Ca^{2+} Influx Induced by the Agonist U46619 Is Inhibited by Hyperpolarization Induced by the K^+ Channel Opener Cromakalim in Canine Coronary Artery

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ABSTRACT — The fura-2 microscopic fluorimetric method was used to examine the effects of the thromboxane A_2 analogue, U46619, on the force of contraction and intracellular calcium concentrations ($[Ca^{2+}]_i$) in canine coronary arteries. Upon cumulative application, U46619 increased $[Ca^{2+}]_i$ and force. Depolarization by 20 mM KCl potentiated the increase in $[Ca^{2+}]_i$ and increased the maximum force induced by U46619. In 5 mM KCl-PSS, the reduction of resting $[Ca^{2+}]_i$ by cromakalim (3×10^{-6} M) was greater than that by verapamil (3×10^{-6} M). Cromakalim and verapamil inhibited the increases in $[Ca^{2+}]_i$ and force induced by U46619 in 5 mM KCl-PSS. In 90 mM KCl-PSS in the presence of U46619, verapamil inhibited the increases in $[Ca^{2+}]_i$ and force, whereas cromakalim did not inhibit them at all. The inhibitory effect of cromakalim was counteracted by depolarization by 20 or 25 mM KCl. Curves in the presence of U46619 which related force to $[Ca^{2+}]_i$ were shifted to the left compared with that in the absence of U46619, suggesting that U46619 increases the Ca^{2+}-sensitivity of the contractile proteins. Thus, U46619 produces Ca^{2+} influx through L-type Ca^{2+} channels, which are deactivated by hyperpolarization induced by cromakalim.

Keywords: Thromboxane A_2 analogue, Cromakalim, Intracellular calcium concentration, Ca^{2+} channels, K^+ channels

Thromboxane A_2 is a strong vasoconstrictor as well as a potent inducer of platelet aggregation, which is thought to be involved in ischemic states such as angina pectoris and myocardial infarction (1, 2). In canine coronary arteries, carbocyclic thromboxane A_2 produces a relatively consistent contraction (3). Its mechanism of action is thought to be related with the release of Ca^{2+} from intracellular storage sites and the Ca^{2+} influx (3). However, the methods used were mainly the measurement of force of contraction. Since the signal transduction after the stimulation of various receptors by agonists involves several mechanisms to induce contractions in vascular smooth muscle (4), the simultaneous measurement of force and intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) is very crucial. We have studied mostly KCl-induced contractions with various vasodilators, including K^+ channel openers, by measuring simultaneously force and $[Ca^{2+}]_i$ using a fura-2 microscopic fluorimetric method (5 - 7). Recently, it has become well-known that the contractions induced by agonists are inhibited effectively by the K^+ channel opener cromakalim (8, 9), the relaxant action of which is generally understood to be due to the indirect closure of L-type Ca^{2+}-channels by its hyperpolarizing action on the cell membrane of vascular smooth muscle. However, the effects of K^+ channel openers on $[Ca^{2+}]_i$ and force of contraction induced by thromboxane A_2 analogues in coronary artery are little investigated. Thus, we wanted to elucidate the mechanisms and characteristics of vasoconstriction induced by U46619 and examine the relaxation produced by the K^+ channel opener cromakalim by measuring $[Ca^{2+}]_i$ and force in canine coronary arteries.

MATERIALS AND METHODS

Preparation of arterial rings

Hearts were obtained from mongrel dogs of either sex, weighing 6 to 15 kg, anesthetized with sodium pentobarbital (30 mg/kg, i.v.). Coronary arterial rings

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(0.6 – 1.2 mm in diameter, about 1 mm in width) were dissected, and connective tissues were carefully removed in a dissecting chamber under a binocular microscope (SZ, Olympus, Tokyo, Japan). The endothelium was removed by gentle rubbing, and the luminal side was turned to face outwards.

Fluorescence measurements

We performed fluorescence measurements as previously described (5). In brief, coronary arterial rings were exposed to 10 μM fura-2 acetoxymethyl ester (fura-2 AM) for about 2 hr at 37°C. The noncytotoxic detergent pluronic F-127 (0.1% W/V) was premixed in the loading physiological salt solution (PSS) to help dissolve the fura-2 AM in PSS. After the fura-2 loading, muscles were rinsed with normal PSS for longer than 1 hr and then used for the experiments. Fluorescence was measured in a fluorimeter equipped with a dual wavelength excitation device (CAM-200 or CAM-220, Japan Spectroscopic, Tokyo, Japan) connected to an inverted microscope (TMD-8, Nikon, Tokyo, Japan). The fluorescence image was obtained by focusing on the smooth muscle cells in the medial layer with a Nikon CF UV (Fluor) 10 × objective lens. The muscle ring was placed horizontally in a temperature-controlled 0.4 ml tissue bath which was mounted on the inverted microscope and perfused with PSS at a rate of 4 ml/min. The muscle ring was stretched to a resting tension of about 5 mN between two tungsten needles, one of which was glued to a transducer element (AE801, AME, Horten, Norway or U-10230, Shinko, Tokyo, Japan). The photosignals and the mechanical activity were measured simultaneously and both were recorded on a chart recorder (Recti-horiz-8K, NEC-San-ei, Tokyo, Japan). They were also digitized by A/D converters and fed into a microcomputer (PC-9801, NEC, Tokyo, Japan) for further calculation and graphical analysis (10). Every experiment commenced with perfusion with 90 mM KCl-PSS for 10 min. At the end of each experiment, the cell membrane was lysed with the detergent Triton X-100 (1%), and autofluorescence signals were determined by quenching fura-2 signals with $MnCl_2$. We subtracted the F_{340} and F_{380} values due to autofluorescence from the corresponding values of F_{340} and F_{380} obtained under conditions of fura-2 loading to derive a recalculated $ratio_{rc}$. Since it is difficult to calculate the absolute concentration of $[Ca^{2+}]_i$ (11, 12), we used the relative F_{340}/F_{380} ratio as an indicator of $[Ca^{2+}]_i$ (5, 13). Changes in the $ratio_{rc}$ (changes in $[Ca^{2+}]_i$) and those in force were expressed as percentages of the differences between basal values and those obtained with a 10-min perfusion with 90 mM KCl-PSS. None of the drugs and chemicals used in the present study, with the exception of fura-2 and $MnCl_2$, affected the fluorescence signals at the concentrations used.

