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Effect of membrane hyperpolarization induced by a K^+ channel opener on histamine-induced Ca^{2+} mobilization in rabbit arterial smooth muscle

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1 The role of membrane hyperpolarization on agonist-induced contraction was investigated in intact and α-toxin-skinned smooth muscles of rabbit mesenteric artery by use of the ATP-sensitive K^+ channel opener, (-)-(3S,4R)-4-(N-acetyl-*N*-hydroxyamino)-6-cyano-3,4-dihydro-2,2-dimethyl-2H-1-benzopyran-3-ol (Y-26763), and either histamine (Hist) or noradrenaline (NA).

2 Hist (3 μM) and NA (10 μM) both produced a phasic, followed by a tonic increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and force. Y-26763 (10 μM) potently inhibited the NA-induced phasic and tonic increase in $[Ca^{2+}]_i$ and force. In contrast, Y-26763 attenuated the Hist-induced phasic increase in $[Ca^{2+}]_i$ and force but had almost no effect on the tonic response. However, ryanodine-treatment of muscles in order to inhibit the function of intracellular Ca^{2+} storage sites altered the action of Y-26763 which now attenuated the Hist-induced tonic increase in $[Ca^{2+}]_i$ and force in a concentration-dependent manner (at concentrations > 1 μM). Glibenclamide (10 μM) attenuated the inhibitory action of Y-26763.

3 Hist (3 μM) depolarized the smooth muscle cells to the same extent as NA (10 μM). In the absence of either agonist, Y-26763 (over 30 nM) hyperpolarized the membrane and glibenclamide inhibited this hyperpolarization. Y-26763 (10 μM) almost abolished the NA-induced membrane depolarization, but only slightly attenuated the Hist-induced membrane depolarization in which the delta (Δ) value (the difference before and after application of Hist) was not modified by any concentration of Y-26763. In ryanodine-treated smooth muscle cells, Y-26763 hyperpolarized the membrane and potently inhibited the membrane depolarization induced by Hist.

4 In ryanodine-treated muscle, Y-26763 had no measurable effect on the Hist-induced $[Ca^{2+}]_i$-force relationship. Y-26763 also had no apparent effect on the myofilament Ca^{2+}-sensitivity in the presence of Hist in α-toxin-skinned smooth muscles.

5 It is concluded that the membrane hyperpolarization induced by Y-26763 may not be enough to inhibit the Hist-activated Ca^{2+} influx. It is also suggested that Hist prevents the membrane hyperpolarization induced by Y-26763, activating an unknown mechanism which is thought to depend on the function of intracellular Ca^{2+} storage sites.

Keywords: ATP-sensitive K^+ channel; K^+ channel opener; Y-26763; agonist-induced Ca^{2+} mobilization; vascular smooth muscle physiology; histamine; noradrenaline

Introduction

K^+ channel openers (KCOs), a variety of compounds which have different chemical structures, hyperpolarize the membrane of vascular smooth muscle cells through an opening of glibenclamide-sensitive K^+ channels (see for a review, Quast, 1993). The membrane hyperpolarization induced by KCOs is thought to relax smooth muscle by inhibiting voltage-dependent Ca^{2+} channels. It is also known that KCOs, such as pinacidil or levcromakalim, attenuate the noradrenaline (NA)-induced synthesis of inositol 1,4,5,-trisphosphate ($InsP_3$) associated with their membrane hyperpolarizing action and thus inhibit NA-induced Ca^{2+} release from intracellular storage sites in smooth muscle of the rabbit mesenteric artery (Ito *et al.*, 1991; 1992b).

In smooth muscle of the rabbit mesenteric artery, NA produces a phasic, followed by a tonic increase in $[Ca^{2+}]_i$ and force (Itoh *et al.*, 1992a). In general, the agonist-induced phasic responses are thought to be due to the agonist-induced Ca^{2+} release whereas the tonic responses are due to an interplay between the agonist-activated Ca^{2+} influx through the plasmalemma and Ca^{2+} uptake into, or Ca^{2+} release from the storage sites (Itoh *et al.*, 1983). Y-26763, one of the KCOs which has the same basic chemical structure as levcromakalim, inhibits both the NA-induced phasic and tonic responses (Itoh *et al.*, 1994). However, it is not yet known whether this agent inhibits the responses induced by other types of Ca^{2+}-mobilizing agonists in this tissue. In preliminary experiments, we found that Y-26763, even at high concentrations, only marginally inhibits the maximum contraction induced by histamine (Hist). This suggests that the vaso-relaxing actions of the agent may differ among vaso-spasmogenic agonists, but the reason for this is unclear.

To this end, using smooth muscle tissue obtained from the third and fourth branches of the rabbit mesenteric artery (diameter 0.06–0.1 mm), the effects of Y-26763 on membrane potential, $[Ca^{2+}]_i$ and force were compared in the presence of either Hist or NA. The effects of Y-26763 were also studied in ryanodine-treated smooth muscle which had functionally lost its agonist-sensitive Ca^{2+} storage sites (Itoh *et al.*, 1992a).

Methods

Male albino rabbits, weighing 1.9–2.5 kg, were anaesthetized with pentobarbitone sodium (40 mg kg^{-1}, i.v.) and then ex-

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sanguinated. A segment from the third or fourth branch of the mesenteric artery distributing to the ileum was excised, immediately immersed in Krebs solution and cleaned of connective tissue. All the following experiments were carried out at room temperature.

Membrane potential measurement

Glass microelectrodes filled with 3 M KCl were made from borosilicate glass tubing (o.d. 1.2 mm with a core inside, Hilgenberg, Germany). The resistance of the electrodes was 40–80 MΩ. The electrode was inserted into smooth muscle cells in the endothelium-denuded tissue from the adventitial side with responses displayed on both a cathode-ray oscilloscope (SS-7602, Iwatsu) and a pen recorder (Recticorder RJG-4024, Nihon-Kohden).

