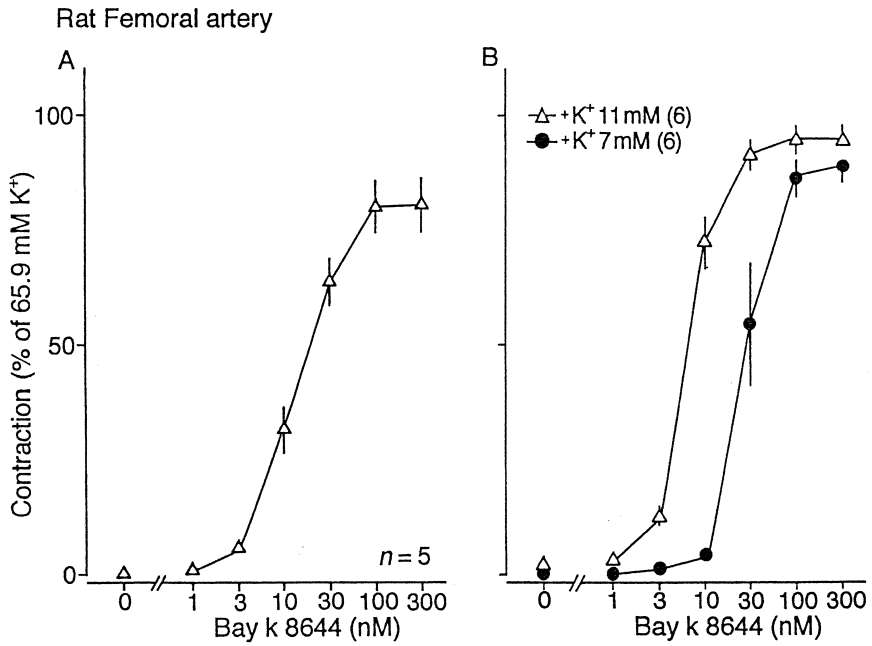

Fig. 3. Effects of ryanodine (A) and elevation of extracellular K^+ concentration (B) on the responses to Bay k 8644 in strips of rat femoral artery. (A) After determination of the maximum contraction induced by 65.9 mM K^+ as in Fig. 1, the strips were treated with 10 μM ryanodine (30 min) plus 20 mM caffeine (10 min). Following washout for 45 min, the contractile responses to Bay k 8644 (1–300 nM) were determined in a cumulative fashion. (B) K^+ at a concentration of 11 mM or 7 mM was added, and then the contractile responses to Bay k 8644 (1–300 nM) were determined in a cumulative fashion. The peak contractions induced by each concentration of Bay k 8644 are expressed as % of the maximum contraction induced by 65.9 mM K^+. At '0' on the abscissa of A and B panels, the ryanodine-induced contraction (A) and the K^+-induced contractions (B) remained before the addition of Bay k 8644 are expressed as % of the maximum contraction induced by 65.9 mM K^+. Data points are means of 5 (A) and 6 (B) preparations, and S.E.M. are shown by vertical bars.

