British Journal of Pharmacology (1998) 123, 869–878

Effect of cilostazol, a phosphodiesterase type III inhibitor, on histamine-induced increase in $[Ca^{2+}]_i$ and force in middle cerebral artery of the rabbit

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1 The effect of cilostazol, an inhibitor of phosphodiesterase type III (PDE III), on the contraction induced by histamine was studied by making simultaneous measurements of isometric force and the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) in endothelium-denuded muscle strips from the peripheral part of the middle cerebral artery of the rabbit.

2 High K^+ (80 mM) produced a phasic, followed by a tonic increase in both $[Ca^{2+}]_i$ and force. Cilostazol (10 μM) did not modify the resting $[Ca^{2+}]_i$, but it did significantly decrease the tonic contraction induced by high K^+ without a corresponding change in the $[Ca^{2+}]_i$ response.

3 Histamine (3 μM) produced a phasic, followed by a tonic increase in both $[Ca^{2+}]_i$ and force. Cilostazol (3 and 10 μM) significantly reduced both the phasic and tonic increases in $[Ca^{2+}]_i$ and force induced by histamine, in a concentration-dependent manner.

4 Rp-adenosine-3′:5′-cyclic monophosphorothioate (Rp-cAMPS, 0.1 mM), a PDE-resistant inhibitor of protein kinase A (and as such a cyclic AMP antagonist), did not modify the increases in $[Ca^{2+}]_i$ and force induced by histamine alone, but it did significantly decrease the cilostazol-induced inhibition of the histamine-induced responses.

5 In Ca^{2+}-free solution containing 2 mM EGTA, both histamine (3 μM) and caffeine (10 mM) transiently increased $[Ca^{2+}]_i$ and force. Cilostazol (1–10 μM) (i) significantly reduced the increases in $[Ca^{2+}]_i$ and force induced by histamine, and (ii) significantly reduced the increase in force but not the increase in $[Ca^{2+}]_i$ induced by caffeine.

6 In ryanodine-treated strips, which had functionally lost the histamine-sensitive Ca^{2+} storage sites, histamine (3 μM) slowly increased $[Ca^{2+}]_i$ and force. Cilostazol (3 and 10 μM) lowered the resting $[Ca^{2+}]_i$, but did not modify the histamine-induced increase in $[Ca^{2+}]_i$, suggesting that functional Ca^{2+} storage sites are required for the cilostazol-induced inhibition of histamine-induced Ca^{2+} mobilization.

7 The $[Ca^{2+}]_i$-force relationship was obtained in ryanodine-treated strips by applying ascending concentrations of Ca^{2+} (0.16–2.6 mM) in Ca^{2+}-free solution containing 100 mM K^+. Histamine (3 μM) shifted the $[Ca^{2+}]_i$-force relationship to the left and increased the maximum Ca^{2+}-induced force. Under the same conditions, whether in the presence or absence of 3 μM histamine, cilostazol (3–10 μM) shifted the $[Ca^{2+}]_i$-force relationship to the right without producing a change in the maximum Ca^{2+}-induced force.

8 It is concluded that, in smooth muscle of the peripheral part of the rabbit middle cerebral artery, cilostazol attenuates the histamine-induced contraction both by inhibiting histamine-induced Ca^{2+} mobilization and by reducing the myofilament Ca^{2+} sensitivity. It is suggested that the increase in the cellular concentration of cyclic AMP that will follow the inhibition of PDE III may play an important role in the cilostazol-induced inhibition of the histamine-contraction.

Keywords: Cilostazol; phosphodiesterase; histamine-induced Ca^{2+} mobilization; myofilament Ca^{2+} sensitivity; agonist-induced Ca^{2+} release; cerebral vessels

Introduction

An increase in adenosine 3′:5′ cyclic-monophosphate (cyclic AMP) and guanosine 3′:5′ cyclic-monophosphate (cyclic GMP) in smooth muscle cells causes a vascular relaxation. The degradation of these cyclic nucleotides by hydrolytic cleavage of the 3′-ribose-phosphate bond is catalyzed by cyclic nucleotide phosphodiesterases (PDEs) (Beavo, 1995; Polson & Strada, 1996). Consequently, activation of adenylyl or guanylyl cyclase and inhibition of PDEs can both lead to an increase in the intracellular concentration of cyclic AMP or cyclic GMP in vascular smooth muscle cells (Komas *et al*., 1991). The classification of PDE enzymes includes at least seven different isoenzyme families. The classification is based on the nucleotide preferentially hydrolyzed and the regulatory properties of the various enzymes, and the isoforms of these enzymes have been found to be variously distributed in cardiac muscle, vascular smooth muscle and platelets (Weishaar *et al*., 1986; Lindgren & Andersson, 1991; Beavo, 1995; Manganiello *et al*., 1995). Among the isoenzymes, type III PDE is a low K_m cyclic AMP PDE which hydrolyzes both cyclic AMP and cyclic GMP and is inhibited by cyclic GMP (Manganiello *et al*., 1995). The presence of this enzyme has been detected in vascular smooth muscle cells (Komas *et al*., 1991).

Various types of selective inhibitors of PDE III have been synthesized in the course of the search for a treatment for acute

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heart failure (e.g. amrinone, milrinone and enoximone) (Silver *et al*., 1988) or as an anti-thrombotic agent (cilostamide) (Caldicott *et al*., 1993; Polson & Strada, 1996). It has been found that these PDE III inhibitors cause vasodilatation, leading to a concomitant reduction in arterial pressure (Polson & Strada, 1996). Cilostazol (6-[4-(1-cyclohexyl-1H-tetrazol-5-yl)butoxy]-3,4-dihydro-2(1H)-quinolinone), a derivative of cilostamide, was found selectively to inhibit PDE III both in platelets (Umekawa *et al*., 1984) and in vascular smooth muscle (Tanaka *et al*., 1988), and this agent is now clinically used as an anti-platelet agent. It has been found that cilostazol has a peripheral vasodilating action with only weak cardiac effects and that the vasodilator action is more powerful on the vertebral artery than on the carotid and superior mesenteric arteries in the anaesthetized dog (Kawamura *et al*., 1985; Kimura *et al*., 1985; Shintani *et al*., 1985). These results suggest that cilostazol might be useful in the treatment of acute cerebral infarction, especially lacunar infarction (Kawamura *et al*., 1985; Kimura *et al*., 1985). However, the effect of cilostazol has not yet been fully clarified in a cerebral resistance artery. Moreover, it is as yet unclear which of the various mechanisms underlying vascular smooth muscle contraction and relaxation (for review, see Kuriyama *et al*., 1995) might be affected by cilostazol.

To study the action of cilostazol in a cerebral resistance artery, its effect was observed on the increases in $[Ca^{2+}]_i$ and force induced by high K^+ or histamine in endothelium-denuded strips from the distal part of the middle cerebral artery (MCA) of the rabbit. The effect of cilostazol on histamine-induced Ca^{2+} influx was also examined in ryanodine-treated muscle strips (which have functionally lost the histamine-sensitive Ca^{2+} storage sites) (Watanabe *et al*., 1996). To assess the effect of cilostazol on the histamine-induced Ca^{2+} release, its effect was observed on the histamine-induced increase in $[Ca^{2+}]_i$ in Ca^{2+}-free solution. The action of cilostazol on myofilament Ca^{2+}-sensitivity was assessed by examining its effect on the $[Ca^{2+}]_i$-force relationship both in the presence and in the absence of histamine in ryanodine-treated muscle strips. Moreover, the role of cyclic AMP in cilostazol-induced responses was studied by examining the effect of Rp-cAMPS, a PDE-resistant inhibitor of protein kinase A (and as such a cyclic AMP antagonist), on cilostazol-induced relaxation.

Methods

Male Japanese White albino rabbits (supplied by Kiyayama Labes Co. Ltd, Japan), weighing 1.9–2.5 kg, were anaesthetized by injection of pentobarbitone sodium (40 mg kg^{-1}, i.v.) and then exsanguinated. The protocols used conformed with guidelines on the conduct of animal experiments issued by Nagoya City University Medical School and by the Japanese government (Law no. 105; Notification no. 6), and were approved by The Committee on the Ethics of Animal Experiments in Nagoya City University Medical School. The brain was removed and placed in a chamber filled with Krebs solution. Intermediate and distal segments of the middle cerebral artery (MCA, diameter approximately 100–120 μm) were dissected under a binocular microscope, and arachnoid membrane and connective tissues were carefully removed. After the artery had been cut along its long axis with small scissors, the endothelium of the strip was carefully removed by gentle rubbing of the internal surface of the vessel with small pieces of razor blade, as described previously (Itoh *et al*., 1992a,b). Satisfactory ablation of the endothelium was pharmacologically verified by the absence of a relaxing effect of 3–10 μM acetylcholine during a histamine-contraction.