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

$[Ca^{2+}]_i$ and isometric force were monitored simultaneously in endothelium-denuded circularly-cut strips (0.3–0.5 mm length, 0.04–0.05 mm width, 0.02–0.03 mm thickness) mounted horizontally on an inverted-microscope (Diaphot TMD, Nikon), as previously described (Itoh *et al.*, 1983; 1992a, b). The resting force was adjusted to obtain a maximum contraction in Krebs solution containing 128 mM K^+. Fura 2 was loaded into smooth muscle cells by application of 1 μM of the acetoxy methyl ester of Fura 2 (Fura 2-AM) for 1 h in Krebs solution at room temperature. Two alternative excitation wavelengths, 340 nm and 380 nm (each slit 5 nm) were applied by a spectro-fluorimeter (Spex, NJ, USA) and the data were analyzed using customized software provided by Spex (DM-3000CM). The ratio of the Fura 2 fluorescence intensities excited by 340 or 380 nm was calculated after subtraction of the background fluorescence and $[Ca^{2+}]_i$ were calculated by the formula described by Grynkiewicz *et al.* (1985) and using an *in vitro* calibration procedure (Poenie *et al.*, 1986). 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 K_d value for Fura 2 was estimated to be 200 nM (Becker *et al.*, 1989).

NA (10 μM) or Hist (3 μM) was applied for 2 min at 20 min intervals in Krebs solution, so that the reproducible responses were obtained. Y-26763 (0.1–10 μM) was applied for 10 min before and during applications of NA or Hist. Guanethidine (5 μM), propranolol (3 μM) and ranitidine (an H_2-receptor blocker, 3 μM) were present throughout the experiments to prevent NA-outflow from sympathetic nerves, β-adrenoceptor stimulation by exogenously applied NA and H_2-receptor stimulation by Hist, respectively. Experiments were also 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 Hist for 2 min and then brought back to Ca^{2+}-containing Krebs solution (Ca^{2+} = 2.6 mM) for 20 min. Y-26763 was applied for 5 min in Krebs solution and was present in the Ca^{2+}-free solution in the presence and absence of Hist.

In some experiments, Ca^{2+} storage sites in smooth muscle cells were functionally removed by application of ryanodine (Fleischer *et al.*, 1985; Itoh *et al.*, 1992a). After recording the control responses induced by 10 μM NA or 3 μM Hist, ryanodine (50 μM) together with 5 mM caffeine was applied for 5 min in Krebs solution followed by 10 min application of a Krebs solution containing 10 μM ryanodine only, and either NA or Hist was again applied in the presence of ryanodine. The effect of Y-26763 on $[Ca^{2+}]_i$-force relationships in the presence of NA or Hist was then studied in these ryanodine-treated muscle strips in Ca^{2+}-free solution containing 2 mM EGTA for 5 min with either 10 μM NA or 3 μM Hist applied for 30 s in the Ca^{2+}-free solution. Finally, Ca^{2+} (0.16–2.6 mM) was applied in an ascending concentration together with either NA or Hist in the presence or absence of Y-26763.

Experiments on chemically skinned smooth muscle

Chemically skinned smooth muscle strips were made by use of 400 units ml^{-1} α-toxin for 25 min in a relaxing solution containing 4 mM EGTA with 1 μM ionomycin (Nishimura *et al.*, 1988; Kitazawa *et al.*, 1991). The composition of the solutions have been described elsewhere (Itoh *et al.*, 1992a, b). The Ca^{2+}-force relationship was determined with 4 mM EGTA and 1 μM ionomycin present to avoid spurious effects due to Ca^{2+} release from intracellular storage sites in the skinned muscle strips. After 25 min treatment of α-toxin with ionomycin, increasing concentrations of Ca^{2+} were cumulatively applied. The slope of the concentration-response for the effect of Ca^{2+} on force is shown by the Hill coefficient (n) 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 eqn. (1) by a non-linear least-squares method.

$$F/F_0 = (C/K)^n/[1 + (C/K)^n] \quad (1)$$

Where C represents the concentration of Ca^{2+}, F is the amplitude of contraction at any given concentration of Ca^{2+} and F_0 is the maximum response evoked by 10 μM Ca^{2+} expressed as a relative force of 1.0.

Solutions

The ionic compositions 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 replacing NaCl with KCl, isosmotically. Ca^{2+}-free Krebs solution was made by substituting an equimolar concentration of $MgCl_2$ for $CaCl_2$ and adding 2 mM EGTA. The solutions were bubbled with 95% O_2 and 5% CO_2 and their pH maintained at 7.3–7.4.

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

For experiments on skinned muscles, the composition of the relaxing solution was as follows: 87 mM potassium methanesulphonate (KMS), 20 mM piperazine-*N-N'-bis*-(2-ethanesulphonic acid) (PIPES), 5.1 mM $Mg(MS)_2$, 5.2 mM adenosine 5'-triphosphate (ATP), 5 mM phosphocreatine, 1 μM propranolol, 3 μM ranitidine and 4 mM EGTA. Various Ca^{2+} concentrations were prepared by adding appropriate amounts of $Ca(MS)_2$ to 4 mM EGTA, based on the calculations given previously (Itoh *et al.*, 1986). The pH of the solution was adjusted to 7.1 at 25°C with KOH and the ionic strength was standardized at 0.2 M by changing the amount of KMS added.

Drugs

Drugs used were Fura 2, Fura 2-AM, EGTA, PIPES and HEPES (Dojin, Japan), NA, guanosine 5'-triphosphate (GTP), nicardipine, ranitidine and glibenclamide (Sigma, U.S.A.), ryanodine (Agri-system, U.S.A.), guanethidine (Tokyo Kasei, Japan), ATP (Na salt; Kohjin, Japan), histamine dihydrochloride (Hist) and propranolol (Nacalai, Japan), α-toxin (Gibco BRL, U.S.A.) and ionomycin (free acid; Calbiochem, U.S.A.). Y-26763 was kindly provided by Yoshitomi Pharmaceutical Ind., Ltd. (Japan).