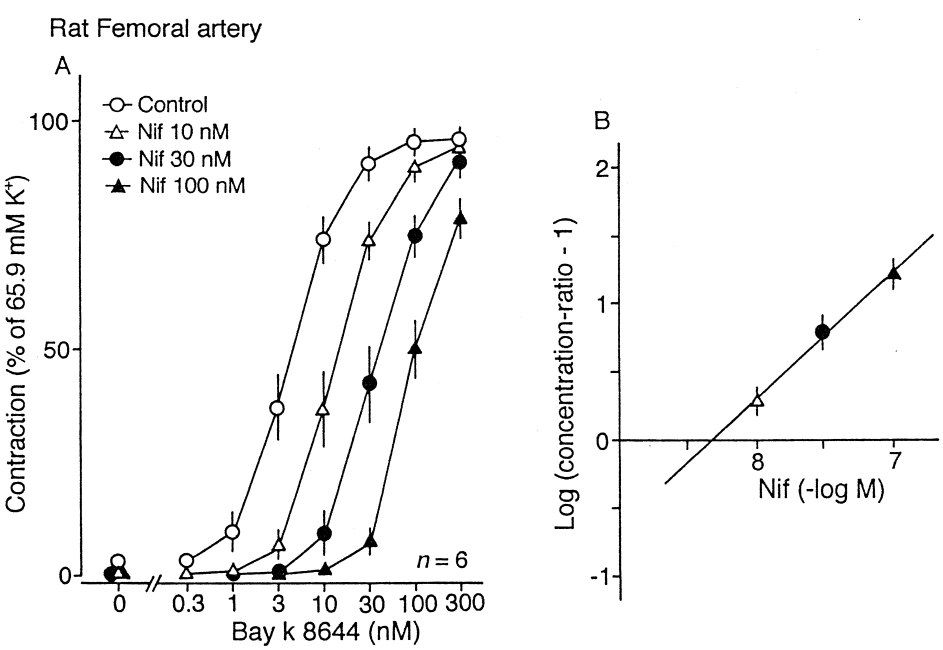

Fig. 4. Effects of nifedipine (Nif) on the concentration–response curve for Bay k 8644 determined in the presence of cyclopiazonic acid (CPA) in strips of rat femoral artery. (A) Control curve for Bay k 8644 in the presence of 10 μM CPA was determined as in Fig. 1B. Nif at 10, 30 or 100 nM was added 20 min before the addition of CPA. For details, see Section 2. (B) Schild plot for nifedipine antagonism of the responses to Bay k 8644 in the presence of CPA. The plot was done using the concentration-ratios calculated from the concentration–response curves for Bay k 8644 shown in A. The line indicates the regression line of best fit three data points. Schild plot of data to yield a pA_2 value of 8.32 ± 0.10 and a slope of 0.94 ± 0.14 (mean ± 95% confidence limits). Data points are means of 6 preparations, and S.E.M. are shown by vertical bars.

3. Results

3.1. Effects of cyclopiazonic acid and thapsigargin on the responses to Bay k 8644

The addition of Bay k 8644 (0.3–300 nM) to the resting state of the strips almost failed to cause a contraction (Fig. 1A). Even at a high concentration (300 nM), Bay k 8644 induced a contraction of $2.8 \pm 0.5\%$ ($n = 11$) of the maximum contraction induced by 65.9 mM K^+ (Table 1A). To examine the Ca^{2+} buffering action of sarcoplasmic reticulum during the responses to Bay k 8644, effects of cyclopiazonic acid on these responses were first determined (Fig. 1B, Fig. 2A, Table 1B). The preincubation of the strips with 10 μM cyclopiazonic acid caused a large transient contraction (Fig. 1B), as already shown in our previous studies (Nomura et al., 1996, 1997). After the cyclopiazonic acid-induced contraction had declined and reached a plateau (35 min), the addition of Bay k 8644 caused a concentration-dependent contraction with a maximum contraction that is comparable to the maximum contraction induced by 65.9 mM K^+ and an EC_{50} value of 4.9 nM. Almost the same strong contraction to Bay k 8644 was observed when cyclopiazonic acid at 3 μM was preincubated (Fig. 1C, Fig. 2A, Table 1B). Cyclopiazonic acid at 3 μM also induced a transient contraction. Even at a low concentration (1 μM), cyclopiazonic acid also augmented the contractions to Bay k 8644 (Fig. 1D, Fig. 2A, Table 1B). The effect of 1 μM cyclopiazonic acid was less than that of 10 or 3 μM cyclopiazonic acid. The contractions to Bay k 8644 in the presence of 1 μM cyclopiazonic acid were slower than those in the presence of 10 or 3 μM cyclopiazonic acid (Fig. 1D vs. Fig. 1B or Fig. 1C).

Effects of thapsigargin on the responses to Bay k 8644 were then determined (Fig. 2B, Table 1C). The preincubation of the strips with 30 nM thapsigargin caused a slow, small transient contraction. After the thapsigargin-induced contraction had declined to the resting level (50 min), the addition of Bay k 8644 caused a strong contraction. Almost the same strong contraction to Bay k 8644 was observed when thapsigargin at 10 nM was preincubated. Thapsigargin at 10 nM induced only a very small contraction. Even at a low concentration (3 nM), thapsigargin also augmented the contractions to Bay k 8644. The effect of 3 nM thapsigargin was less than that of 30 or 10 nM thapsigargin. The contractions to Bay k 8644 became slower as the concentration of thapsigargin decreased. The maximum effect of thapsigargin on the contractions to Bay k 8644 was the same as that of cyclopiazonic acid, because the EC_{50} value for Bay k 8644 was not significantly different between the preincubation with 10 μM cyclopiazonic acid and with 30 nM thapsigargin (Table 1).