Protocol

Preparations were exposed to cumulative concentrations of U46619 (10^{-9} to 3×10^{-7} M) every 5 min, following washout with the drug-free 5 mM KCl-PSS for 30 min. The perfusion with 20 mM KCl-PSS or drug containing PSS was followed by the cumulative application of U46619 every 5 min. In the experiments with cromakalim (3×10^{-6} M) and verapamil (3×10^{-6} M), at the end of exposure to cumulative concentrations of U46619 (10^{-8} to 3×10^{-7} M), the rings were perfused with 90 mM KCl containing U46619 (3×10^{-7} M) for 10 min.

Drugs and solutions

The composition of normal or 5 mM KCl physiological salt solution (PSS) was as follows: 140 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 2.5 mM $MgCl_2$, 11.1 mM glucose, 3 mM HEPES (pH 7.4). The solution was equilibrated with 100% O_2 at 37°C. High KCl-PSS was made by substituting NaCl with equimolar KCl. Ca^{2+}-free PSS was made by removing $CaCl_2$ from normal PSS and by adding 1 mM EGTA to the solution.

Drugs and chemicals were obtained from the following sources: cromakalim (Beecham, Harlow, U.K.), verapamil hydrochloride (Eisai, Tokyo, Japan), U46619 (9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$; Sigma, St. Louis, MO, U.S.A.), EGTA (glycol-etherdiamine-tetraacetic acid), HEPES (*N*-2-hydroxyethyl-piperazine-*N*'-2-ethanesulphonic acid), fura-2 AM and pluronic F-127 (Dojin, Kumamoto, Japan), DMSO (dimethyl sulphoxide) and Triton X-100 (Wako, Tokyo, Japan). U46619 was dissolved in ethanol to a concentration of 1 mM. Cromakalim was dissolved in 70% ethanol to a concentration of 10 mM. Fura-2 AM was dissolved in DMSO at the concentration of 1 mM. Pluronic F-127 was dissolved in 25% w/v in DMSO. Triton X-100 was dissolved in 1% w/v in PSS. Other drugs or chemicals were dissolved in distilled water and diluted to the desired concentrations by PSS.

Analysis of concentration-response curves for U46619 and statistical analysis

To examine the potencies of U46619 and the maximum responses produced by U46619 in 5 mM KCl-PSS and in 20 mM KCl-PSS, the concentration-response curves for U46619 in increasing $[Ca^{2+}]_i$ and force of contraction were computer-fitted to the equation (1).

$$E = M \times A^P / (A^P + K^P) \qquad (1)$$

where E is the effect normalized by the differences between basal values and those of 90 mM KCl at 10 min, M is the maximum responses, A is the concentration of U46619, K is the EC_{50} value of U46619 and p is the slope parameter. EC_{50} values were presented as pD_2 ($pD_2 = -\log EC_{50}$).

Experimental values are given as means ± S.E.M. Statistical significance of results was evaluated by Student's t-test. A P value smaller than 0.05 was considered to indicate a significant difference.

RESULTS

Effects of depolarization on changes in $[Ca^{2+}]_i$ and force induced by U46619

Figure 1a shows typical recordings of $[Ca^{2+}]_i$ and force induced by exposure to cumulative concentrations of U46619 and the influence of membrane depolarization by 20 mM KCl-PSS in a canine coronary arterial ring. Data obtained from 6 preparations are summarized in Fig. 1b. U46619 (10^{-9} to 3×10^{-8} M) increased both $[Ca^{2+}]_i$ and force in a concentration-dependent manner. The increases in $[Ca^{2+}]_i$ reached a maximum level at about 20% (expressed as percentage of the response by 90 mM KCl) at 3×10^{-8} M U46619, but the increase in force occurred in a concentration-dependent manner. Upon depolarization by 20 mM KCl-PSS, $[Ca^{2+}]_i$ increased by about 20%, but the basal force remained unchanged. The increases in $[Ca^{2+}]_i$ and force produced by U46619 became apparent from 10^{-9} M U46619, and the increase in force was markedly potentiated in 20 mM KCl-PSS (Fig. 1, a and b). The summarized data obtained from analysis of concentration-response curves for U46619 to increase $[Ca^{2+}]_i$ and force are presented in Table 1. In 20 mM KCl-PSS, the pD_2 value of U46619 to increase $[Ca^{2+}]_i$ was increased, whereas that to increase force was not changed. The maximum effect of U46619 to