Statistics

The values recorded are expressed as mean ± standard deviation (s.d.) except for the ED_{50} values which are mean ±

standard error (s.e.). A one-way repeated-measures ANOVA followed by Scheffe's F test for *post hoc* analysis and paired or unpaired t tests were used for the statistical analysis. Probabilities less than 5% ($P<0.05$) were considered significant.

Results

Effects of Y-26763 on membrane depolarization and increase in $[Ca^{2+}]_i$ and force induced by NA

In smooth muscle cells of rabbit mesenteric artery, the resting membrane potential was -53.5 ± 1.7 mV ($n=6$). Y-26763 (>30 nM) hyperpolarized the membrane in a concentration-dependent manner, and the maximum hyperpolarization obtained with 10 μM Y-26763 was -71.6 ± 1.0 mV ($n=5$, $P<0.05$ from control by unpaired t test). In the presence of 10 μM NA, Y-26763 (10 μM) hyperpolarized the membrane from -44.5 ± 3.2 mV ($n=12$) to -68.2 ± 3.6 mV ($n=6$, $P<0.05$), and greatly attenuated the agonist-induced membrane depolarization (Figure 1a).

The resting $[Ca^{2+}]_i$ was 106 ± 12 nM for smooth muscle strips of the rabbit mesenteric artery set to a tension of 0.5 ± 0.3 mg ($n=4$). NA (10 μM) produced a phasic, followed by a tonic increase in both $[Ca^{2+}]_i$ and force (Figure 2a). Y-26763 (0.1–10 μM) lowered the resting $[Ca^{2+}]_i$ and strongly inhibited both the phasic and tonic increases in $[Ca^{2+}]_i$ and force induced by NA, in a concentration-dependent manner (Figure 2b).

Effects of Y-26763 on membrane depolarization and increase in $[Ca^{2+}]_i$ and forced induced by Hist

In smooth muscle of the rabbit mesenteric artery, Hist (0.3–10 μM) concentration-dependently produced contraction (ED_{50} values = 0.8 ± 0.2 μM, $n=4$) with a just submaximal response obtained at 3 μM. The contraction induced by 3 μM Hist was blocked by 3 μM mepyramine.

In the presence of 3 μM ranitidine, Hist (3 μM) depolarized the membrane and the magnitude of membrane depolarization induced by 3 μM Hist was not significantly different from that induced by 10 μM NA. Y-26763 (10 μM) hyperpolarized the membrane from -41.5 ± 2.3 mV ($n=10$) to -53.7 ± 5.4 mV in the presence of 3 μM Hist together with 3 μM ranitidine ($n=29$, $P<0.05$; Figure 1b). The extent of membrane hyperpolarization induced by 10 μM Y-26763 was smaller in the presence of 3 μM Hist than in 10 μM NA ($P<0.05$, Figure 1a and b).

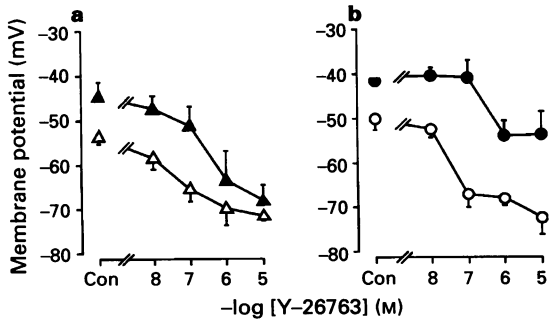

Figure 1 Concentration-dependent effects of Y-26763 on membrane potential in the presence of either 10 μM noradrenaline (NA, a) or 3 μM histamine (Hist, b) in smooth muscle cells of rabbit mesenteric artery. Both NA and Hist were applied before and during applications of Y-26763 (10 nM–10 μM). Membrane potentials were measured after 30 min application of Y-26763 and 2–3 min after application of NA or Hist in the presence and absence of Y-26763. (▲) and (●), in the presence of NA and Hist, respectively; (△) and (○), in the absence of the agonists.

As for NA (Figure 2), Hist (3 μM) produced a phasic, followed by a tonic increase in both $[Ca^{2+}]_i$ and force (Figure 3a), although the amplitude of tonic contraction induced by Hist was larger than that by NA. Y-26763 (0.1–10 μM) attenuated the Hist-induced phasic response to some extent, but had no significant effect on the Hist-induced tonic increase in $[Ca^{2+}]_i$ and force ($P=0.12$ and 0.17, respectively, one-way repeated measures ANOVA; Figure 3b). Glibenclamide (10 μM) prevented all the inhibitory actions of Y-26763 on increases in $[Ca^{2+}]_i$ and force induced by each NA and Hist (data not shown).

The effects of nicardipine (0.3 μM) on the increase in $[Ca^{2+}]_i$ and force induced by 3 μM Hist were examined and compared with those of Y-26763 (10 μM). Nicardipine did not affect either the resting $[Ca^{2+}]_i$ (from 108 ± 26 nM to 103 ± 27 nM, $n=4$) or the phasic increase in $[Ca^{2+}]_i$ (from 465 ± 108 nM to 430 ± 135 nM) and relative force (from 0.84 ± 0.19 to 0.74 ± 0.32) induced by Hist, but attenuated the tonic increase in $[Ca^{2+}]_i$ and force induced by Hist (Table 1). In the presence of nicardipine, Y-26763 had no effect on the Hist-induced tonic increase in $[Ca^{2+}]_i$ and force, and in the presence of Y-26763, nicardipine attenuated the Hist-induced tonic increase in $[Ca^{2+}]_i$ and force (Table 1).