3.2. Effects of ryanodine on the responses to Bay k 8644

Effects of the functional elimination of sarcoplasmic reticulum Ca^{2+} buffering by ryanodine on the contractions to Bay k 8644 were determined according to the method as described previously (Asano et al., 1996a). After the elimination of sarcoplasmic reticulum Ca^{2+} buffering by 10 μM ryanodine plus 20 mM caffeine, the addition of Bay k 8644 to the strips caused a strong contraction (Fig. 3A, Table 1D). This effect of ryanodine was less than that of 10 μM cyclopiazonic acid or 30 nM thapsigargin, because the EC_{50} value for Bay k 8644 was significantly larger in the effect of ryanodine than in that of 10 μM cyclopiazonic acid or 30 nM thapsigargin (Table 1).

3.3. Effects of elevation of extracellular K^+ concentration on the responses to Bay k 8644

Bay k 8644 also induced a strong contraction when the extracellular K^+ concentration was elevated (Fig. 3B,

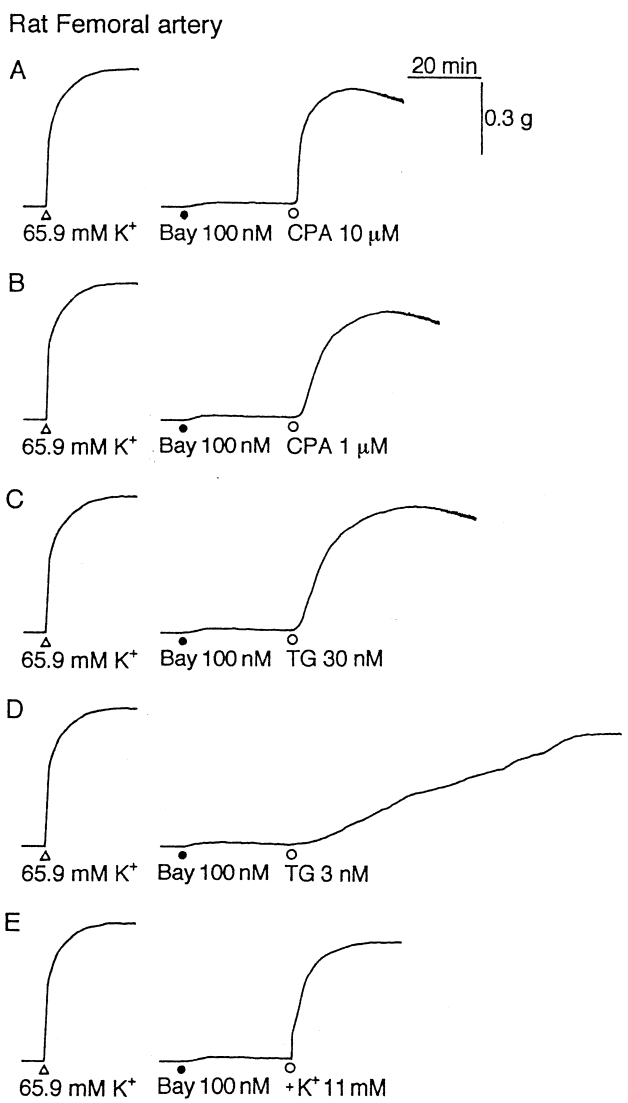

Fig. 5. Typical recordings of the contractile responses to cyclopiazonic acid (CPA), thapsigargin (TG) and K^+ (addition of 11 mM) determined after the addition of Bay k 8644 in strips of rat femoral artery. After determination of the maximum contraction induced by 65.9 mM K^+, the contractile response to 100 nM Bay k 8644 was determined and then CPA, TG or K^+ was added.

Table 2
Contractile responses to cyclopiazonic acid (CPA), thapsigargin, ryanodine and the elevated K^+ determined after the addition of Bay k 8644 in strips of rat femoral artery

Contractile agents[a]	Contractile responses		
	n	Magnitude (%)[b]	$t_{1/2}$ (min)[c]
(A) CPA 10 μM	5	87.5 ± 2.4	2.0 ± 0.04
CPA 3 μM	4	87.3 ± 1.1	3.1 ± 0.5[d]
CPA 1 μM	4	80.2 ± 3.2	6.0 ± 0.7[d]
(B) Thapsigargin 30 nM	4	92.7 ± 6.2	7.1 ± 0.9
Thapsigargin 10 nM	4	92.9 ± 0.3	17.8 ± 1.8[e]
Thapsigargin 3 nM	4	84.5 ± 2.1	45.5 ± 8.5[e]
(C) Ryanodine 10 μM	4	86.1 ± 3.2	2.5 ± 0.3
(D) K^+ 11 mM	4	86.6 ± 1.6	1.9 ± 0.3
K^+ 7 mM	4	85.0 ± 1.9	4.0 ± 0.5[f]