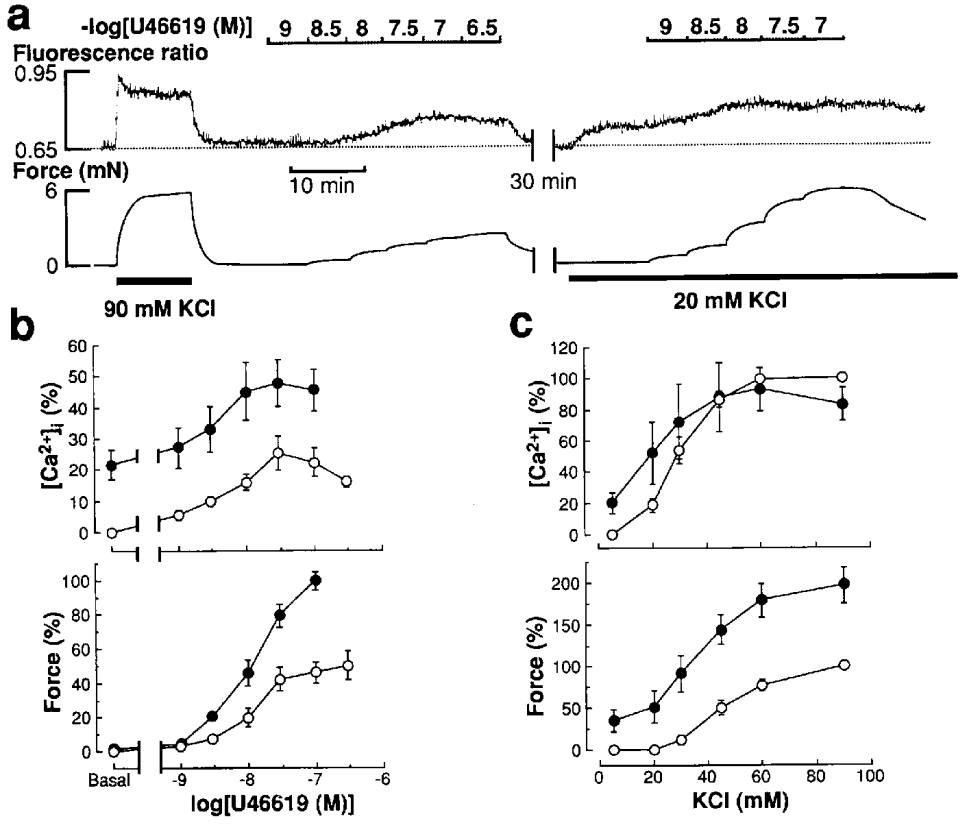

Fig. 1. Effects of U46619 on $[Ca^{2+}]_i$ and force in canine coronay arteries. (a) Effects of depolarization by 20 mM KCl-PSS on changes in $[Ca^{2+}]_i$ and force of contraction induced by U46619. Typical traces of $[Ca^{2+}]_i$ (indicated as fluorescence ratio) and force under resting (5 mM KCl-PSS) and depolarized (20 mM KCl-PSS) conditions. The concentrations of U46619 (10^{-9} to 3×10^{-7} M) were increased comulatively. (b) The summarized data obtained from 6 preparations. The increases in $[Ca^{2+}]_i$ and force of contraction in 5 mM KCl-PSS (○) and in 20 mM KCl-PSS (●) are expressed as a percentage of each effect to 90 mM KCl-PSS at 10 min. Vertical bars show S.E.M. (c) Effects of U46619 on increases in $[Ca^{2+}]_i$ and force of contraction produced by various concentrations of KCl (5, 20, 30, 45, 60, 90 mM). Control (○), in the presence of 10^{-8} M U46619 (●). Data are expressed as means ± S.E.M. (n = 6).

Table 1. The summarized results of the analysis of concentration-response curves for U46619 on the $[Ca^{2+}]_i$ and force of contraction in canine coronary arteries

	Increase in $[Ca^{2+}]_i$			Increase in force		
	Maximum effect (%)	pD_2	p	Maximum effect (%)	pD_2	p
5 mM KCl	30.1 ± 6.9	8.08 ± 0.14	1.27 ± 0.17	47.6 ± 6.9	7.89 ± 0.13	1.74 ± 0.23
20 mM KCl	33.1 ± 5.2	8.55 ± 0.10*	1.30 ± 0.09	113.2 ± 10.9*,a	7.87 ± 0.13a	1.28 ± 0.12

The concentration-effect curves for U46619 were analyzed by computer-fitting to the logistic equation (1) described in the methods. $pD_2 = -\log EC_{50}$, and p is slope parameter. *: $P < 0.05$, compared with 5 mM KCl values; a: $P < 0.05$, compared with the values of $[Ca^{2+}]_i$. Values are presented as means ± S.E.M. of 6 experiments.

increase the force of contraction was increased; however, that to increase $[Ca^{2+}]_i$ was not changed. Thus, in 20 mM KCl-PSS, the pD_2 values of U46619 to increase $[Ca^{2+}]_i$ and force became different ($P < 0.05$).