Y-26763 attenuates the increase in $[Ca^{2+}]_i$ and force induced by Hist in ryanodine-treated strips

The effect of Y-26763 on the Hist-induced increase in $[Ca^{2+}]_i$ and force was further studied in ryanodine-treated muscle

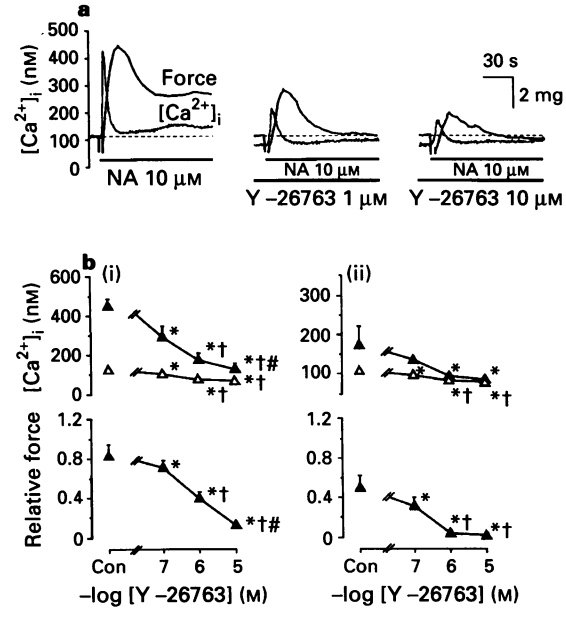

Figure 2 Concentration-dependent effects of Y-26763 on the increase in $[Ca^{2+}]_i$ and force induced by NA in smooth muscle of rabbit mesenteric artery. (a) Actual records of simultaneous measurement of $[Ca^{2+}]_i$ (thinner lines) and force (thicker lines) obtained from a single smooth muscle strip. NA (10 μM) was applied for 2 min (indicated by horizontal bars) at 20 min intervals in the presence or absence of Y-26763. Y-26763 was applied for 10 min before and during application of NA (indicated by horizontal bars). The broken lines indicate the resting $[Ca^{2+}]_i$ in the absence of Y-26763. (b) Concentration-dependent effects of Y-26763 on the phasic (i) and tonic (ii, measured at 2 min after application of NA) responses induced by NA (▲) and on the resting $[Ca^{2+}]_i$ (△). $[Ca^{2+}]_i$, upper panel; force, lower panel. The maximum amplitude of contraction induced by 128 mM K^+ in the absence of Y-26763 was normalized as a relative force of 1.0 for each strip. Mean of 4 cases with s.d. *, † and # indicate values which are significantly different from those in control, 0.1, and 1.0 μM Y-26763, respectively ($P<0.05$, one-way repeated-measures ANOVA and Scheffe's F test).

strips. In ryanodine-treated strips, Y-26763 more potently inhibited the NA-induced increase in $[Ca^{2+}]_i$ and force than in non-treated strips, and abolished the NA-induced responses at concentrations over 0.3 μM (data not shown). Figure 4 shows the effects of Y-26763 on the increase in $[Ca^{2+}]_i$ and force induced by Hist in ryanodine-treated muscle strips. Following ryanodine treatment, the resting $[Ca^{2+}]_i$ increased from 126±27 nM to 189±21 nM ($n=4$; $P<0.05$, paired t test), and 3 μM Hist failed to induce the phasic increase in $[Ca^{2+}]_i$ and force. Under these conditions, the $[Ca^{2+}]_i$ and force induced by Hist increased slowly and the time to peak was delayed (Figure 4a). Y-26763 (0.1–10 μM) lowered the resting $[Ca^{2+}]_i$ and inhibited the maximum increase in $[Ca^{2+}]_i$ and force induced by Hist (Figure 4b).

In ryanodine-treated strips, nicardipine (0.3 μM) lowered the resting $[Ca^{2+}]_i$ and greatly attenuated the increase in $[Ca^{2+}]_i$ and force induced by either NA or Hist. In the presence of nicardipine, Y-26763 (10 μM) further lowered the resting $[Ca^{2+}]_i$ and abolished these agonist-induced increases in $[Ca^{2+}]_i$ and force (Table 2).

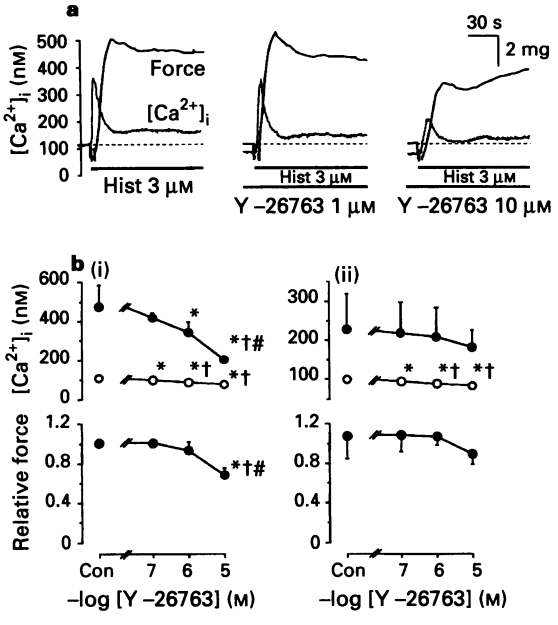

Figure 3 Concentration-dependent effects of Y-26763 on the increase in $[Ca^{2+}]_i$ and force induced by histamine (Hist). (a) The results were obtained from the same strip as that used in Figure 2a. Broken lines represent the resting $[Ca^{2+}]_i$ in the absence of Y-26763. Hist (3 μM) and Y-26763 were applied, as described in Figure 2a. (b) Concentration-dependent effects of Y-26763 on the phasic (i) and tonic (ii, measured at 2 min after application of Hist) responses induced by Hist (●) and on the resting $[Ca^{2+}]_i$ (○). The maximum amplitude of contraction induced by 128 mM K^+ in the absence of Y-26763 was normalized as a relative force of 1.0 for each strip. Mean of 4 cases with s.d. *, † and # indicate values which are significantly different from those in control, 0.1, and 1.0 μM Y-26763, respectively ($P<0.05$, one-way repeated-measures ANOVA and Scheffe's F test).