[a]Experimental conditions were the same as in Fig. 5.
[b]The magnitude of sustained contraction induced by CPA, thapsigargin, ryanodine or K^+ is expressed as % of the maximum contraction induced by 65.9 mM K^+.
[c]The time (min) to attain a half of the sustained contraction was calculated. Data are expressed as means ± S.E.M., and *n* indicates the number of preparations used.
[d]Significantly different from the respective value of CPA 10 μM ($P < 0.05$).
[e]Significantly different from the respective value of thapsigargin 30 nM ($P < 0.05$).
[f]Significantly different from the respective value of K^+ 11 mM ($P < 0.05$).

Table 1E). The addition of 11 mM K^+ to the bath (total K^+ concentration; 16.9 mM) caused a small contraction and augmented the contractions to Bay k 8644. This EC_{50} value for Bay k 8644 was similar to the case of either 10 μM cyclopiazonic acid or 30 nM thapsigargin, but the contractions were slower in the case of K^+ than in other cases (Fig. 3B, Table 1E). The addition of 7 mM K^+ did not cause a contraction but augmented the contractions to Bay k 8644 (Fig. 3B, Table 1E).

3.4. Effects of nifedipine on the strong contraction to Bay k 8644

The strong contraction to Bay k 8644 in the presence of cyclopiazonic acid was inhibited concentration-dependently by nifedipine. The addition of nifedipine at 10, 30 and 100 nM inhibited the cyclopiazonic acid-induced contraction, as already shown in our previous studies (Nomura et al., 1996, 1997), and concomitantly caused a rightward displacement of the concentration–response curve for Bay k 8644 (Fig. 4A). The Schild plot analysis of the antagonism by nifedipine of the contraction to Bay k 8644 indicated a competitive type of antagonism with a pA_2 value of 8.32 for nifedipine and a slope of 0.94 (Fig. 4B).

3.5. Contractile responses to cyclopiazonic acid, thapsigargin, ryanodine and the elevated K^+ determined after the addition of Bay k 8644

The augmenting effects of cyclopiazonic acid, thapsigargin and ryanodine on the contractions to Bay k 8644 shown above suggest that the Ca^{2+} influx during the stimulation with Bay k 8644 is completely buffered by Ca^{2+} uptake into the sarcoplasmic reticulum. This possibility was then examined in the following three experiments.

When cyclopiazonic acid, thapsigargin or ryanodine was added after the small contraction induced by 100 nM Bay k 8644 had reached a plateau, a large sustained contraction was observed (Fig. 5, Table 2). Bay k 8644 at 100 nM itself induced a peak contraction of 3.4 ± 0.3% ($n = 37$) of the maximum contraction induced by 65.9 mM K^+, and the contraction reached a steady-state level (2.3 ± 0.2%, $n = 37$) at 25–30 min after the addition. When cyclopiazonic acid at 10 μM was post-added, a large sustained contraction was observed (Fig. 5A, Table 2A). When cyclopiazonic acid at 3 or 1 μM was post-added, similar magnitude of the contraction was observed, but the contraction became slower as the concentration of cyclopiazonic acid decreased (Fig. 5A, B, Table 2A). Similar results were obtained when thapsigargin at 30, 10 and 3 nM was post-added (Fig. 5C, D, Table 2B). The thapsigargin-induced contraction became slower as the concentration of thapsigargin decreased. When ryanodine at 10 μM was post-added, a large sustained contraction was also observed (Table 2C). The time course of the ryanodine-induced contraction was similar to the case of 10 μM cyclopiazonic acid (Table 2). Similar results were obtained

Table 3
Effects of Bay k 8644 on basal Ca^{2+} influx and net Ca^{2+} entry in rat femoral artery

$^{45}Ca^{2+}$ incubation[a]		Ca^{2+} taken up by the tissue		
Time (min)	Condition	*n*	μmol/kg wet tissue (% of control)	
5	Control	19	87.0 ± 2.3	
	Bay k 8644 100 nM	19	103.9 ± 2.7[b]	(119.4)
30	Control	19	155.0 ± 4.0	
	Bay k 8644 100 nM	19	175.0 ± 4.1[b]	(112.9)