We examined the influences of U46619 (10^{-8} M) on changes in $[Ca^{2+}]_i$ and force induced by cumulative concentrations of KCl in PSS (5, 20, 30, 45, 60 and 90 mM). The results are shown in Fig. 1c. In 5 mM KCl-PSS, U46619 (10^{-8} M) produced about a 20% increase in $[Ca^{2+}]_i$ and about a 40% increase in force of contraction. The depolarization by 20 or 30 mM KCl induced a larger increase in $[Ca^{2+}]_i$ in the presence of U46619 than in its absence; however, the increases in $[Ca^{2+}]_i$ induced by higher concentrations (45, 60 and 90 mM) of KCl were not different between in the absence and presence of U46619. Thus, in the presence of U46619, the curve which relates $[Ca^{2+}]_i$ to KCl concentrations was shifted to the left. The force of contraction induced by depolarization by KCl was enhanced in the presence of U46619. Although the increase in $[Ca^{2+}]_i$ in higher concentrations (45, 60 and 90 mM) of KCl was almost the same as the control (5 mM), the force was much larger in the presence of U46619 than in its absence (Fig. 1c).

Effects of cromakalim and verapamil on changes in $[Ca^{2+}]_i$ and force induced by U46619

After exposure to cumulative concentrations of U46619 (10^{-8} to 3×10^{-7} M), the preparation was depolarized by 90 mM KCl-PSS in the presence of 3×10^{-7} M U46619. The increase in $[Ca^{2+}]_i$ produced by 90 mM KCl-PSS was almost the same as that in the absence of U46619, whereas the force of contraction was almost twice that in its absence (Fig. 2, a and b). After washout with 5 mM KCl-PSS for 30 min, cromakalim (3×10^{-6} M) was administered. As in a previous study (6), cromakalim reduced the basal $[Ca^{2+}]_i$ without changing the force. Cromakalim almost abolished the increases in $[Ca^{2+}]_i$ and force induced by U46619 (Fig. 2a). In the presence of cromakalim, 90 mM KCl-PSS containing U46619 at 3×10^{-7} M which had been reached cumulatively produced increases in $[Ca^{2+}]_i$ and force comparable to those in the absence of cromakalim (Fig. 2a, Table 2).

In the presence of verapamil (3×10^{-6} M), $[Ca^{2+}]_i$ became slightly lower than the basal level. The decrease in $[Ca^{2+}]_i$ produced by verapamil was smaller than that by cromakalim. Verapamil greatly reduced the increases in $[Ca^{2+}]_i$ and force induced by U46619 (Fig. 2b). In the presence of verapamil, 90 mM KCl-PSS containing U46619 (3×10^{-7} M) produced increases in $[Ca^{2+}]_i$ and force which were significantly smaller than those in the absence of verapamil ($P < 0.01$, Table 2). The summarized data concerning the effects of cromakalim and verapamil and their influences on the concentration-response relations of U46619 are shown in Fig. 2, c and d.

Modification by depolarization of the inhibitory effects of cromakalim on changes in $[Ca^{2+}]_i$ an force induced by U46619

Because, as described in the previous section, the application of 90 mM KCl abolished the inhibitory effects of cromakalim (3×10^{-6} M) on U46619 induced changes in $[Ca^{2+}]_i$ and force, we wanted to determine the concentration of KCl and the extent of depolarization to counteract the hyperpolarization induced by cromakalim. Figure 3a shows the modification by 20 mM KCl-PSS of the effects of cromakalim (3×10^{-6} M) on changes in $[Ca^{2+}]_i$ and force induced by U46619 (10^{-8} to 3×10^{-7} M). The $[Ca^{2+}]_i$ reduced by cromakalim below the basal level was increased to near the basal level. The increases in $[Ca^{2+}]_i$ and force by U46619 in the presence of cromakalim in 20 mM KCl-PSS were slightly smaller than those of the control. The summarized data to represent the modification by 20 and 25 mM KCl of the effects of cromakalim and its influences on U46619 induced changes in $[Ca^{2+}]_i$ and