Y-26763 attenuates the Hist-induced membrane depolarization much more in ryanodine-treated cells than in non-treated cells

The effects of Y-26763 on membrane potential in the presence and absence of 3 μM Hist in ryanodine-treated cells are presented in Figure 5. Ryanodine treatment did not affect either the resting membrane potential (−49.4±1.6 mV, $n=9$) or the membrane depolarization induced by 3 μM Hist (−40.2±2.6 mV, $n=6$). In the absence of Hist, the magnitude of the membrane hyperpolarization induced by Y-26763 was not modified by the ryanodine treatment (Figure 5a). However, in the presence of 3 μM Hist, Y-26763 (10 μM) hyperpolarized the membrane much more in ryanodine-treated cells (to −66.1±4.7 mV, $n=5$) than in cells not treated with ryanodine (to −53.7±5.4 mV, $n=29$; $P<0.05$, unpaired t test), and thus strongly attenuated the Hist-induced membrane depolarization (Figure 5b).

Y-26763 has no effect on the $[Ca^{2+}]_i$-force relationship in ryanodine-treated intact smooth muscles

The effect of Y-26763 on the NA or Hist-induced $[Ca^{2+}]_i$-force relationship is presented in Figure 6. The relationship was obtained by cumulative applications of solutions with various concentrations of Ca^{2+} (from 0 to 2.6 mM) containing either NA (10 μM) or Hist (3 μM) in ryanodine-treated muscle strips. In the absence of Y-26763, the sensitivity of the contraction at given Ca^{2+} concentrations seemed to be higher in the presence of 3 μM Hist than in 10 μM NA. Y-26763 did not shift the relationship between $[Ca^{2+}]_i$ and force in the presence of either NA or Hist.

Y-26763 has no effect on the $[Ca^{2+}]_i$-force relationship in α-toxin-treated skinned smooth muscles

The direct action of Y-26763 on contractile proteins was also studied by measuring the Ca^{2+}-force relationship in the presence of 1 μM Hist together with 30 μM GTP in α-toxin-

Table 1 Effects of nicardipine with or without Y-26763 on the tonic increase in $[Ca^{2+}]_i$ and force induced by 3 μM histamine (Hist) in smooth muscle strips of the rabbit mesenteric artery

	$[Ca^{2+}]_i$ (nM)		Relative force
	Resting	*Tonic*	*(tonic)*
A Control	108±26	225±32	0.72±0.12
Nicardipine	103±27	162±28*	0.29±0.11*
Nicardipine + Y-26763	86±28†	159±33	0.29±0.10
B Control	112±14	324±27	1.28±0.16
Y-26763	89±14*	307±27	1.36±0.21
Y-26763 + nicardipine	87±15	195±19#	0.80±0.16#

The maximum amplitude of contraction induced by 128 mM K^+ in the absence of Y-26763 and nicardipine was normalized as a relative force of 1.0 for each strip. Hist (3 μM) was applied for 2 min at 20 min intervals in Krebs solution. In **A**, nicardipine (0.3 μM) was given as pretreatment for 5 min, and then Hist was applied in the presence of nicardipine. Subsequently, Y-26763 (10 μM) was applied in the presence of nicardipine for 10 min. Hist was finally applied in the presence of nicardipine with Y-26763. In **B**, Y-26763 (10 μM) was firstly applied for 10 min, and then Hist was applied in the presence of Y-26763. Nicardipine (0.3 μM) was subsequently applied in the presence of Y-26763 for 5 min, and finally Hist was applied in the presence of Y-26763 with nicardipine. Each value represents mean±s.d. ($n=4$). *, † and # represent significant difference from the corresponding control, nicardipine (without Y-26763) and Y-26763 (without nicardipine) data, respectively ($P<0.05$, paired t test).