$[Ca^{2+}]_i$ and force measurement

To enable simultaneous recording of isometric force and $[Ca^{2+}]_i$, fine circularly-cut strips (0.3–0.5 mm long, 0.04–0.05 mm wide, 0.02–0.03 mm thick) were prepared as described previously (Itoh *et al*., 1992a,b). The strip was transferred to a chamber of 0.3 ml volume and mounted horizontally on an invert-microscope (Diaphoto TMD with special optics for epifluorescence, Nikon). The resting force was adjusted to obtain a maximum contraction in Krebs solution containing 80 mM K^+.

To enable loading of Fura 2 into smooth muscle cells of the strip, 1.5 μM acetoxymethyl ester of Fura 2 (Fura 2-AM) was applied for 1.5 h in Krebs solution at room temperature. After this period, the solution containing Fura 2-AM was washed out with Krebs solution for 1 h to ensure sufficient de-esterification of Fura 2-AM in the cells. The position of the strip was adjusted to the centre of the field and a mask placed in an intermediate image plane to reduce background fluorescence (0.04 mm square). The Fura 2 fluorescence emission at 510 nm passed through the lens (20 times CF Fluor objective lens, Nikon) was collected in a photomultiplier tube (R 928, side-on type, Hamamatsu Photonics, Japan). Two alternative excitation wavelengths, 340 nm and 380 nm (each slit 5 nm) were applied by a spectro-fluorimeter (CAM 220, Japan Spectroscopic Co. Ltd., Tokyo, Japan) and the data were analysed by use of software developed in our laboratory. The ratio of the Fura 2 fluorescence intensities excited by 340 or 380 nm was calculated after subtraction of the background fluorescence and the $[Ca^{2+}]_i$ was calculated by use of a formula given by Grynkiewicz *et al*. (1985) and an *in vitro* calibration procedure (Poenie *et al*., 1986). The background fluorescence excited by 340 and 380 nm u.v. light was measured after the experiment following the application of a solution containing 50 μM ionomycin, 20 mM $MnCl_2$, 110 mM KCl and 10 mM MOPS. The ratio of maximum (F_{max}) to minimum (F_{min}) fluorescence was determined in the calibration solution after subtraction of background, and the 380 nm signal of Fura 2 was assumed to decrease by 15% in the cell due to the possible intracellular viscosity effects of the dye (Poenie *et al*., 1986; Itoh *et al*., 1992a,b). The F_{max} values were within the range 5.75 to 7.89 and F_{min} values were 0.19–0.27. The K_d value for Fura 2 was estimated to be 200 nM (Itoh *et al*., 1992a). To minimize the leakage of Fura 2 from the cells, the present experiments were conducted at room temperature, and u.v. light was applied during the recording period only (to minimize the photobleaching of Fura 2), as previously described (Itoh *et al*., 1992a,b; Shafiq *et al*., 1992; Itoh *et al*., 1993). The *in vitro* calibration procedure used in the present experiments may not provide an accurate measurement of $[Ca^{2+}]_i$ due to differences in the behaviour of the dye in the cell cytosol and in free solution. Any values of $[Ca^{2+}]_i$ given here should therefore be treated as approximations.

Histamine (3 μM), high K^+ (80 mM) or caffeine (10 mM) was applied for 2 min at 20 min intervals in Krebs solution, so that reproducible responses could be obtained. When cilostazol (1–10 μM) was used, it was present for 10 min before and throughout the applications of histamine, high K^+ or caffeine.

To observe the effect of cilostazol on the histamine-induced release of Ca^{2+} from the storage sites, experiments were carried out in Ca^{2+}-free solution containing 2 mM ethyleneglycol-bis-(β-aminoethyl)-N,N,N′,N′-tetraacetic acid (EGTA)

with 5.9 mM K^+. After 2 min in Ca^{2+}-free solution, the strips were stimulated by 3 μM histamine for 2 min and then brought back to Ca^{2+}-containing Krebs solution (Ca^{2+} = 2.6 mM) for 20 min. When cilostazol (1–10 μM) was used, it was applied for 10 min in Krebs solution and was present in the Ca^{2+}-free solution and during the application of histamine.

In some experiments, Ca^{2+} storage sites were functionally removed by the application of ryanodine (Itoh *et al*., 1992a). In brief, ryanodine (50 μM) together with 10 mM caffeine was applied for 5 min in Krebs solution followed by a 10 min application of Krebs solution containing 10 μM ryanodine alone. Subsequently, 3 μM histamine was applied for 2 min at 30 min intervals in the presence of 10 μM ryanodine to obtain reproducible responses. When cilostazol (10 μM) was used, it was applied for 10 min before and was present during the application of histamine.

The effect of cilostazol on the $[Ca^{2+}]_i$-force relationship was studied in the presence and absence of 3 μM histamine in ryanodine-treated muscle strips. Ryanodine-treatment of the muscle strips was carried out as described above. Next, Ca^{2+}-free solution containing 2 mM EGTA with 5.9 mM K^+ was applied for 1 min, and then Ca^{2+}-free solution containing 100 mM K^+ with 2 mM EGTA was applied for 1 min in the presence or absence of 3 μM histamine. Starting from this point, various concentrations of Ca^{2+} (0.16–2.6 mM) without EGTA were cumulatively applied for 2 min in an ascending order together with 100 mM K^+ in the presence or absence of 3 μM histamine. Finally, Ca^{2+}-free solution containing 2 mM EGTA with 100 mM K^+ was applied for 1 min in the presence or absence of 3 μM histamine, followed by a 1 min application of Ca^{2+}-free solution containing 2 mM EGTA with 5.9 mM K^+ but with no histamine. The strip was then placed in Krebs solution for 20 min. These procedures were repeated in the presence of cilostazol (10 μM) which was applied for 10 min in Krebs solution and was then present during the application of the various concentrations of Ca^{2+}.

Calculation of Hill coefficient

The slope of the concentration-response relationship for the effect of $[Ca^{2+}]_i$ on force is shown by the Hill coefficient (n_H) and mid-point position ($pK = (-\log K)$, where K is the dissociation constant). These parameters were obtained by fitting the data points for each curve to equation (1) by a non-linear least-squares method.

$$F/F_o = (C/K)^{n_H}/[1 + (C/K)^{n_H}] \quad (1)$$

C represents the $[Ca^{2+}]_i$, F is the amplitude of contraction at any given $[Ca^{2+}]_i$ and F_o is the maximum response (expressed as a relative force of 1.0).

Solutions

The ionic composition of the Krebs solution was as follows (mM): Na^+ 137.4, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.6, HCO_3^- 15.5, $H_2PO_4^-$ 1.2, Cl^- 134 and glucose 11.5. The concentration of K^+ was modified by the isotonic replacement of NaCl with KCl. Ca^{2+}-free Krebs solution was made by substituting an equimolar concentration of $MgCl_2$ for $CaCl_2$ and adding 2 mM EGTA. All the solutions used in the present experiments contained guanethidine (5 μM, to prevent sympathetic nerve activity), indomethacin (10 μM, to prevent the production of cyclo-oxygenase products) and ranitidine (3 μM, to prevent H_2-receptor activation by histamine). The solutions were bubbled with 95% O_2 and 5% CO_2 and their pH was maintained at 7.3–7.4.

The calibration solution for Ca^{2+} measurement contained 11 mM EGTA, 110 mM KCl, 1 mM $MgCl_2$, 20 μM Fura 2 and 20 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES) (pH 7.1) with or without 11 mM $CaCl_2$.