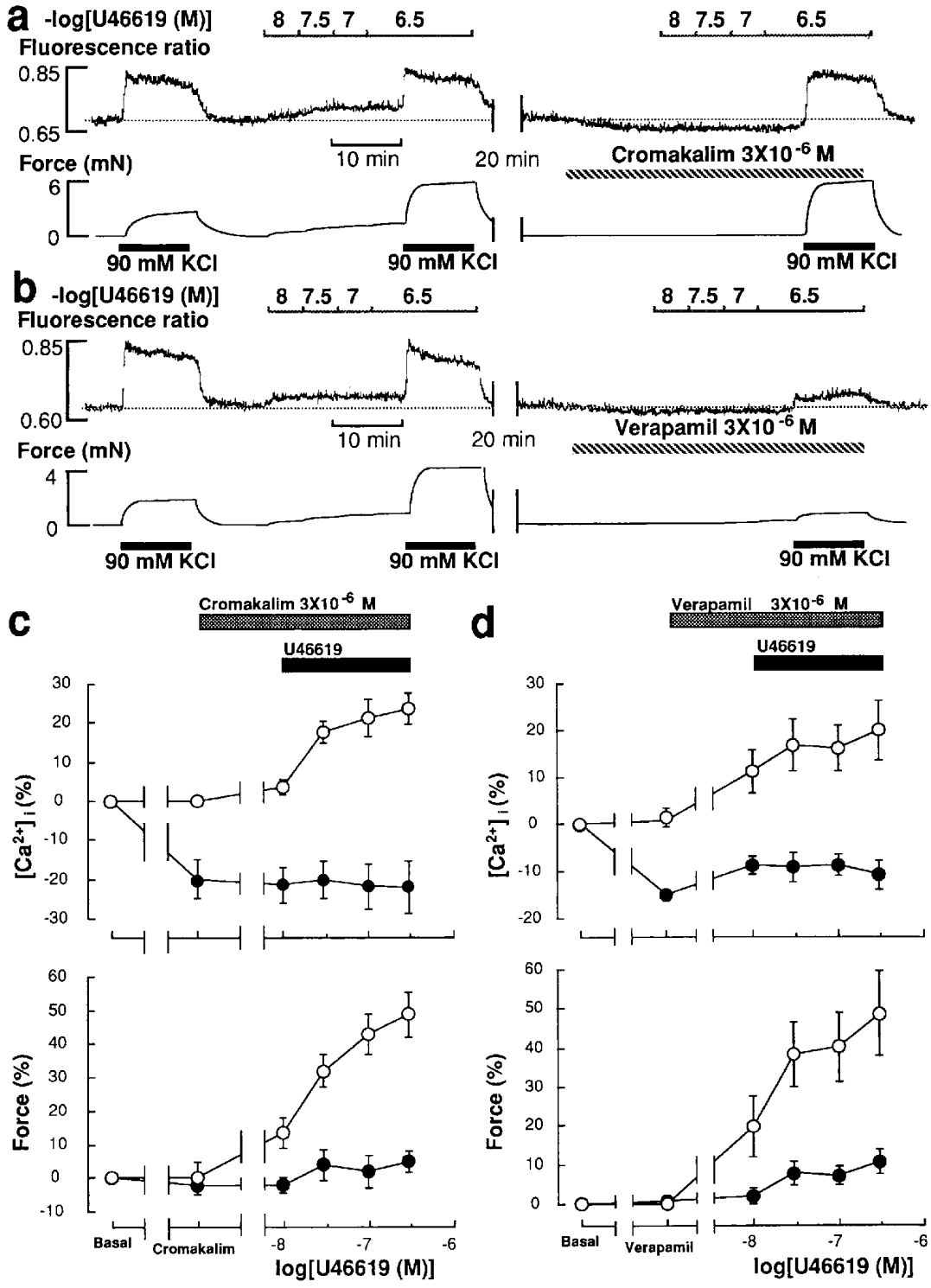

Fig. 2. Effects of cromakalim and verapamil on changes in $[Ca^{2+}]_i$ and force of contraction induced by U46619. (a) Typical traces of $[Ca^{2+}]_i$ and force under resting conditions (5 mM KCl-PSS) and in the presence of cromakalim (3×10^{-6} M). The concentrations of U46619 (10^{-8} to 3×10^{-7} M) were increased cumulatively. (b) Typical traces of $[Ca^{2+}]_i$ and force under resting conditions (5 mM KCl-PSS) and in the presence of verapamil (3×10^{-6} M). (c) The summarized data obtained from 6 preparations without and with cromakalim. Control (○) and in the presence of 3×10^{-6} M cromakalim (●). (d) The summarized data obtained from 6 preparations without and with verapamil. Control (○) and in the presence of 3×10^{-6} M verapamil (●). Data are expressed as means ± S.E.M.

Table 2. Effects of cromakalim and verapamil on increases in $[Ca^{2+}]_i$ and force by 90 mM KCl in the presence of U46619

	n	Increase in $[Ca^{2+}]_i$ (%)	Increase in force (%)
Control	5	95 ± 14	256 ± 39
+Cromakalim 3×10^{-6} M		85 ± 8	239 ± 40
Control	6	85 ± 13	240 ± 40
+Verapamil 3×10^{-6} M		23 ± 6*	56 ± 13*

*: $P < 0.01$, compared with the control. n: number of experiments. Control values are not signficantly different from each other.