[a]Arteries were incubated for 5 min (basal Ca^{2+} influx) or 30 min (net Ca^{2+} entry) in either solution to which $^{45}Ca^{2+}$ had been added. Data are μmol Ca^{2+}/kg wet tissue, which are expressed as means ± S.E.M., and *n* indicates the number of measurements. The % of the respective 'Control' value is shown in parentheses.
[b]Significantly different from the respective 'Control' ($P < 0.05$).

when K^+ was post-added (Fig. 5E, Table 2D). The contractions induced by cyclopiazonic acid, thapsigargin, ryanodine and K^+ determined after the addition of Bay k 8644 were significantly greater than the respective control contraction determined in the absence of Bay k 8644 and were similar to the augmented maximum contraction to Bay k 8644 (Table 2 vs. Table 1).

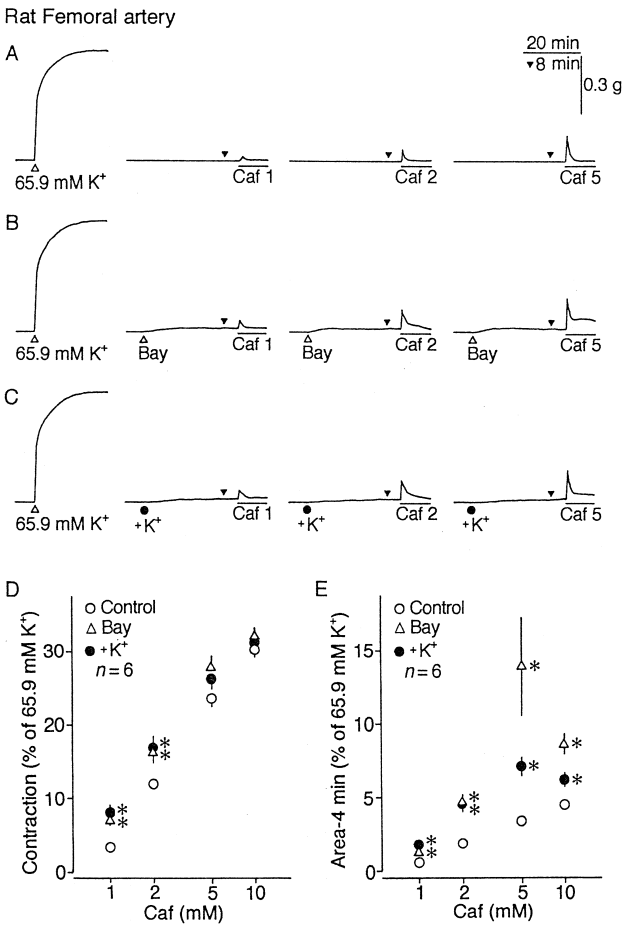

Fig. 6. Contractile responses to caffeine during the stimulation with 100 nM Bay k 8644 or 11 mM K^+. (A,B,C) Typical recordings. After determination of the maximum contraction induced by 65.9 mM K^+, the control contractions induced by caffeine (1–5 mM) were determined (A). The caffeine-induced contractions during the stimulation with 100 nM Bay k 8644 (B) or 11 mM K^+ (C) are shown. Bay k 8644 or K^+ was added 30 min before the addition of caffeine. The caffeine-induced contractions were recorded after the chart speed was changed to 2.5-fold (▼). Caffeine was added for 4 min. (D) Peak contractions induced by caffeine during the stimulation with 100 nM Bay k 8644 or 11 mM K^+ shown in A, B and C. The peak contractions induced by each concentration of caffeine (1–10 mM) are expressed as % of the maximum contraction induced by 65.9 mM K^+. (E) Area for caffeine-induced contractions (4 min) during the stimulation with 100 nM Bay k 8644 or 11 mM K^+ shown in A, B and C. The area induced by each concentration of caffeine (1–10 mM) was calculated and expressed as % of the area for 65.9 mM K^+-induced contraction (first 4 min). The area was calculated under the same chart speed. Data points are means of 6 preparations, and S.E.M. are shown by vertical bars. ∗Significantly different from the respective 'Control' value ($P < 0.05$).