Drugs

Drugs used were Fura 2, Fura 2-AM, EGTA and HEPES (Dojin, Kumamoto, Japan), caffeine (Wako Pure Chemical, Tokyo, Japan), adenosine-3′ : 5′-cyclic monophosphorothioate Rp-isomer (Rp-cAMPS) sodium salt (Biolog Life Sci. Inst., Bremen, F.R.G.), histamine dihydrochloride (Nacalai, Kyoto, Japan), indomethacin and ranitidine (Sigma, St. Louis, MO, U.S.A.), ryanodine (Agri-system, Wind Gap, PA, U.S.A.), guanethidine (Tokyo Kasei, Tokyo, Japan) and acetylcholine hydrochloride (Daiichi Pharmaceutical Co. Ltd, Tokyo, Japan). Cilostazol was kindly provided by Otsuka Pharmaceutical Co. Ltd. (Tokushima, Japan). The stock solution of cilostazol (0.1 M) was made with N′N-dimethylformide (DMF), and this stock was directly diluted in Krebs solution (the DMF concentration was 0.01% when 10 μM cilostazol was used). At this concentration, DMF itself modified neither the resting $[Ca^{2+}]_i$ nor the increase in $[Ca^{2+}]_i$ and force induced by histamine or high K^+.

Statistics

The values recorded are expressed as mean ± s.e. A one-way repeated-measures ANOVA followed by Scheffe's F test for *post hoc* analysis, as well as paired or unpaired t tests were used for the statistical analysis. Probabilities less than 5% ($P < 0.05$) were considered significant.

Results

Effect of cilostazol on the increases in $[Ca^{2+}]_i$ and force induced by high K^+ and histamine

Figure 1 shows the effect of cilostazol on the increases in $[Ca^{2+}]_i$ and force induced by 80 mM K^+. The resting $[Ca^{2+}]_i$ was 88 ± 7 nM with a resting force of 0.2 ± 0.2 μN ($n = 4$). Application of 80 mM K^+ produced a rapid increase in $[Ca^{2+}]_i$ to a peak reached within 4–5 s (phasic phase, to 594 ± 171 nM) followed by a decay to a steady level (tonic phase, to 299 ± 48 nM) ($n = 4$). Following the initial increase in $[Ca^{2+}]_i$, force developed with a phasic phase (to 119.6 ± 16.3 μN) and a subsequent tonic phase (to 86.4 ± 8.6 μN) (Figure 1). The tonic increases in $[Ca^{2+}]_i$ and force were measured 2 min after the start of the application of high K^+. Cilostazol (10 μM) did not modify either the resting $[Ca^{2+}]_i$ (94 ± 11 nM) or the phasic (to 513 ± 110 nM) and tonic (to 277 ± 34 nM) increases in $[Ca^{2+}]_i$ induced by 80 mM K^+, but did significantly reduce the tonic, but not the phasic, increase in force induced by high K^+. The levels reached during the high K^+-induced tonic increase in force were 86.4 ± 8.6 μN and 61.6 ± 6.9 μN in the absence and presence of 10 μM cilostazol, respectively; these values are significantly different from each other ($n = 4$, $P < 0.05$).

Nicardipine (0.3 μM), an L-type Ca^{2+} channel blocker, did not significantly change the resting $[Ca^{2+}]_i$ (from 85 ± 3 nM to 86 ± 2 nM, $P > 0.1$, $n = 4$), but it almost completely blocked the phasic and tonic increases in both $[Ca^{2+}]_i$ and force induced by 80 mM K^+ ($n = 4$, $P < 0.05$). In the absence of nicardipine, the levels reached during the phasic increases in $[Ca^{2+}]_i$ and force induced by high K^+ were 478 ± 25 nM and 119.3 ± 6.5 μN,

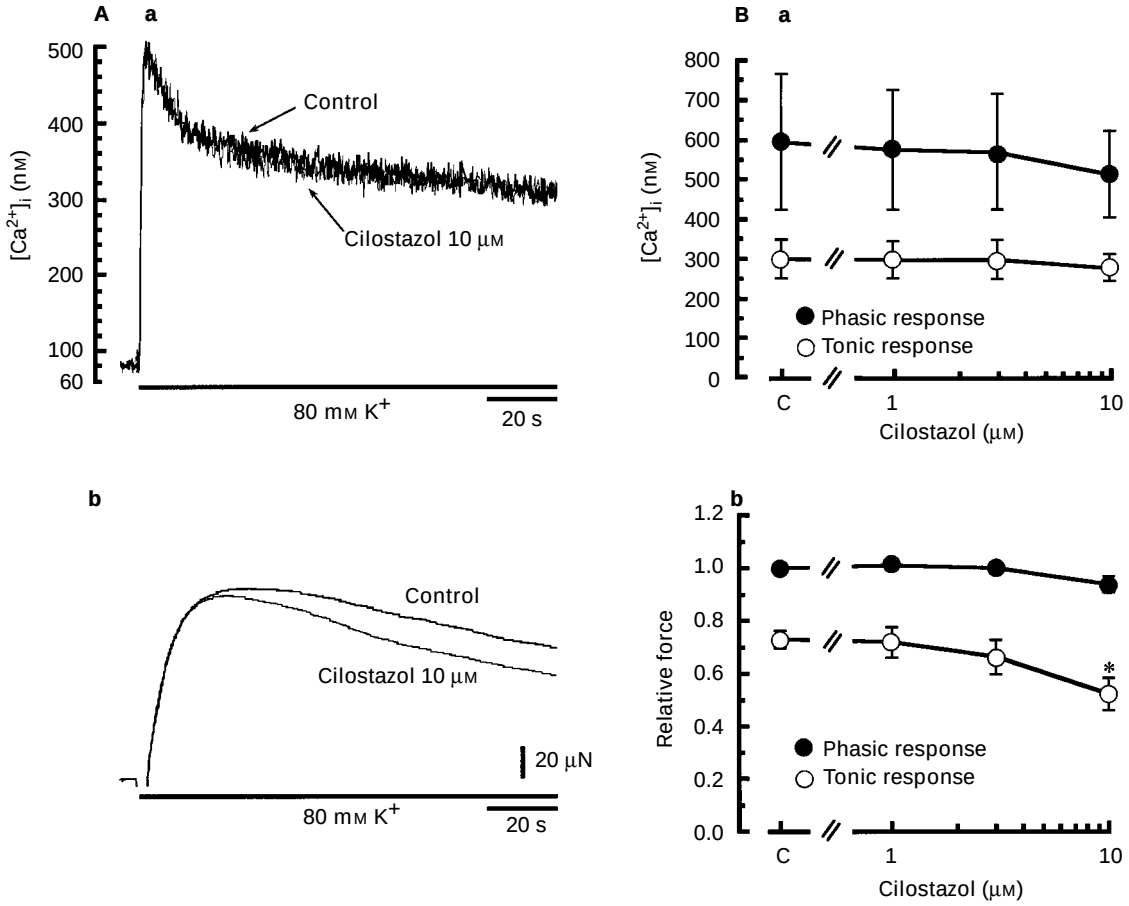

Figure 1 Effect of cilostazol on increases in $[Ca^{2+}]_i$ and force induced by 80 mM K^+ in smooth muscle strips from the middle cerebral artery of the rabbit. (A) Actual traces of simultaneous measurements of $[Ca^{2+}]_i$ (Aa) and force (Ab) obtained from a single smooth muscle strip. High K^+ (80 mM) was applied for 2 min (indicated by horizontal bars) at 20 min intervals in the presence or absence of cilostazol. Cilostazol was present for 10 min before and throughout the application of 80 mM K^+. (B) Concentration-dependence of effects of cilostazol on high K^+-induced increases in $[Ca^{2+}]_i$ (a) and force (b). The tonic responses were measured 2 min after the start of the application of high K^+. The maximum amplitude of contraction induced by 80 mM K^+ in the absence of cilostazol was normalized as a relative force of 1.0 for each strip. Mean of data from 4 strips, with s.e. shown by vertical lines. *Indicates values that are significantly different from control ($P<0.05$, one-way repeated-measures ANOVA and Scheffé's F test).

respectively, and those reached during the tonic increases (measured 2 min after the application of high K^+) were 272 ± 11 nM and 85.5 ± 7.3 μN, respectively. In the presence of nicardipine, the corresponding values were 96 ± 6 nM and 2.1 ± 0.8 μN (phasic increases) and 93 ± 5 nM and 1.5 ± 1.0 μN (tonic increases).