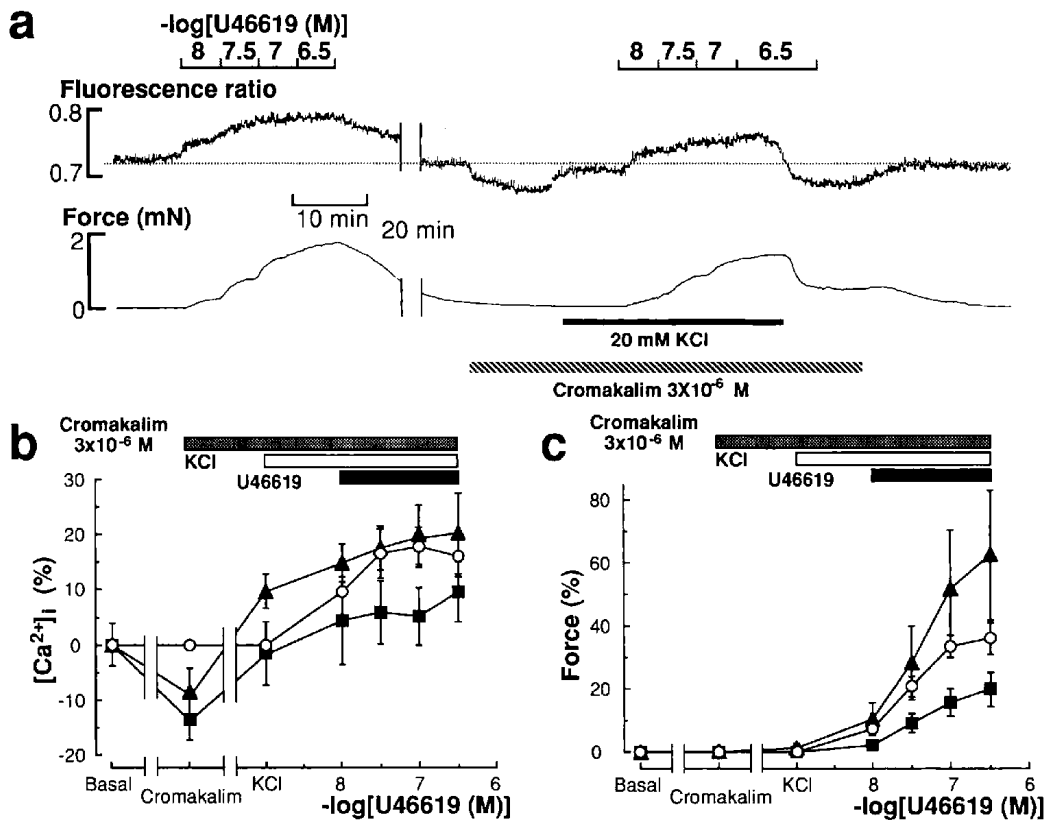

Fig. 3. Modification by depolarization of the inhibitory effects of cromakalim on increases in $[Ca^{2+}]_i$ and force induced by U46619. (a) Typical traces of $[Ca^{2+}]_i$ and force under resting conditions (5 mM KCl-PSS) and in the presence of cromakalim (3×10^{-6} M) and 20 mM KCl-PSS. The concentrations of U46619 (10^{-8} to 3×10^{-7} M) were increased cumulatively. (b), (c) The summarized data obtained from 6 preparations each. Control (○, n = 12), in the presence of 20 mM KCl-PSS (■, n = 6) and 25 mM KCl-PSS (▲, n = 6).

force are shown in Fig. 3, b and c. The concentration-response curve for U46619 in the presence of cromakalim in 20 mM KCl-PSS was located below the control curve and that in its presence in 25 mM KCl-PSS was located above the control curve. Therefore, the optimal concentration of KCl in PSS which counteracts both the hyperpolarizing action and the inhibitory effects of cromakalim (3×10^{-6} M) on changes in $[Ca^{2+}]_i$ and force induced by U46619 is between 20 and 25 mM.

Effects of U46619 on the relationship between $[Ca^{2+}]_i$ and force

Figure 4 shows the relationship between $[Ca^{2+}]_i$ and force in the absence (control) and in the presence of U46619 (10^{-8} or 3×10^{-7} M). Data were obtained by

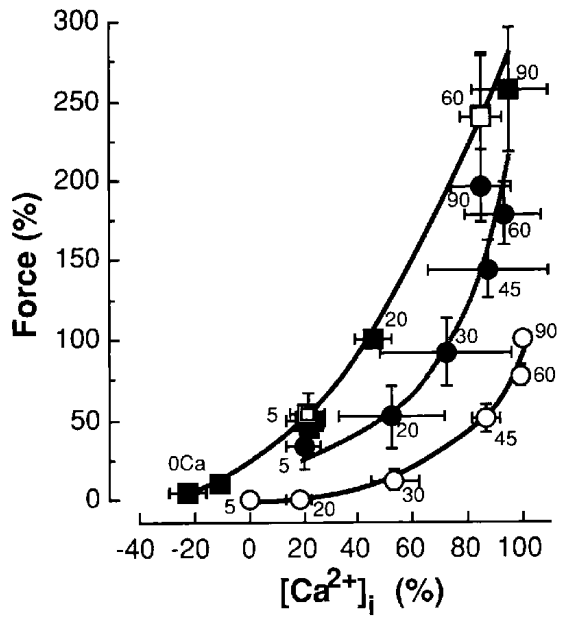

Fig. 4. Relationships between $[Ca^{2+}]_i$ and force in the presence and the absence of U46619. ○: in the absence of U46619 (control). Data are adopted from Fig. 1c. ● and ■: in the presence of 10^{-8} M (adopted from Fig. 1c) and 3×10^{-7} M U46619, respectively. □ and ⊡: in the presence of 3×10^{-6} M cromakalim and 3×10^{-6} M verapamil in 90 mM KCl-PSS with 3×10^{-7} M U46619, respectively. The numbers (5, 20, 30, 45, 60, and 90) indicate the KCl concentration (mM) of PSS, and 0Ca is Ca^{2+}-free PSS. Vertical and horizontal bars are S.E.M. (n = 5–6). The curves were fitted by eye.

changing KCl concentrations in PSS (5, 20, 30, 45, 60 and 90 mM) or removing extracellular Ca^{2+} in the absence and in the presence of U46619. The curves in the presence of U46619 were shifted to the left and upward in a concentration-dependent manner. This result shows that U46619 has an action to increase the Ca^{2+}-sensitivity of the contractile proteins. In Fig. 4, there are data points showing the relationship between $[Ca^{2+}]_i$ and force in the presence of cromakalim (3×10^{-6} M) or verapamil (3×10^{-6} M) in 90 mM KCl-PSS with 3×10^{-7} M U46619. These points are not different from those in the absence of cromakalim or verapamil. Thus, in 90 mM KCl-PSS, cromakalim and verapamil do not change the Ca^{2+}-sensitivity of the contractile proteins.