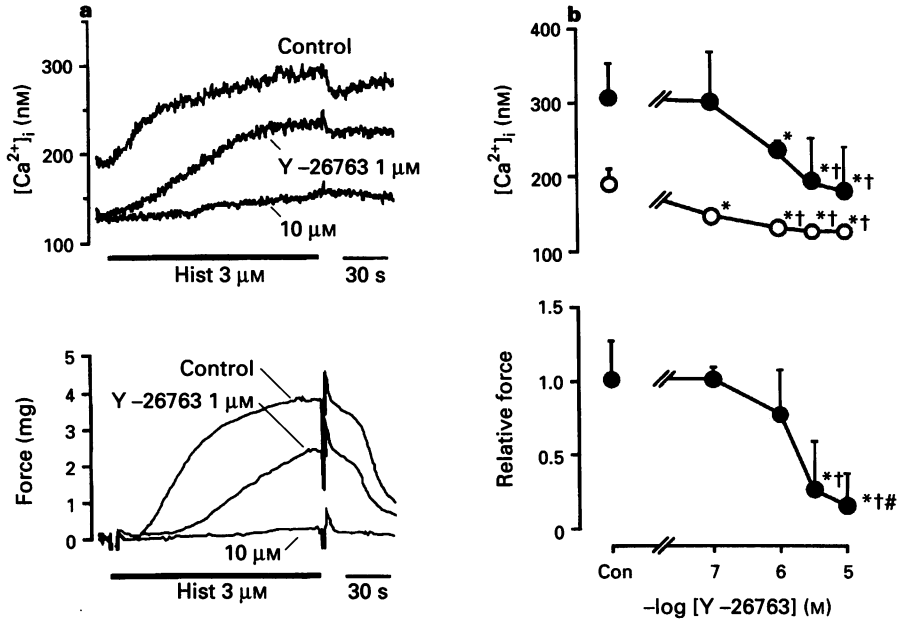

Figure 4 Concentration-dependent effects of Y-26763 on increases in $[Ca^{2+}]_i$ and force induced by histamine (Hist) in ryanodine-treated smooth muscle strips. (a) Traces in the absence and presence of Y-26763 were obtained from a single smooth muscle strip. After muscle strips were treated with ryanodine, Hist (3 μM) was applied for 2 min at 20 min intervals in Krebs solution (as indicated by bars). Y-26763 was given as pretreatment for 10 min and was present during application of Hist. Upper panel, $[Ca^{2+}]_i$; lower panel, force. (b) Shows concentration-dependent effects of Y-26763 on the increase in $[Ca^{2+}]_i$ (upper panel) and force (lower panel) induced by Hist. $[Ca^{2+}]_i$ and force were measured at 2 min after an application of Hist. (○) $[Ca^{2+}]_i$ level before application of Hist; (●) maximum responses induced by Hist. The maximum amplitude of contraction induced by 128 mM K^+ was normalized as a relative force of 1.0 for each strip. Mean of 4 cases with s.d. *, † and # indicate values which are significantly different from those in control, 0.1, and 1.0 μM Y-26763, respectively ($P<0.05$, one-way repeated-measures ANOVA and Scheffe's F test).

Table 2 Effects of nicardipine with or without Y-26763 on the increase in $[Ca^{2+}]_i$ and force induced by noradrenaline (NA, **A**) or histamine (Hist, **B**) in ryanodine-treated smooth muscle strips

	$[Ca^{2+}]_i$ (nM)		
	Resting	*Maximum*	*Relative force*
A NA (10 μM)			
Control	224±11	275±18#	0.65±0.13
Nicardipine	180±14*	198±37*	0.04±0.02*
Nicardipine+Y-26763	150±14†	162±29†	0.01±0.01†
B Hist (3 μM)			
Control	217±15	272±27#	0.84±0.08
Nicardipine	181±13*	188±12*#	0.14±0.03*
Nicardipine+Y-26763	149±12†	163±13†#	0.07±0.03†

The maximum amplitude of contraction induced by 128 mM K^+ was normalized as a relative force of 1.0 for each strip. Nicardipine (0.3 μM) was applied for 5 min before and during applications of NA or Hist. Subsequently, Y-26763 (10 μM) was given in the presence of nicardipine, and then NA or Hist was finally applied in the presence of nicardipine with Y-26763. Each value represents mean±s.d. ($n=4$). * and † represent significant differences from the corresponding control and nicardipine (without Y-26763) data, respectively ($P<0.05$, paired t test). # represents significant differences from the corresponding data of the resting $[Ca^{2+}]_i$ ($P<0.05$, paired t test).

treated skinned muscle strips. In the absence of Hist, the minimum concentration of Ca^{2+} that produced contraction was 0.1 μM and the maximum contraction was obtained at 10 μM. The concentration of Ca^{2+} required for half maximal contraction (ED_{50}) was 0.49±0.03 μM ($n=4$). Hist shifted the Ca^{2+}-force relationship to the left with a slight increase in the amplitude of the maximum Ca^{2+}-induced contraction. Y-26763 did not modify the Ca^{2+}-force relationship in the presence of Hist. In the presence of Hist, the ED_{50} values for Ca^{2+} were 0.37±0.07 μM ($n=4$) and 0.36±0.08 μM ($n=4$) before and in the presence of 10 μM Y-26763, respectively.

Y-26763 inhibits the Hist-induced increase in $[Ca^{2+}]_i$ in Ca^{2+}-free solution

The effect of Y-26763 on Hist-induced Ca^{2+} release was examined to observe the increase in $[Ca^{2+}]_i$ induced by 3 μM Hist in Ca^{2+}-free solution (containing 2 mM EGTA with 5.9 mM K^+). Changeover of the Krebs solution to the Ca^{2+}-free solution was rapid with no increase in $[Ca^{2+}]_i$ induced by 128 mM K^+ after 15 s application. Following application of the Ca^{2+}-free solution, the resting $[Ca^{2+}]_i$ rapidly decreased from 116±18 nM to 56±12 nM ($n=4$) within 1 min and then

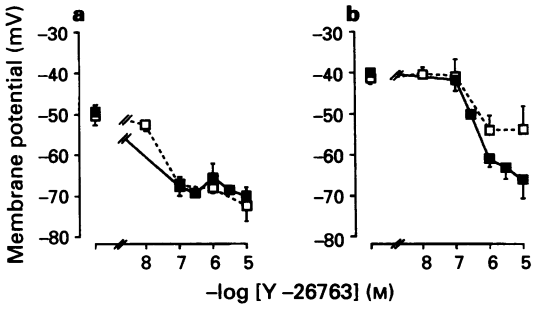

Figure 5 Concentration-dependent effects of Y-26763 on membrane potential in the presence (b) and absence (a) of histamine (Hist, 3 μM) in ryanodine-treated smooth muscle cells. Hist was applied before and during applications of Y-26763. Membrane potentials were measured after 30 min application of Y-26763 (10 nM – 10 μM) and 2–3 min after application of Hist in the presence and absence of Y-26763. (■) and (□), in ryanodine-treated and non-treated cells, respectively. Mean ± s.d. collected from 3–5 animals (n = 3–23).