Histamine (3 μM) produced a phasic, followed by a tonic increase in both $[Ca^{2+}]_i$ and force (Figure 2). The levels reached during the phasic increases in $[Ca^{2+}]_i$ and force were 312 ± 21 nM and 99.7 ± 13.1 μN, respectively, and those reached during the tonic increases (measured 2 min after the application of histamine) were 189 ± 10 nM and 111.1 ± 19.5 μN, respectively ($n=6$). When cilostazol (3 and 10 μM) was present for 10 min before and throughout the application of 3 μM histamine, this agent significantly reduced the phasic and tonic increases in both $[Ca^{2+}]_i$ and force, in a concentration-dependent manner. For example, in the presence of 10 μM cilostazol, the levels reached during the histamine-induced phasic increases in $[Ca^{2+}]_i$ and force were 172 ± 13 nM and 56.0 ± 12.5 μN, respectively, and during the tonic increases in $[Ca^{2+}]_i$ and force were 134 ± 13 nM and 45.7 ± 13.4 μN, respectively ($n=6$). These values were significantly different from their respective controls ($P<0.05$).

Nicardipine (0.3 μM) did not modify the phasic increase in either $[Ca^{2+}]_i$ or force ($P>0.1$, $n=4$), but it did significantly reduce the tonic responses induced by 3 μM histamine ($P<0.05$, $n=4$). In control, the levels reached during the phasic increases in $[Ca^{2+}]_i$ and force induced by histamine were 327 ± 21 nM and 87.3 ± 8.5 μN, respectively, and those reached during the tonic increases were 195 ± 16 nM and 95.0 ± 2.8 μN, respectively ($n=4$). In the presence of nicardipine, the corresponding values were 301 ± 21 nM and 89.5 ± 8.9 μN (phasic increases) ($P>0.1$, $n=4$), and 139 ± 10 nM and 62.1 ± 1.6 μN (tonic increases).

Effect of Rp-cAMPS on the increases in $[Ca^{2+}]_i$ and force induced by histamine in the presence and absence of cilostazol

Before the application of Rp-cAMPS, the levels reached during the histamine-induced phasic and tonic increases in $[Ca^{2+}]_i$ were 343 ± 14 nM and 212 ± 9 nM, respectively, and the values reached for relative force were 0.866 ± 0.045 and 0.908 ± 0.095, respectively, when the maximum amplitude of contraction induced by 80 mM K^+ (control responses) was normalized as a relative force of 1.0 for each strip (Figure 3). Following the application of 0.1 mM Rp-cAMPS for 60 min,

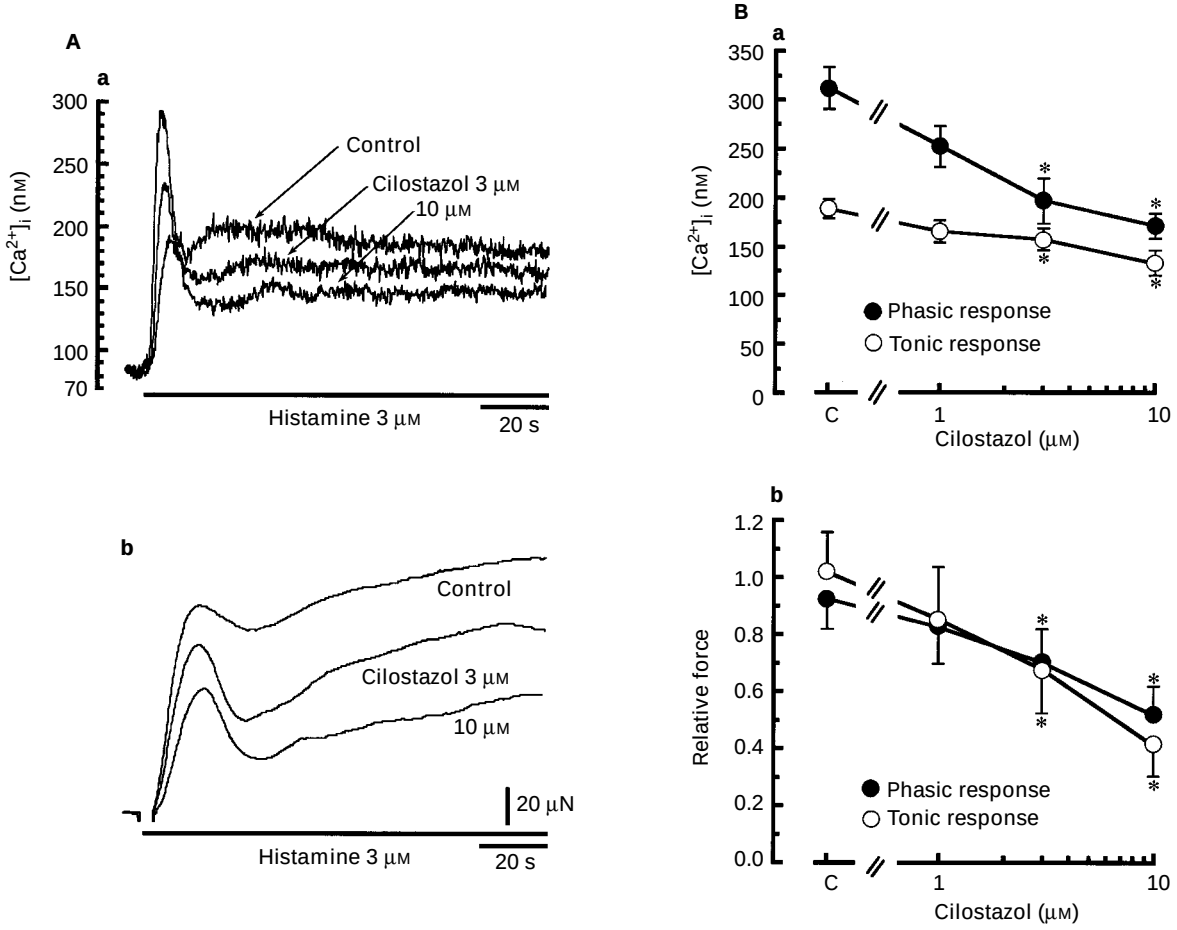

Figure 2 Effect of cilostazol on increases in $[Ca^{2+}]_i$ and force induced by 3 μM histamine. (A) Actual traces of simultaneous measurements of $[Ca^{2+}]_i$ (Aa) and force (Ab) obtained from a single smooth muscle strip (the same strip as that used for Figure 1A). Histamine (3 μM) was applied for 2 min, as indicated by the horizontal bars. The experimental protocol was similar to that described in Figure 1. (B) Concentration-dependence of effects of cilostazol on histamine-induced increases in $[Ca^{2+}]_i$ (a) and force (b). The tonic responses were measured 2 min after the start of the application of histamine. The maximum amplitude of contraction induced by 80 mM K^+ in the absence of cilostazol was normalized as a relative force of 1.0 for each strip. Mean of data from 4 strips, with s.e. shown by vertical lines. *Indicates values that are significantly different from control ($P<0.05$, one-way repeated-measures ANOVA and Scheffé's F test).

the resting $[Ca^{2+}]_i$ was not significantly changed (from 95 ± 8 nM in control to 106 ± 10 nM after the application, $n=4$, $P>0.05$). In the presence of Rp-cAMPS (0.1 mM), the levels reached during the histamine-induced phasic and tonic increases in $[Ca^{2+}]_i$ were 323 ± 37 nM and 222 ± 26 nM, respectively, and the values reached for phasic and tonic relative force (normalized with respect to the maximum contraction induced by 80 mM K^+ under control conditions for each strip) were 0.884 ± 0.058 and 0.950 ± 0.027, respectively. These values were not significantly different from the corresponding values in the absence of Rp-cAMPS ($n=4$, $P>0.05$). However, 0.1 mM Rp-cAMPS did significantly decrease the inhibitory action of 10 μM cilostazol on the phasic and tonic increases in $[Ca^{2+}]_i$ and force induced by 3 μM histamine. Before the application of Rp-cAMPS, in the presence of 10 μM cilostazol the levels reached during the histamine-induced phasic and tonic increases in $[Ca^{2+}]_i$ were 192 ± 15 nM and 157 ± 5 nM, respectively, and the values reached for relative force were 0.441 ± 0.041 and 0.380 ± 0.130, respectively. In the presence of 0.1 mM Rp-cAMPS with 10 μM cilostazol, the corresponding values were 275 ± 16 nM and 189 ± 7 nM, respectively, and 0.776 ± 0.068 and 0.670 ± 0.138, respectively. These values (with and without Rp-cAMPS) were significantly different from each other ($n=4$, $P>0.05$) (Figure 3).