3.6. Effects of Bay k 8644 on basal Ca^{2+} influx and net Ca^{2+} entry

The increase in cellular Ca^{2+} level during the stimulation with Bay k 8644 was also measured by using $^{45}Ca^{2+}$ (Table 3). The basal Ca^{2+} influx in the resting state of arteries measured with a 5-min $^{45}Ca^{2+}$ incubation was significantly increased by 100 nM Bay k 8644. With a 30-min $^{45}Ca^{2+}$ incubation, the net Ca^{2+} entry was also significantly increased by 100 nM Bay k 8644.

3.7. Effects of Bay k 8644 and the elevated K^+ on the contractile responses to caffeine

Contractile responses to caffeine after the addition of 100 μM Bay k 8644 or 11 mM K^+ were augmented when compared with the control responses (Fig. 6). Since the pattern of the contraction induced by caffeine was different between the Bay k 8644- or K^+-added strips and the control strips, both the peak contraction and the area for caffeine-induced contraction (4 min) were calculated. The peak contraction induced by 1 and 2 mM caffeine was greater in the added strips than in the control strips (Fig. 6D). The area for contraction induced by caffeine (1–10 mM) was greater in the added strips than in the control strips (Fig. 6E).

4. Discussion

The present study clearly showed that in rat femoral artery, Bay k 8644 itself almost failed to induce a contraction, but the inhibition of sarcoplasmic reticulum Ca^{2+} buffering by cyclopiazonic acid, thapsigargin or ryanodine markedly augmented the contractions to Bay k 8644. We consider that these responses to Bay k 8644 can be explained by 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). According to this hypothesis, Ca^{2+} that entered the cell across the plasmalemma is taken up into the superficial sarcoplasmic reticulum by Ca^{2+}-ATPase before it can reach the myofilaments. To initiate a contraction, the existence of Ca^{2+} entry that exceeds the Ca^{2+} buffering action of sarcoplasmic reticulum is needed. It is thus possible that the Ca^{2+} influx induced by Bay k 8644 is completely buffered by sarcoplasmic reticulum before it can reach the myofilaments. This sarcoplasmic reticulum can buffer a large amount of Ca^{2+} that induces a maximum contraction.

To eliminate the Ca^{2+} buffering action of sarcoplasmic reticulum, cyclopiazonic acid, thapsigargin and ryanodine were used in the present study. Cyclopiazonic acid and thapsigargin have been reported to inhibit Ca^{2+} uptake into sarcoplasmic reticulum by inhibiting sarcoplasmic reticulum Ca^{2+}-ATPase (Seidler et al., 1989; Thastrup et

al., 1990; Deng and Kwan, 1991; Uyama et al., 1992; Nomura et al., 1996). Therefore, under the inhibition of the Ca^{2+} uptake, Ca^{2+} that entered the cell across the plasmalemma bypasses the sarcoplasmic reticulum and reaches the myofilaments resulting in a contraction. This is clearly shown in the present study. It is thus likely that during the stimulation with Bay k 8644, a large amount of Ca^{2+} enters the cell and is taken up into the sarcoplasmic reticulum. This conclusion is drawn from the following observations: (1) Bay k 8644 almost failed to induce a contraction, (2) under the inhibition of sarcoplasmic reticulum Ca^{2+} uptake by cyclopiazonic acid or thapsigargin, Bay k 8644 induced a strong contraction, (3) although the concentrations of cyclopiazonic acid and thapsigargin to augment the contractions to Bay k 8644 were different, the maximally augmented contractions to Bay k 8644 were the same for cyclopiazonic acid and thapsigargin, (4) Bay k 8644 increased the basal $^{45}Ca^{2+}$ influx and net $^{45}Ca^{2+}$ entry, and (5) Bay k 8644 increased the amount of Ca^{2+} stored in the caffeine-releasable sarcoplasmic reticulum. To obtain the maximally augmented contractions to Bay k 8644, cyclopiazonic acid at 3 μM or thapsigargin at 10 nM was needed.

As shown in Fig. 1 and Table 1, cyclopiazonic acid or thapsigargin itself induced a contraction, as already shown in our previous studies (Nomura et al., 1996, 1997). This contraction was observed at relatively high concentrations of each agent when compared with the concentration to cause the augmentation. This contraction reflects the basal Ca^{2+} entry in the resting state of the artery, as already discussed in our previous studies (Nomura et al., 1996, 1997). It is thus likely that the sarcoplasmic reticulum can buffer both basal and stimulated Ca^{2+} entry.