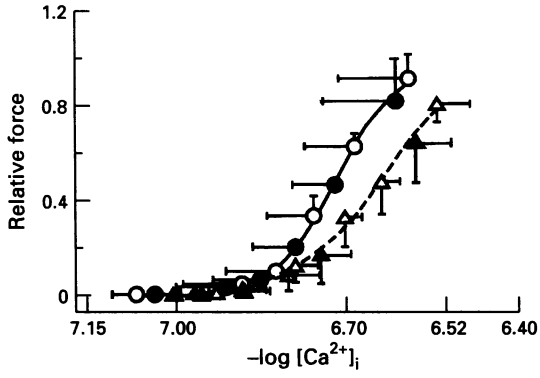

Figure 6 Effects of Y-26763 on $[Ca^{2+}]_i$-force relationship in the presence of either 10 μM noradrenaline (NA) (△, ▲) or 3 μM histamine (Hist) (○, ●) in ryanodine-treated muscle strips. (△) and (○), control; (▲), in the presence of 0.1 μM Y-26763 (n = 4); (●), in the presence of 0.3 μM Y-26763 (n = 4). Following application of Ca^{2+}-free solution (containing 2 mM EGTA) for 5 min, either 10 μM NA or 3 μM Hist was applied for 30 s in the Ca^{2+}-free solution and various concentrations of Ca^{2+} (from 0.16–2.6 mM) were then cumulatively applied at 2 min intervals in the presence of either NA or Hist. Subsequently, Ca^{2+} free solution was applied to relax the strip, followed by an application of Krebs solution for 20 min. This protocol was repeated in the presence of Y-26763. Y-26763 was applied for 10 min before and during application of various concentrations of Ca^{2+}. The maximum amplitude of contraction induced by 128 mM K^+ was normalized as a relative force of 1.0 for each strip. The curves were obtained by fitting the data points in control condition to eqn. (1) by a non-linear least-squares method according to the equation: $F/F_0 = (C/K)^n/[1+(C/K)^n]$. n, the Hill coefficient; K, the dissociation constant. C and F/F_0 represent $[Ca^{2+}]_i$ and relative force, respectively. The fitted parameters of n and K were respectively 5.0 and 0.24 μM in the presence of NA (r = 0.998) and 7.9 and 0.19 μM in the presence of Hist (r = 0.999). Each value represents mean ± s.e. (n = 4).

remained at this new steady level. Under these conditions, Hist transiently increased $[Ca^{2+}]_i$ to 368 ± 98 nM (n = 4). In Ca^{2+}-free solution, Y-26763 (10 μM) lowered the resting $[Ca^{2+}]_i$ to 46 ± 12 (n = 4, $P < 0.05$ from control by paired t test) and attenuated the Hist-evoked increases in $[Ca^{2+}]_i$ (126 ± 26 nM, n = 4; $P < 0.05$). Glibenclamide (10 μM) blocked the inhibitory action of Y-26763 on both the resting $[Ca^{2+}]_i$ and the Hist-evoked response in Ca^{2+}-free solution.

Discussion

In smooth muscle of rabbit mesenteric artery, NA (10 μM) depolarizes the membrane and increases $[Ca^{2+}]_i$ and force with

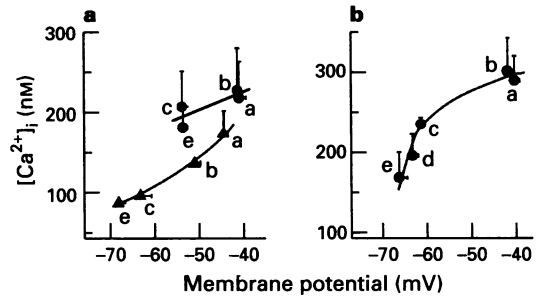

Figure 7 Relationships between membrane potential and $[Ca^{2+}]_i$ in the presence of Y-26763 with noradrenaline (NA) or histamine (Hist) in ryanodine-non-treated (a) and ryanodine-treated (b) smooth muscles. Membrane potentials and the tonic increases in $[Ca^{2+}]_i$ at given concentrations of Y-26763 are plotted from the data shown in Figures 1, 2b(ii), 3b(ii), 4b and 5b. [a] In the absence of Y-26763; [b] in the presence of 0.1 μM Y-26763; [c] in 1 μM Y-26763; [d] in 3 μM Y-26763; [e] in 10 μM Y-26763. (▲) and (●) represent in the presence of NA and Hist, respectively. Mean ± s.e. collected from 3–5 animals (n = 3–23).

two phases: the transient phasic response is provoked by Ca^{2+} release from intracellular storage sites, whereas the subsequently maintained tonic phase is induced by an activation of Ca^{2+} influx through the plasmalemma (Itoh *et al.*, 1983; 1992a). We previously reported that KCOs such as pinacidil and Y-26763 hyperpolarize the membrane and inhibit the NA-induced membrane depolarization and thus attenuate these NA-induced increases in $[Ca^{2+}]_i$ and force (Itoh *et al.*, 1992b; 1994). We also found that Y-26763 (10 μM) almost completely whereas nicardipine (0.3–1 μM) only partially inhibited the NA-induced tonic increase in $[Ca^{2+}]_i$ and force (Itoh *et al.*, 1994), suggesting that the membrane hyperpolarization induced by Y-26763 inhibits the NA-induced increases in $[Ca^{2+}]_i$ and force through mechanisms additional to simple inhibition of L-type Ca^{2+} channels.