Effect of cilostazol on the increases in $[Ca^{2+}]_i$ and force induced by histamine and caffeine in Ca^{2+}-free solution

The effect of cilostazol on histamine-induced Ca^{2+} release was examined by observing the increase in $[Ca^{2+}]_i$ induced by 3 μM histamine in Ca^{2+}-free solution containing 2 mM EGTA (Figure 4). The changeover from normal Krebs solution to the Ca^{2+}-free solution was rapid with no increase in $[Ca^{2+}]_i$ induced by 80 mM K^+ after a 15 s application. Following the application of Ca^{2+}-free solution, the resting $[Ca^{2+}]_i$ decreased rapidly from 81 ± 2 nM to 58 ± 6 nM within 1 min; it then remained at this new steady level ($n=4$). Under these conditions, 3 μM histamine transiently increased $[Ca^{2+}]_i$ (to 251 ± 29 nM) and force (to 83.5 ± 30.3 μN). In Ca^{2+}-free solution, cilostazol ($1-10$ μM) significantly reduced these histamine-evoked increases in $[Ca^{2+}]_i$ and force. For example, in the presence of 10 μM cilostazol, the levels reached during the histamine-induced increases in $[Ca^{2+}]_i$ and force were 134 ± 7 nM and 47.2 ± 19.7 μN, respectively. These values were significantly different from their corresponding controls ($P<0.05$).

Caffeine (10 mM) produced a transient increase in $[Ca^{2+}]_i$ (to 607 ± 157 nM) and force (to 87.8 ± 44.7 μN) in Ca^{2+}-free solution containing 2 mM EGTA. Cilostazol (10 μM) significantly reduced the maximum caffeine-induced increase in

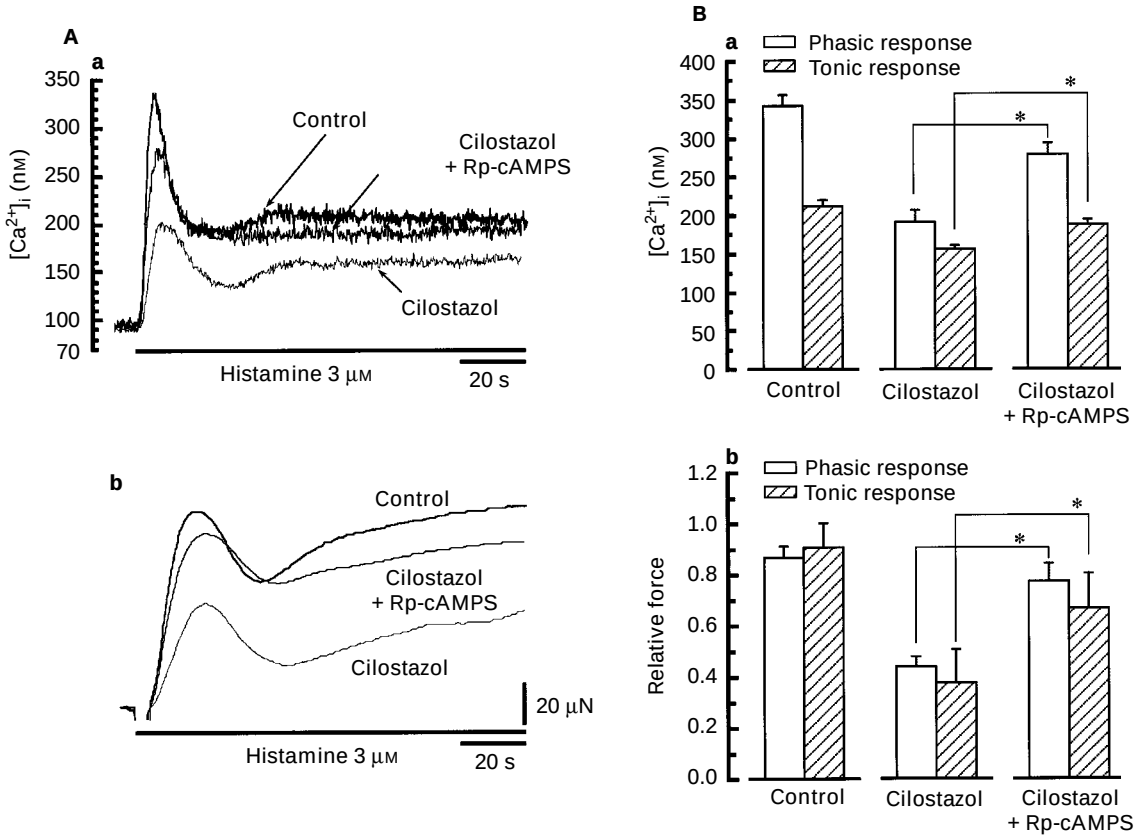

Figure 3 Effect of Rp-cAMPs (0.1 mM) on increases in $[Ca^{2+}]_i$ and force induced by 3 μM histamine in the presence or absence of cilostazol (10 μM). (A) Actual traces of simultaneous measurements of $[Ca^{2+}]_i$ (Aa) and force (Ab) obtained from a single smooth muscle strip. Histamine (3 μM) was applied for 2 min, as indicated by the horizontal bars. (B) Effect of Rp-cAMPS on inhibition by cilostazol of increases in $[Ca^{2+}]_i$ (a) and force (b) induced by histamine. The tonic responses were measured 2 min after the start of the application of histamine. The maximum amplitude of contraction induced by 80 mM K^+ in the absence of cilostazol was normalized as a relative force of 1.0 for each strip. Mean of data from 4 strips, with s.e. shown by vertical lines. *Indicates values that are significantly different from control ($P<0.05$, one-way repeated-measures ANOVA and Scheffé's F test).

force (0.880 ± 0.018 times control, $P<0.05$) without a corresponding change in the $[Ca^{2+}]_i$ response (to 579 ± 129 nM, $P>0.1$) ($n=4$).

Effect of cilostazol on the histamine-induced increases in $[Ca^{2+}]_i$ and force in ryanodine-treated smooth muscle strips

Following the application of ryanodine (see Methods for the protocol), the resting $[Ca^{2+}]_i$ was significantly increased (from 101 ± 13 nM to 146 ± 18 nM, $n=4$) and subsequently applied 3 μM histamine failed to induce a phasic increase in $[Ca^{2+}]_i$ and force. In ryanodine-treated strips, the increases in $[Ca^{2+}]_i$ (to 191 ± 17 nM) and force (to 106.0 ± 28.8 μN) that were induced by 3 μM histamine occurred slowly and the time to peak was thus greatly increased ($n=4$) (Figure 5). Cilostazol (3 and 10 μM) slightly lowered the resting $[Ca^{2+}]_i$ (to 127 ± 18 with 3 μM cilostazol and to 117 ± 16 nM with 10 μM, $n=4$, $P<0.05$). At 3 μM, cilostazol significantly decreased the maximum level reached during the histamine-induced increase in force (to 85.4 ± 28.4 μN, $n=4$, $P<0.05$) without inducing a significant change in the maximum $[Ca^{2+}]_i$ level reached (to 180 ± 21 nM, $P>0.05$). At 10 μM, this agent significantly decreased the maximum level reached during the histamine-induced increases in both $[Ca^{2+}]_i$ (to 164 ± 18 nM, $n=4$, $P<0.05$) and force (64.7 ± 28.8 μN, $P<0.05$) (Figure 5). However, the 'delta $[Ca^{2+}]_i$ increase' induced by 3 μM histamine (delta $[Ca^{2+}]_i$ increase = the maximum $[Ca^{2+}]_i$ − resting $[Ca^{2+}]_i$) was not significantly modified by either 3 or 10 μM cilostazol ($P>0.05$). These values were 45 ± 8 nM in control, 53 ± 5 nM in the presence of 3 μM cilostazol and 47 ± 7 nM with 10 μM cilostazol ($n=4$).

Nicardipine (0.3 μM) significantly lowered the resting $[Ca^{2+}]_i$ (from 141 ± 11 nM to 116 ± 10 nM, $n=4$, $P<0.05$) and greatly diminished the maximum increases in both $[Ca^{2+}]_i$ and force induced by 3 μM histamine in ryanodine-treated strips ($n=4$, $P<0.05$). In control, the maximum levels reached during the histamine-induced increases in $[Ca^{2+}]_i$ and force were 179 ± 14 nM and 111.8 ± 9.5 μN, respectively ($n=4$), and in the presence of nicardipine, the coresponding values were 124 ± 8 nM and 6.2 ± 5.1 μN, respectively.