Ryanodine was used as another tool to eliminate the Ca^{2+} buffering action of sarcoplasmic reticulum. The mechanism of action of ryanodine is the binding of this alkaloid to Ca^{2+}-induced Ca^{2+} release channels in sarcoplasmic reticulum, and then locks them open. The initial action of ryanodine could thus be acceleration of Ca^{2+} release. After full development of the ryanodine action, the sarcoplasmic reticulum becomes empty and cannot accumulate Ca^{2+} any longer, so that the sarcoplasmic reticulum cannot buffer Ca^{2+} that enters the cell, nor release Ca^{2+} from there (for details, see Asano et al., 1996a,b). Ryanodine also augmented the contractions to Bay k 8644, again suggesting the strong Ca^{2+} buffering action of sarcoplasmic reticulum during the stimulation with Bay k 8644.

As shown in Table 1, the augmenting effect of ryanodine was less than that of cyclopiazonic acid or thapsigargin. These results indicate that to augment the contractions to Bay k 8644, the inhibition of sarcoplasmic reticulum Ca^{2+}-ATPase is more effective than the open-lock of Ca^{2+}-induced Ca^{2+} release channels. This is probably due to the activity of sarcoplasmic reticulum Ca^{2+}-ATPase under the open-lock of the channels. Under the open-lock of the channels, Ca^{2+} that entered the cell during the Bay k 8644-stimulation is once taken up into the leaky sarcoplasmic reticulum, and most Ca^{2+} that leaked from the sarcoplasmic reticulum reach the myofilaments resulting in a contraction, but some Ca^{2+} appear to cycle through the leaky sarcoplasmic reticulum; Ca^{2+} that leaked around the outer surface of the sarcoplasmic reticulum was again taken up into the sarcoplasmic reticulum by Ca^{2+}-ATPase. Moreover, some Ca^{2+} leaked from the sarcoplasmic reticulum appear to be extruded from the cell via the Na^{+}-Ca^{2+} exchanger and the Ca^{2+}-ATPase of the plasmalemma, since the Ca^{2+}-induced Ca^{2+} release channels are involved in this Ca^{2+} extrusion process, as proposed in the 'superficial buffer barrier' hypothesis (Van Breemen and Saida, 1989; Chen et al., 1992; Van Breemen et al., 1995; Laporte and Laher, 1997). Thus, the three agents which, either the inhibition of Ca^{2+}-ATPase or through the open-lock of Ca^{2+}-induced Ca^{2+} release channels, inhibited the Ca^{2+} buffering action of sarcoplasmic reticulum and resulted in a strong contraction in response to Bay k 8644. The Ca^{2+} buffering action of sarcoplasmic reticulum during the stimulation of arterial strips with Bay k 8644 was proposed by Hwang and Van Breemen (1985) and was confirmed in the present study using the inhibitors of sarcoplasmic reticulum Ca^{2+} buffering.

Numerous studies have shown that the preactivation of arterial strips with K^{+} is needed for Bay k 8644 to induce a strong contraction (for details, see Asano et al., 1987; Wanstall and O'Donnell, 1989). The present study also confirmed a need of such preactivation in rat femoral 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), the effectiveness of the sarcoplasmic reticulum Ca^{2+} buffering depends on the state of Ca^{2+} loading of the sarcoplasmic reticulum; empty sarcoplasmic reticulum will buffer all stimulated Ca^{2+} entry and overloaded sarcoplasmic reticulum cannot buffer the entered Ca^{2+}. At rest or during depolarization, Ca^{2+} that entered the cell is taken up into the sarcoplasmic reticulum before it reaches the myofilaments. Because the preactivation with K^{+} causes an influx of extracellular Ca^{2+} through L-type Ca^{2+} channels, the entered Ca^{2+} can be taken up into the sarcoplasmic reticulum. When the sarcoplasmic reticulum Ca^{2+} buffering does not reach the maximum level (e.g., 7 mM K^{+} addition in the present study), some Ca^{2+} that entered the cell during the Bay k 8644-stimulation are still taken up into the sarcoplasmic reticulum, resulting in a relatively weak contraction in response to Bay k 8644. However, when the Ca^{2+} buffering reaches the maximum level (e.g., 11 mM K^{+} addition in the present study), the entered Ca^{2+} cannot be taken up into the sarcoplasmic reticulum, resulting in a strong contraction to Bay k 8644. Thus, the Bay k 8644-induced contractions under the preactivation with K^{+} can also be explained by the 'superficial buffer barrier' hypothesis. This assumption is also supported by

the following findings; (1) during the addition of 11 mM K^+, the amount of Ca^{2+} stored in the caffeine-releasable sarcoplasmic reticulum was increased, and (2) the contractile responses of femoral arterial strips to K^+ were significantly potentiated by 10 μM cyclopiazonic acid or 10 μM ryanodine, as already shown in our previous study (Nomura et al., 1994).