As for NA, Hist produced a phasic, followed by a tonic increase in $[Ca^{2+}]_i$ and force. The former response is possibly due to the Hist-induced Ca^{2+} release from intracellular storage sites, because the response was maintained in Ca^{2+}-free solution and abolished in ryanodine-treated smooth muscle. The latter response may be caused by Hist-activated Ca^{2+} influx since the response was greatly inhibited by nicardipine and abolished in Ca^{2+}-free solution. Y-26763 (10 μM) had no effect on the tonic increase in $[Ca^{2+}]_i$ and force induced by 3 μM Hist, although this agent inhibited the phasic responses to some extent (Figure 3). In contrast, Y-26763 (10 μM) strongly attenuated the NA-induced phasic and tonic response (Figure 2). The magnitude of membrane depolarization induced by 3 μM Hist was similar to that produced by 10 μM NA, but the effect of Y-26763 on membrane potential was different in the presence of NA and Hist, in that in the presence of 10 μM NA, Y-26763 (10 μM) hyperpolarized the membrane close to the level in the absence of NA (−70 mV), and in the presence of 3 μM Hist this agent hyperpolarized the membrane close to the resting potential (−50 mV, Figure 1). Since Ca^{2+} mobilizing agonists shift the voltage-dependency of L-type Ca^{2+} channels to a more negative potential in vascular smooth muscle cells (Nelson *et al.*, 1988), Hist might activate voltage-dependent Ca^{2+} channels even at the resting membrane potential. This might explain our observation that nicardipine attenuated the Hist-induced tonic increases in $[Ca^{2+}]_i$ and force in the presence of Y-26763 (Table 1). Thus, these results suggest that in the presence of Hist, the level of the membrane potential achieved by Y-26763 may not be enough to inhibit the Hist-induced Ca^{2+} influx.

In smooth muscle of the rabbit mesenteric artery, ryanodine causes the loss of function of NA-sensitive Ca^{2+} store sites (Itoh *et al.*, 1992a). In the present experiments, we found that following application of ryanodine, both NA and Hist failed to

produce the phasic increase in $[Ca^{2+}]_i$ and force and only produced a monotonic response. These results suggest that ryanodine causes the loss of function to both NA and Hist. Thus, in ryanodine-treated smooth muscle, it is likely that both NA and Hist increase $[Ca^{2+}]_i$ and force through agonist-activated Ca^{2+}-influx. In ryanodine-treated smooth muscle cells, Y-26763 (10 μM) almost completely abolished the Hist-induced increase in $[Ca^{2+}]_i$ and force (Figure 4). Moreover, after ryanodine treatment, the membrane potential levels obtained by 10 μM Y-26763 in the presence and absence of Hist were the same (−70 mV and −72 mV, respectively), indicating that the membrane depolarization induced by Hist was completely blocked by 10 μM Y-26763 (Figure 5b). These results suggest that in ryanodine-treated cells, the membrane hyperpolarization induced by Y-26763 was sufficient to attenuate the Hist-induced Ca^{2+}influx. This effect of Y-26763 differs from that observed in ryanodine-non-treated smooth muscle strips.

To try to understand the role of membrane hyperpolarization on regulation of agonist-activated $[Ca^{2+}]_i$ increase, the relationship between membrane potential and $[Ca^{2+}]_i$ in the presence of 3 μM Hist or 10 μM NA was plotted using the data shown in Figures 1–5. In muscle not treated with ryanodine (Figure 7a), the relationship in the presence of Hist appears different from that in NA, with hyperpolarization having less effect on $[Ca^{2+}]_i$ increased by Hist than by NA. In ryanodine-treated muscle (Figure 7b), when the membrane potential increased to more than −60 mV (which could not be achieved even in maximum concentration of Y-26763 in ryanodine-non-treated muscle) in the presence of Hist, $[Ca^{2+}]_i$ dramatically decreased within a membrane potential range of −60 mV to −70 mV. These indicate that in ryanodine-non-treated smooth muscle, the membrane hyperpolarization attained by Y-26763 in the presence of Hist may not be enough to inhibit the Hist-activated Ca^{2+} influx, thus causing no inhibition of the Hist-induced tonic increase in $[Ca^{2+}]_i$. Alternatively, Hist may inhibit the membrane hyperpolarization induced by Y-26763 through an activation of ryanodine-sensitive intracellular Ca^{2+} storage sites. It has recently been reported that in bladder smooth muscle cells of the guinea-pig, carbachol activates protein kinase C and then inhibits the ATP-sensitive K^+ channel (Bonev & Nelson, 1993). It is also known that Ca^{2+} released from the storage sites activates receptor-operated non-selective cation channels in longitudinal smooth muscle cells of the guinea-pig ileum (Inoue & Isenberg, 1990). These mechanisms may be activated by Hist and then attenuate the membrane hyperpolarization induced by Y-26763.

In the present experiments Y-26763 had no effect on the $[Ca^{2+}]_i$-force relationship when activated by either 10 μM NA or 3 μM Hist under membrane polarized (*i.e.* non-permeabilized) conditions (Figure 6). Moreover, this agent had no direct effect on the Ca^{2+}-force relationship when activated by 3 μM Hist with GTP in α-toxin-skinned smooth muscle strips. These results indicate that neither Y-26763 itself nor the membrane hyperpolarization induced by the agent modify the myofilament Ca^{2+}-sensitivity when activated by Hist, suggesting that Y-26763 inhibits the agonist-induced Ca^{2+} mobilization, and then attenuates the agonist-induced contractions.

In smooth muscle of the rabbit mesenteric artery, both levcromakalim and pinacidil attenuate NA-induced $InsP_3$ synthesis and then inhibit NA-induced Ca^{2+} release in a membrane potential-dependent manner (Ito *et al.*, 1991; Itoh *et al.*, 1992b). Y-26763 attenuates the NA induced Ca^{2+} release in the membrane-polarized condition (Itoh *et al.*, 1994). In the present experiments, Y-26763 inhibited the Hist-induced $[Ca^{2+}]_i$ increase in Ca^{2+}-free solution and this inhibition was prevented by glibenclamide. The results suggest that as for NA, the membrane hyperpolarization induced by Y-26763 inhibits the Hist-induced Ca^{2+} release.

In conclusion, the membrane hyperpolarization induced by Y-26763 may not be enough to inhibit the Hist-activated Ca^{2+} influx, thus causing no inhibition of the Hist-induced tonic contraction. It is also suggested that Hist inhibits the membrane hyperpolarization induced by Y-26763 activating an unknown mechanism which is thought to depend on the function of the intracellular Ca^{2+} storage sites.

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