Effect of cilostazol on the $[Ca^{2+}]_i$-force relationship in the presence and absence of histamine in ryanodine-treated strips

The $[Ca^{2+}]_i$-force relationship was obtained by applying various concentrations of Ca^{2+} ($[Ca^{2+}]_o$) in Ca^{2+}-free solution containing 100 mM K^+ with or without 3 μM histamine in ryanodine-treated strips (see Methods for the protocol). After the application of Ca^{2+}-free solution with 5.9 mM K^+ for 1 min, the resting $[Ca^{2+}]_i$ decreased to 74 ± 6 nM ($n=6$). Subsequent application of Ca^{2+}-free solution with 100 mM K^+ increased neither $[Ca^{2+}]_i$ nor force. Cumulative application

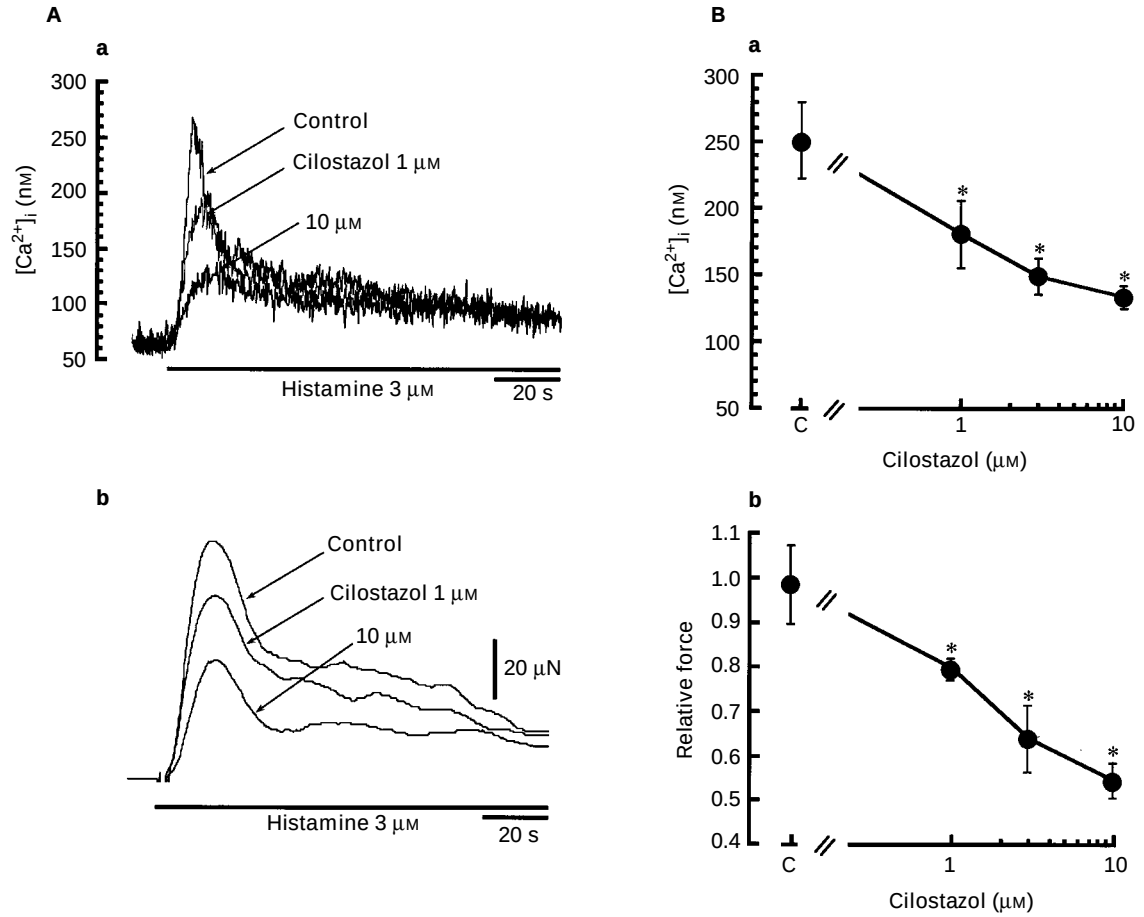

Figure 4 Effect of cilostazol on increases in $[Ca^{2+}]_i$ and force induced by 3 μM histamine in Ca^{2+}-free solution containing 2 mM EGTA with 5.9 mM K^+. (A) Actual traces obtained from a single smooth muscle strip; $[Ca^{2+}]_i$ (Aa) and force (Ab). Histamine (3 μM) was applied for 2 min, as indicated by the horizontal bars. Cilostazol was present for 10 min before and throughout the application of histamine. (B) Concentration-dependence of effect of cilostazol on the histamine-induced maximum increases in $[Ca^{2+}]_i$ (a) and force (b). The maximum amplitude of each force response induced by histamine in the absence of cilostazol was normalized as a relative value of 1.0 for each strip. Mean of data from 4 strips, with s.e. shown by vertical lines. *Indicates values that are significantly different from control ($P<0.05$, one-way repeated-measures ANOVA and Scheffé's F test).

of various concentrations of $[Ca^{2+}]_o$ progressively increased the level of $[Ca^{2+}]_i$ and force. The total extracellular Ca^{2+} ($[Ca^{2+}]_o$) required to obtain the half-maximum increase in $[Ca^{2+}]_i$ (ED_{50}) was 422 ± 38 μM ($n=6$) in the absence, and 509 ± 61 μM ($n=6$) in the presence of 3 μM histamine; these values are not significantly different from each other (unpaired t test, $P>0.05$). The corresponding values of $[Ca^{2+}]_o$ for the half-maximum increase in force (ED_{50}) were 378 ± 12 μM and 294 ± 21 μM, respectively; these values are significantly different from each other (unpaired t test, $P<0.05$). The maximum values for $[Ca^{2+}]_i$ obtained on application of 2.6 mM Ca^{2+} were 273 ± 16 nM and 252 ± 13 nM in the absence and presence of 3 μM histamine, respectively (unpaired t test, $P>0.05$), and the corresponding values for maximum force were 179.0 ± 20.2 μN and 226.9 ± 20.7 μN, respectively (unpaired t test, $P<0.05$). Under these conditions, cilostazol (10 μM) significantly reduced the increases in force, but not the increases in $[Ca^{2+}]_i$ induced by any given $[Ca^{2+}]_o$ (0.16–2.6 mM) whether the experiment was conducted in the presence or in the absence of histamine. In some additional experiments, we confirmed that the control $[Ca^{2+}]_i$- force relationship was essentially the same some 50 min after washout of cilostazol (whether or not histamine had been used). Thus, over the time course of the above experiments there was no detectable instability in the response of the tissue to $[Ca^{2+}]_i$.

Figure 6 summarizes the effect of cilostazol on the $[Ca^{2+}]_i$-force relationship in the presence and absence of 3 μM histamine. Histamine shifted the $[Ca^{2+}]_i$-force relationship to the left and increased the maximum amplitude of contraction induced by 2.6 mM Ca^{2+}. The $[Ca^{2+}]_i$ required for half-maximum force was 175 ± 3 nM in the absence of 3 μM histamine and 148 ± 9 nM in its presence ($P<0.05$). Whether applied in the presence or absence of histamine, cilostazol (10 μM) significantly shifted the $[Ca^{2+}]_i$-force relationship to the right without any change in the maximum increase in force. In the presence of 10 μM cilostazol, the values of $[Ca^{2+}]_i$ required for half-maximum force were 199 ± 4 nM and 170 ± 5 nM in the absence and presence of histamine, respectively; these values were significantly different ($P<0.05$) from the corresponding controls (i.e. in the absence of 10 μM cilostazol).