If the entered Ca^{2+} during the Bay k 8644-stimulation is always taken up into the sarcoplasmic reticulum, it can be expected that the post-addition of cyclopiazonic acid, thapsigargin or ryanodine causes an inhibition of SR Ca^{2+} buffering, and the entering Ca^{2+} can reach the myofilaments resulting in a strong contraction. Moreover, the post-addition of K^+ is also expected to cause a strong contraction, because the sarcoplasmic reticulum is already overloaded with Ca^{2+} during the stimulation with Bay k 8644, so that the Ca^{2+} influx by the K^+-depolarization bypasses the sarcoplasmic reticulum and reaches the myofilaments. This assumption is clearly proved by the results shown in Fig. 5 and Table 2. The magnitude of the sustained contraction was the same for cyclopiazonic acid, thapsigargin, ryanodine and K^+. Moreover, the magnitude was also the same for higher and lower concentrations of cyclopiazonic acid (10 μM vs. 1 μM), thapsigargin (30 nM vs. 3 nM) or K^+ (11 mM vs. 7 mM). The difference in the contractions induced by cyclopiazonic acid, thapsigargin, ryanodine and K^+ was only noted in the time course of the contraction.

As shown in the present study, Bay k 8644 induced a strong contraction that was comparable to the maximum contraction in this artery under the inhibition of sarcoplasmic reticulum Ca^{2+} buffering by cyclopiazonic acid, thapsigargin or ryanodine and the Ca^{2+} overloading of sarcoplasmic reticulum by the elevated K^+. Thus, the sarcoplasmic reticulum of rat femoral artery can buffer a large amount of Ca^{2+} that induces a maximum contraction in this artery. This is probably due to the assumption that the Ca^{2+} influx induced by Bay k 8644 is large but slow, so that the sarcoplasmic reticulum Ca^{2+} buffering would be effective to blunt the contraction. It thus appears that the sarcoplasmic reticulum of this artery acts as a buffer barrier to the Ca^{2+} influx via L-type Ca^{2+} channels. As shown in Fig. 6, during the stimulation with Bay k 8644 or the elevated K^+, the entered Ca^{2+} was taken up into the caffeine-releasable sarcoplasmic reticulum. This result is in good agreement with the previous report where the effects of Bay k 8644 were examined in rabbit aorta (Hwang and Van Breemen, 1985).

We have already shown that Bay k 8644 itself induced a strong contraction without the preactivation in the femoral artery from spontaneously hypertensive rats (Asano et al., 1986) and dog cerebral arteries (Asano et al., 1987). These arteries have been shown to possess the high cellular Ca^{2+} level probably due to maintained Ca^{2+} influx in the resting state. Thus, the potent contractions to Bay k 8644 in these arteries may reflect the Ca^{2+} overloading of sarcoplasmic reticulum (for details, see Asano et al., 1996a,b; Nomura et al., 1997).

In the present study, nifedipine antagonized the strong contraction to Bay k 8644 in the presence of cyclopiazonic acid. The Schild plot analysis for antagonism by nifedipine of the contraction to Bay k 9644 clearly showed a competitive antagonism, suggesting that Bay k 8644 induced a contraction by acting dihydropyridine receptors of L-type Ca^{2+} channels. The pA_2 value for nifedipine (8.32) is in good agreement with the pA_2 values for this antagonist obtained in other arteries (Asano et al., 1986, 1987).

From the present study, we conclude that in rat femoral arterial smooth muscle, (1) the Ca^{2+} influx induced by Bay k 8644 is completely buffered by Ca^{2+} uptake into the sarcoplasmic reticulum, (2) therefore under the inhibition of sarcoplasmic reticulum Ca^{2+} buffering by cyclopiazonic acid, thapsigargin or ryanodine, Bay k 8644 induces a strong contraction, and (3) this sarcoplasmic reticulum can buffer a large amount of Ca^{2+} that induces a maximum contraction in this artery. Furthermore, the preactivation of arterial strips with the elevated K^+ causes a Ca^{2+} overloading of sarcoplasmic reticulum and results in a strong contraction in response to Bay k 8644.

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