DISCUSSION

The present study demonstrated the following results: In canine coronary arteries, U46619 administered cumulatively produces an increase in $[Ca^{2+}]_i$ due to Ca^{2+} influx. The Ca^{2+} influx induced by U46619 is enhanced by depolarization and abolished by the Ca^{2+} channel blocker verapamil. The Ca^{2+} infulx caused by U46619 is also inhibited by the K^+ channel opener cromakalim, which is counteracted by the depolarization produced by an increase in KCl concentration. U46619 increases the Ca^{2+}-sensitivity of the contractile proteins.

Thromboxane A_2 is a potent constrictor of vascular smooth muscle as well as a strong inducer of platelet aggregation (14–16). U46619 is a stable and full agonist analogue of thromboxane A_2 (17, 18). Recently, the human thromboxane A_2 receptor was cloned, and its amino-acid sequence shows that it is one of the G-protein coupled receptors (19). The vasoconstrictor mechanisms of thromboxane A_2 analogues have been proposed to involve their ability to cause Ca^{2+} release from intracellular stores and increase transmembrane Ca^{2+} influx (3, 20). However, the cumulative manner of the application of U46619 (10^{-9}–3×10^{-7} M) seems not to induce a marked release of Ca^{2+} in this study. The application of U46619 at a higher concentration (3×10^{-7} M) at once induced a Ca^{2+} release in the absence of extracellular Ca^{2+} (data not shown). In the present study, the increase in $[Ca^{2+}]_i$ induced by U46619 was potentiated by depolarization by 20 mM KCl, and the curve which related $[Ca^{2+}]_i$ to extracellular KCl concentrations was shifted to the left by U46619 (Fig. 1). Verapamil inhibited the increases in $[Ca^{2+}]_i$ and force caused by the cumulative applications of U46619 (Fig. 2). In our previous study (21), Ca^{2+} influx induced by histamine was also abolished by verapamil. These results suggest that the Ca^{2+} influx induced by agonists including U46619 is verapamil-sensitive and associated with that through voltage-dependent L-type Ca^{2+} channels (4).

The inhibitory effect of cromakalim on changes in $[Ca^{2+}]_i$ and force induced by U46619 was counteracted by increasing KCl concentration in PSS (Figs. 2 and 3). The optimal concentration of KCl was between 20 and 25 mM. An increase in KCl concentration is known to increase the K^+ conductance of the plasma membrane (22). Thus, the counteracting influence of the increase in KCl concentration seems to be mainly due to its membrane depolarization effect. Therefore, the inhibitory action of cromakalim on Ca^{2+} influx seems to be due to deactivation of L-type Ca^{2+} channels by hyperpolarization of the plasma membrane as is the case with the KCl-depolarization-induced Ca^{2+} influx (6). The same inhibitory mechanisms of cromakalim were reported in the noradrenaline-induced contraction of rabbit aorta (23, 24).

The increase in the Ca^{2+} sensitivity of contractile proteins by various agonists has been reported in pig coronary artery (21), rabbit pulmonary artery (25) and

rat aorta (13). The relationship between $[Ca^{2+}]_i$ and force shows that U46619 has an action to increase the Ca^{2+}-sensitivity of contractile proteins (Fig. 4). In the simultaneous application of 90 mM KCl with U46619 at 3×10^{-7} M, the increase in $[Ca^{2+}]_i$ and force were not changed by cromakalim, whereas verapamil greatly reduced the increases in both $[Ca^{2+}]_i$ and force induced by 90 mM KCl without changing the relationship between $[Ca^{2+}]_i$ and force (Fig. 4). Since the relationships between $[Ca^{2+}]_i$ and force are not changed by cromakalim and verapamil, they do not change the increased Ca^{2+} sensitivity of contractile proteins by U46619 in 90 mM KCl-PSS (Fig. 2, b and d). One possible mechanism of Ca^{2+}-sensitizing is the activation of protein kinase C (26, 27) mediated by the formation of diacylglycerol after the agonist activation of phospholipase C (13, 21, 28) or phospholipase D (29).

In summary, U46619 produces Ca^{2+} influx through L-type channels, which are deactivated by hyperpolarization induced by cromakalim in canine coronary artery.

Acknowledgments

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REFERENCES

1 Lewy, R.I., Wiener, L., Walinsky, P., Lefer, A.M., Silver, M.J. and Smith, J.B.: Thromboxane release during pacing-induced angina pectoris: Possible vasoconstrictor influence on the coronary vasculature. Circulation **61**, 1165–1171 (1980)

2 Halushka, P.V. and Lefer, A.M.: Thromboxane A_2 in health and disease. Fed. Proc. **46**, 131–132 (1987)

3 Toda, N.: Mechanism of action of carbocyclic thromboxane A_2 and its interaction with prostaglandin I_2 and verapamil in isolated arteries. Circ. Res. **51**, 675–682 (1982)

4 Nelson, M.T., Patlak, J.B., Worley, J.F. and Standen, N.B.: Calcium channels, potassium channels, and voltage dependence of arterial smooth muscle tone. Am. J. Physiol. **259**, C3–C18 (1990)

5 Yanagisawa, T., Kawada, M. and Taira, N.: Nitroglycerin relaxes canine coronary arterial smooth muscle without reducing intracellular Ca^{2+} concentrations measured with fura-2. Br. J. Pharmacol. **98**, 469–482 (1989)

6 Yanagisawa, T., Teshigawara, T. and Taira, N.: Cytoplasmic calcium and the relaxation of canine coronary arterial smooth muscle produced by cromakalim, pinacidil and nicorandil. Br. J. Pharmacol. **101**, 157–165 (1990)