Discussion

Effects of cilostazol on histamine-induced Ca^{2+} mobilization

In the present experiments, in endothelium-denuded muscle strips taken from the peripheral part of the middle cerebral

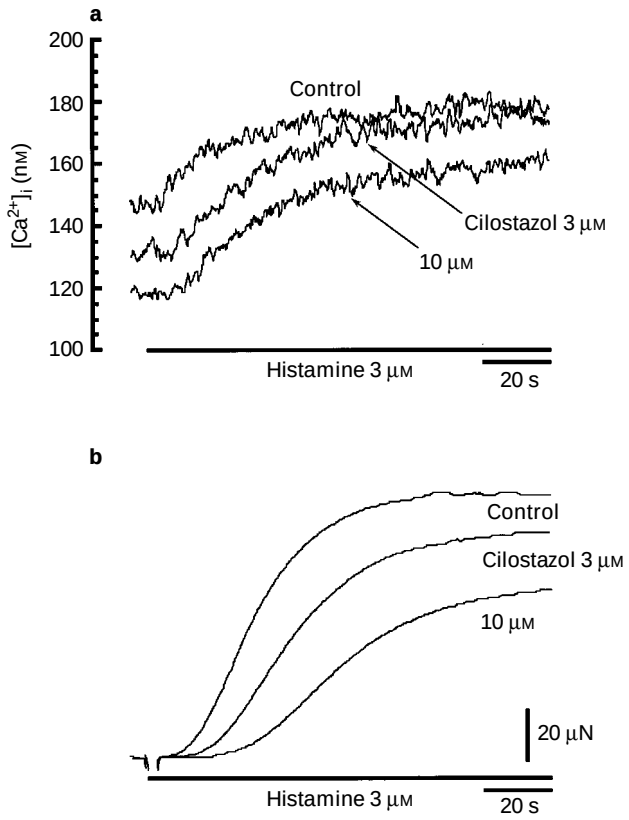

Figure 5 Effect of cilostazol on increases in $[Ca^{2+}]_i$ (a) and force (b) induced by 3 μM histamine in ryanodine-treated strips. Histamine was applied for 2 min at 30 min intervals in Krebs solution. Ryanodine (50 μM) plus 10 mM caffeine was applied for 5 min in Krebs solution followed by a 10 min application of Krebs solution containing 10 μM ryanodine alone. Histamine was then applied with ryanodine still present and in the presence or absence of cilostazol. The results illustrated were obtained from a single smooth muscle strip and were reproducible in another 3 strips.

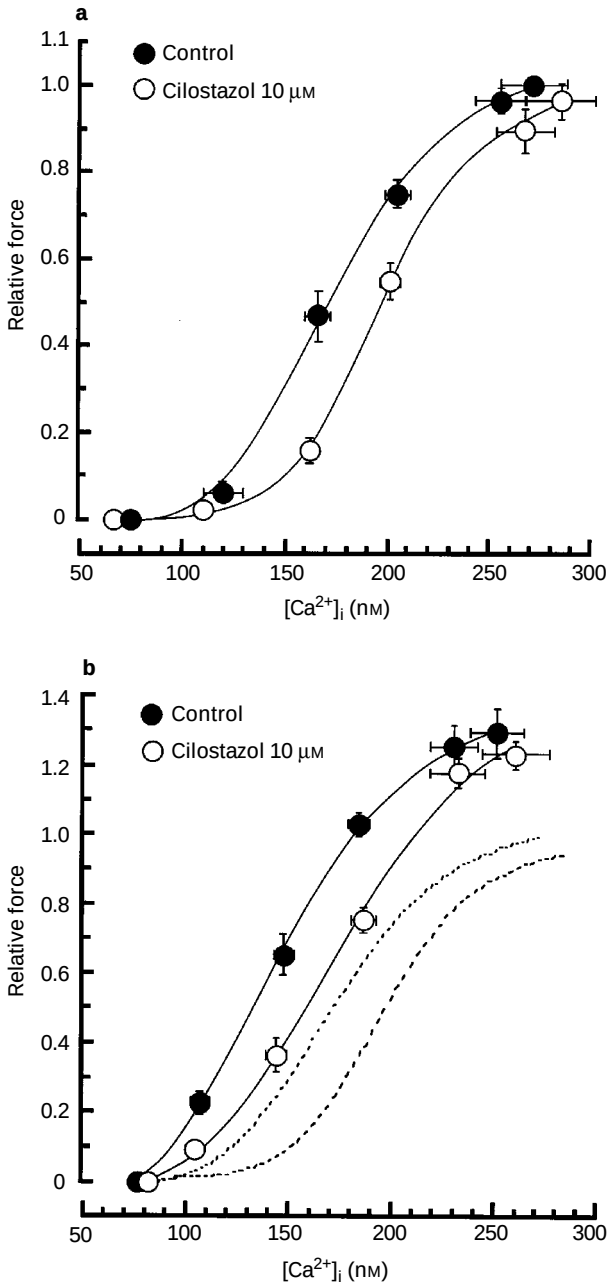

Figure 6 Effect of cilostazol on $[Ca^{2+}]_i$-force relationship in the absence (a) and presence (b) of 3 μM histamine in high K^+, ryanodine-treated smooth muscle strips. The $[Ca^{2+}]_i$-force relationships in the presence or absence of histamine were obtained as described in Methods. The curves were obtained by fitting the data points to equation (1) by a non-linear least-squares method (see Methods). The curves shown in part (a) are repeated (and rescaled) in (b) as a dotted line (control) and a broken line (cilostazol) for comparison purposes. Note the different scales in the two parts of the figure. Each symbol represents the mean of data from 6 strips, with s.e. shown by vertical and horizontal bars.

artery (MCA) of the rabbit, cilostazol (1 – 10 μM) had no effect on the resting $[Ca^{2+}]_i$ but it did significantly reduce the increase in $[Ca^{2+}]_i$ induced by histamine, but not that induced by high K^+. In contrast, nicardipine (0.3 μM), an L-type Ca^{2+} channel blocker, completely blocked both the phasic and tonic increases in $[Ca^{2+}]_i$ induced by high K^+, but only partly inhibited the increases in $[Ca^{2+}]_i$ induced by histamine, as shown previously in the smooth muscle of the rabbit mesenteric artery (Watanabe *et al*., 1996). Cilostazol selectively inhibits PDE III and increases the cellular concentration of cyclic AMP, but not that of cyclic GMP, in rabbit arterial tissues (Umekawa *et al*., 1984; Tanaka *et al*., 1988). In the present experiments, Rp-cAMPS, a specific inhibitor of cyclic AMP (Dostmann, 1995) by virtue of its competitive inhibition of protein kinase A, did not modify the increase in $[Ca^{2+}]_i$ induced by histamine alone, but it did significantly decrease the cilostazol-induced inhibition of the histamine-induced $[Ca^{2+}]_i$ response. This confirms the role played by an increase in cyclic AMP level, and the subsequent activation of protein kinase A and hence protein phosphorylation, in the cilostazol-induced inhibition of the histamine response. These results also suggest that the concentration of cyclic AMP in the presence of cilostazol (1 – 10 μM) may not be high enough to inhibit the high K^+-activated Ca^{2+} influx through L-type Ca^{2+} channels in the smooth muscle of the rabbit MCA. An alternative explanation is that, in this tissue, cyclic AMP may not inhibit the activity of the L-type Ca^{2+} channel, as found in a patch-clamp study of intestinal smooth muscle cells (Ohya *et al*., 1987). This hypothesis is also supported by the previous finding that in smooth muscle of the porcine coronary artery, NKH 477, a water-soluble forskolin derivative, greatly enhanced the cellular concentration of cyclic AMP, but did not modify either the phasic or tonic increases in $[Ca^{2+}]_i$ induced by high K^+, although it did significantly reduce both the phasic and tonic increases in $[Ca^{2+}]_i$ induced by acetylcholine (Shafiq *et al*., 1992).

In ryanodine-treated strips from the rabbit MCA, histamine failed to produce a phasic increase in $[Ca^{2+}]_i$. In fact, it produced only a mono-tonic response which was abolished in Ca^{2+}-free solution, suggesting that in such strips histamine increases $[Ca^{2+}]_i$ only through activation of Ca^{2+} influx. Under these conditions, cilostazol did not modify the histamine-induced increase in $[Ca^{2+}]_i$. These results indicate that the inhibitory effect exerted by cilostazol on the histamine-induced increase in $[Ca^{2+}]_i$ requires functional Ca^{2+} storage sites. Using a water-soluble forskolin derivative and dibutyryl cyclic AMP, we previously found that agents that increase cyclic AMP inhibit agonist-induced increases in $[Ca^{2+}]_i$ through activation of Ca^{2+} uptake into the storage sites in smooth muscle cells, both in the rabbit mesenteric and porcine coronary arteries (Shafiq *et al*., 1992; Ito *et al*., 1993). These results suggest that cilostazol may not inhibit histamine-activated Ca^{2+} influx but instead enhance Ca^{2+} uptake into the storage sites, thus attenuating the histamine-induced increase in $[Ca^{2+}]_i$ seen here in smooth muscle of the rabbit MCA.

In ryanodine-treated muscle strips, the resting $[Ca^{2+}]_i$ was increased, and cilostazol (3 and 10 μM) slightly lowered it. Nicardipine (0.3 μM) also lowered the resting $[Ca^{2+}]_i$ and its action was more potent than that of cilostazol. This effect of nicardipine is in contrast with our previous finding in the rabbit mesenteric artery, in which the ryanodine-enhancement of the resting $[Ca^{2+}]_i$ was insensitive to nicardipine (Itoh *et al*., 1992a; 1994). These results suggest that under conditions in which the function of Ca^{2+}-storage sites is lost, the contribution made by L-type Ca^{2+} channels to the regulation of resting $[Ca^{2+}]_i$ in smooth muscle cells may differ between MCA and mesenteric artery. In ryanodine-treated smooth muscle cells, capacitative Ca^{2+} influx (Putney, 1990) may be activated. In some additional experiments, we examined the effect of cilostazol on capacitative Ca^{2+} entry in smooth muscle of the rabbit MCA using cyclopiazonic acid (CPA), an inhibitor of the Ca^{2+}-ATPase in the Ca^{2+}-storage sites. CPA (10 μM) slowly increased $[Ca^{2+}]_i$ in Krebs solution (from 84 ± 3 nM to 125 ± 9 nM), and cilostazol (10 μM) did not modify this CPA-induced $[Ca^{2+}]_i$ increase (from 85 ± 9 nM to 130 ± 7 nM, $n = 3$, $P > 0.1$). These results suggest that cilostazol does not affect capacitative Ca^{2+} influx in the smooth muscle cells of the rabbit MCA.

Histamine produced phasic increases in $[Ca^{2+}]_i$ and force that were transient and large whether it was applied in the presence or absence of extracellular Ca^{2+} in smooth muscle of the rabbit MCA. Caffeine also transiently increased $[Ca^{2+}]_i$ and force whether in the presence or absence of extracellular Ca^{2+}, suggesting that both caffeine and histamine release Ca^{2+} from the intracellular storage sites. In fact, it has been found that in various types of vascular smooth muscle, caffeine releases Ca^{2+} from storage sites that are closely related to the histamine-sensitive ones, but that the effects are exerted via different mechanisms (Itoh *et al*., 1992a; Watanabe *et al*., 1996). In Ca^{2+}-free solution, cilostazol (3 and 10 μM) significantly reduced the increase in $[Ca^{2+}]_i$ induced by histamine, but not that induced by caffeine. This result is compatible with that of our previous study, in which both NKH 477 (a forskolin derivative) and dibutyryl-cyclic AMP inhibited the Ca^{2+} release induced by noradrenaline (NA), but not that induced by caffeine, in the smooth muscle of the rabbit mesenteric artery (Ito *et al*., 1993). Under these conditions, the maximum value for the rate of rise of $[Ca^{2+}]_i$ was almost 2 times larger for the response induced by caffeine than for that induced by NA (Itoh *et al*., 1992a; Itoh *et al*., 1993). In these earlier experiments, we also found the following: (1) These two agents, both of which increase cyclic AMP, did not change either the NA-induced production of inositol 1,4,5-trisphosphate (IP_3) in intact muscle strips or the IP_3-induced Ca^{2+} release in skinned smooth muscle. (2) The extent of the inhibition induced by these cyclic AMP-increasing agents on the NA-induced increase in $[Ca^{2+}]_i$ in Ca^{2+}-free solution containing various concentrations of K^+ was inversely related to the maximum rate of rise of $[Ca^{2+}]_i$ induced by NA (Ito *et al*., 1993). This suggested that a sufficiently large increase in the rate of NA-induced Ca^{2+} release could conceal the inhibitory action on NA-induced Ca^{2+} mobilization of agents that increase cyclic AMP. (3) CPA significantly decreased the inhibitory action of dibutyryl-cyclic AMP on the NA-induced Ca^{2+} release (Ito *et al*., 1993). In some additional experiments using smooth muscle of the rabbit MCA, we observed the effect of 10 μM cilostazol on the maximum increase in $[Ca^{2+}]_i$ induced by 3 μM histamine in the presence of 10 μM CPA in Krebs solution. In the presence of 10 μM CPA, histamine increased $[Ca^{2+}]_i$ by 231 ± 47 nM or 220 ± 48 nM in the absence or presence of 10 μM cilostazol, respectively, these values were not significantly different ($n = 3$). In the light of these findings, we speculate that cilostazol attenuates histamine-induced Ca^{2+} release through activation of Ca^{2+} uptake into the storage sites.

Effect of cilostazol on myofilament Ca^{2+} sensitivity

Cilostazol significantly reduced the high K^+-induced tonic increase in force without a corresponding change in the induced increase in $[Ca^{2+}]_i$. Similarly, this agent significantly decreased the caffeine-induced contraction without a corresponding change in the induced $[Ca^{2+}]_i$ increase. Under conditions in which the membrane was depolarized (following the application of 100 mM K^+) and the function of Ca^{2+} storage sites was lost (following the application of ryanodine), cilostazol shifted the $[Ca^{2+}]_i$-force relationship to the right without inducing a change in the maximum force evoked by 2.6 mM $[Ca^{2+}]_o$. These results indicate that in the smooth muscle of the rabbit MCA, cilostazol reduces the sensitivity of the contractile proteins to Ca^{2+} and, by this means, inhibits the contraction induced by high K^+ or caffeine.

Histamine shifted the $[Ca^{2+}]_i$-force relationship to the left and increased the maximum force without inducing a change in the maximum increase in $[Ca^{2+}]_i$ induced by 2.6 mM $[Ca^{2+}]_o$, suggesting that histamine enhances the myofilament Ca^{2+}-sensitivity in the smooth muscle of the rabbit MCA. In the presence of histamine, cilostazol again shifted the $[Ca^{2+}]_i$-force relationship to the right without inducing a change in the maximum force evoked by 2.6 mM $[Ca^{2+}]_o$, indicating that cilostazol reduces the myofilament Ca^{2+}-sensitivity whether it is applied in the presence or absence of histamine.

It is known that platelet aggregation is closely related to the development of both cerebral thrombosis and transient ischaemia attack (TIA) (Uchiyama *et al*., 1983). In long-term use, aspirin has been demonstrated to be useful since it is an inhibitor of platelet aggregation that reduces the recurrence of TIA and of reversible ischaemia-induced neurological deficiency (Fields *et al*., 1977). The therapeutic value of cerebral vasodilating agents in the treatment of acute cerebral infarction is controversial because of the presence of the luxury perfusion and intracerebral steal phenomena (Capon *et al*., 1977). However, it has been suggested that the miserly perfusion in lacunar infarction may play a role in determining the extent of the infarction (Baron *et al*., 1983). This reduced perfusion could be improved by cerebral vasodilating agents. A prophylactic strategy against recurrent ischaemic attacks is

thought to be important in cases of lacunar infarction, and when the cause of lacunar infarction is a thrombosis in the perforating artery, rather than an embolism from an extracranial arterial lesion. Cilostazol is known to be a strong anti-platelet agent and, in the present experiments, it strongly inhibited the agonist-induced contraction in peripheral parts of the middle cerebral artery. These results suggest that cilostazol could be useful when given prophylactically for the reduction or prevention of recurrent attacks of cerebral thrombosis, especially in cases of lacunar infarction.

It is concluded that, in a cerebral resistance artery, cilostazol attenuates the histamine-induced contraction through both inhibition of Ca^{2+} mobilization and a lowering of the myofilament Ca^{2+} sensitivity. An increase in cyclic AMP (produced via the inhibition by cilostazol of the cyclic GMP-inhibited PDE) may play a role in this response.

We thank Dr R.J. Timms for critical reading of the manuscript. This work was partly supported by a Grant-In-Aid from the Ministry of Education of Japan. Cilostazol was a gift from Otsuka Pharmaceutical Co. Ltd. (Tokushima, Japan).

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(Received October 1, 1997
Revised November 13, 1997
Accepted November 24, 1997)