7 Okada, Y., Yanagisawa, T. and Taira, N.: An analysis of the nitrate-like and K channel opening actions of KRN2391 in canine coronary arterial smooth muscle. Br. J. Pharmacol. **104**, 829–838 (1991)

8 Hamilton, T.C., Weir, S.W. and Weston, A.H.: Comparison of the effects of BRL 34915 and verapamil on electrical and mechanical activity in rat portal vein. Br. J. Pharmacol. **88**, 103–111 (1986)

9 Cook, N.S.: The pharmacology of potassium channels and their therapeutic potential. Trends Pharmacol. Sci. **9**, 21–28 (1988)

10 Himpens, B. and Somlyo, A.P.: Free-calcium and force transients during depolarization and pharmacomechanical coupling in guinea-pig smooth muscle. J. Physiol. (Lond.) **395**, 507–530 (1988)

11 Konishi, M., Olson, A., Hollingworth, S. and Baylor, S.M.: Myoplasmic binding of fura-2 investigated by steady-state fluorescence and absorbance measurements. Biophys. J. **54**, 1089–1104 (1988)

12 Karaki, H.: Ca^{2+} localization and sensitivity in vascular smooth muscle. Trends Pharmacol. Sci. **10**, 320–325 (1989)

13 Sato, K., Ozaki, H. and Karaki, H.: Multiple effects of caffeine on contraction and cytosolic free Ca^{2+} levles in vascular smooth muscle of rat aorta. Naunyn Schmiedebergs Arch. Pharmacol. **338**, 443–448 (1988)

14 Hamberg, M., Svensson, J. and Samuelsson, B.: Thromboxanes: A new group of biologically active compounds derived from prostaglandin endoperoxides. Proc. Natl. Acad. Sci. U.S.A. **72**, 2994–2998 (1975)

15 Ellis, E.F., Oelz, O., Roberts, L.J., II, Payne, N.A., Sweetman, B.J., Nies, A.S. and Oates, J.A.: Coronary arterial smooth muscle contraction by a substance released from platelets: Evidence that it is thromboxane A_2. Science **193**, 1135–1137 (1976)

16 Moncada, S. and Vane, J.R.: Unstable metabolites of arachidonic acid and their role in haemostasis and thrombosis. Br. Med. Bull. **34**, 129–135 (1978)

17 Hanasaki, K., Nakano, K., Kasai, H., Arita, H., Ohtani, K. and Doteuchi, M.: Specific receptors for thromboxane A_2 in cultured vascular smooth muscle cells of rat aorta. Biochem. Biophys. Res. Commun. **150**, 1170–1175 (1988)

18 Tymkewycz, P.M., Jones, R.L., Wilson, N.H. and Marr, C.G.: Heterogeneity of thromboxane A_2 (TP-) receptors: evidence from antagonist but not agonist potency measurements. Br. J. Pharmacol. **102**, 607–614 (1991)

19 Hirata, M., Hayashi, Y., Ushikubi, F., Yokota, Y., Kageyama R., Nakanishi, S. and Narumiya, S.: Cloning and expression of cDNA for a human thromboxane A_2 receptor. Nature **349**, 617–620 (1991)

20 Kanmura, Y., Itoh, T. and Kuriyama, H.: Mechanisms of vasoconstriction induced by 9,11-epithio-11,12-methanothromboxane A_2 in the rabbit coronary artery. Circ. Res. **60**, 402–409 (1987)

21 Mori, T., Yanagisawa, T. and Taira, N.: Histamine increases vascular tone an intracellular calcium level using both intracellular and extracellular calcium in porcine coronary arteries. Japan. J. Pharmacol. **52**, 263–271 (1990)

22 Sperelakis, N.: Origin of the cardiac resting potential. *In* The Handbook of Physiology, Section 2: The Cardiovascular Sys-

tem, Edited by Berne, R.M., Vol. 1, p. 187–267, American Physiological Society, The William & Wilkins Co., Baltimore (1979)

23 Cook, N.S., Weir, S.W. and Danzeisen, M.C.: Anti-vasoconstrictor effects of the K^+ channel opener cromakalim on the rabbit aorta—comparison with the calcium antagonist isradipine. Br. J. Pharmacol. **95**, 741–752 (1988)

24 Bray, K.M., Weston, A.H., Duty, S., Newgreen, D.T., Longmore, J., Edwards, G. and Brown, T.J.: Differences between the effects of cromakalim and nifedipine on the agonist-induced responses in rabbit aorta. Br. J. Pharmacol. **102**, 337–344 (1991)

25 Himpens, B., Kitazawa, T. and Somlyo, A.P.: Agonist-dependent modulation of Ca^{2+} sensitivity in rabbit pulmonary artery smooth muscle. Pflügers Arch. **417**, 21–28 (1990)

26 Nishizuka, Y.: Studies and perspectives of protein kinase C. Science **233**, 305–312 (1986)

27 Nishizuka, Y.: The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature **334**, 661–665 (1988)

28 Mori, T., Yanagisawa, T. and Taira, N.: Phorbol 12,13-dibutyrate increases vascular tone but has a dual action on intracellular calcium levels in porcine coronary arteries. Naunyn Schmiedebergs Arch. Pharmacol. **341**, 251–255 (1990)

29 Exton, J.H., Taylor, S.J., Augert, G. and Bocckino, S.B.: Cell signalling through phospholipid breakdown. Mol. Cell. Biochem. **104**, 81–86 (1991)