Effects of pinacidil on contractile proteins in high K^+ -treated intact, and in β -escin-treated skinned smooth muscle of the rabbit mesenteric artery

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1 The effects of pinacidil were investigated on changes in cellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and tension in intact and chemically skinned smooth muscle strips of the rabbit mesenteric artery.

2 High K^+ (128 mM) produced a large phasic followed by a tonic increase in $[Ca^{2+}]_i$ and tension in intact muscle strips. Pinacidil at 10 μM but not 1 μM , inhibited the phasic and tonic contractions induced by 128 mM K^+ without a corresponding change in $[Ca^{2+}]_i$.

3 In β -escin-treated skinned smooth muscle, the minimum Ca^{2+} concentration that produced contraction was 0.1 μM and the maximum contraction was obtained at 10 μM . Pinacidil at 10 μM but not 1 μM , shifted the pCa-tension relation curve to the right and also inhibited the maximum contraction induced by Ca^{2+} . The concentrations of Ca^{2+} required for half maximal tension were 0.9 μM in control and 1.5 μM in the presence of 10 μM pinacidil. Calmodulin (2 μM) increased the contraction induced by 0.3 μM Ca^{2+} (but not by 10 μM Ca^{2+}) in the skinned strips. Pinacidil (10 μM) inhibited the contraction induced by 0.3 μM or 10 μM Ca^{2+} in the presence of 2 μM calmodulin.

4 Noradrenaline (NA, 10 μM) with guanosine triphosphate (GTP, 3 μM), guanosine 5'-O-(3-thiotriphosphate) (GTP γ S, 3 μM) or 12-O-tetradecanoylphorbol-13-acetate (TPA, 0.1 μM) all enhanced the contraction induced by 0.3 μM Ca^{2+} . Pinacidil (10 μM) inhibited the contraction induced by 0.3 μM Ca^{2+} more strongly in the presence of the above agents than in their absence.

5 Following application of 2 mM adenosine-5'-O-(3-thiotriphosphate) (ATP γ S) with 0.3 μM Ca^{2+} , 4 mM MgATP produced contraction in skinned strips in Ca^{2+} -free solution containing 4 mM EGTA (' Ca^{2+} -independent contraction'). The amplitude of the Ca^{2+} -independent contraction was almost the same as that obtained with 10 μM Ca^{2+} . Pinacidil (10 μM) had no effect on the amplitude of the Ca^{2+} -independent contraction nor did it have any effect on the contraction induced by a solution containing no MgATP ('rigor contraction').

6 It is concluded that pinacidil (10 μM) acts directly on the contractile apparatus to inhibit Ca^{2+} -induced contraction in smooth muscle of the rabbit mesenteric artery. The site of action of pinacidil may be between Ca^{2+} -calmodulin complex formation and phosphorylation of the myosin light chain.

Keywords: Pinacidil; skinned smooth muscle; cellular Ca^{2+} concentration; rabbit mesenteric artery; contractile proteins; high K^+

Introduction

Pinacidil (($\pm$)-N-cyano-4-pyridyl-N-1,2,2-trimethyl-propyl-guanodine monohydrate) is a novel vascular relaxant which hyperpolarizes the membrane of vascular smooth muscle cells at concentrations between 0.1 μM and 10 μM , in a concentration-dependent manner (Southerton *et al.*, 1988; Videbaek *et al.*, 1988; Quast & Cook, 1989; Standen *et al.*, 1989; Nakashima *et al.*, 1991). These authors suggested that the membrane hyperpolarization inhibits the vasospasmogenic agent-induced contraction by increasing the threshold for activation of the voltage-dependent Ca^{2+} channel. In our preliminary experiments, however, we found that pinacidil, in concentrations over 10 μM , inhibited the contractions induced by 128 mM K^+ in smooth muscle strips of the rabbit mesenteric artery. Glibenclamide, an inhibitor of the ATP-sensitive K^+ channel, did not modify this action of pinacidil. Since under these conditions pinacidil cannot produce membrane hyperpolarization in smooth muscle cells (Nakashima *et al.*, 1991), this suggested that an effect of pinacidil other than its membrane hyperpolarizing action may underlie its inhibition of high K^+ -induced contraction.

To clarify the action of pinacidil, we studied the effects of this agent on the increases in $[Ca^{2+}]_i$ and tension induced by 128 mM K^+ in intact smooth muscle and on Ca^{2+} -induced contraction in chemically skinned muscle.

Methods

Male albino rabbits (1.9–2.5 kg) were anaesthetized by injection of pentobarbitone sodium (40 mg kg⁻¹, i.v.), and were then exsanguinated. The third branch of the mesenteric artery was excised immediately and cleaned by removal of the connective tissue in Krebs solution at room temperature. To prevent NA outflow from sympathetic nerve terminals, 3 μM guanethidine was added to the Krebs solution for over 2 h.

Ca^{2+} and tension measurement

To enable recording of isometric tension, 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.*, 1983). Endothelial cells were removed by gentle rubbing of the internal surface of the vessels with small knives. The absence of function of the endothelial cells was confirmed by the inability of acetylcholine (1 μM) or A23187 (0.1 μM) to cause relaxation during contractions induced by NA. 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).

To enable loading of Fura-2 into smooth muscle cells of the strip, 1 μM acetoxymethyl ester of Fura-2 (Fura-2AM) was applied for 1 h in Krebs solution at room temperature (20–23°C). After this period, the solution containing Fura-2AM

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was washed out with Krebs solution for 1 h to ensure sufficient esterification of Fura-2AM in the cells. The position of the strip was adjusted to the centre of the field with a mask placed in an intermediate image plane to reduce background fluorescence (0.04 mm square). The Fura-2 fluorescence emission at 510 nm using an interference filter (centred at 510 nm and full width at half transmission, 20 nm) was collected through the lens (20 times CF Fluor objective lens, Nikon) and collected in a photomultiplier tube (R928, side-on type, Hamamatsu Photonics, Japan) via a dichroic mirror (DM-400, Nikon) which was substituted for the photochanger in a Nikon Diaphoto-TMD microscope. Two alternative excitation wavelengths, 340 nm and 380 nm (each slit 5 nm) were applied by a spectro-fluorimeter (Spex, NJ, U.S.A.) and the data were analyzed with customized software provided by Spex (DM-3000CM).

The ratio of Fura-2 fluorescence intensities excited by 340 or 380 nm was calculated after subtraction of the background fluorescence. Background fluorescence (including the autofluorescence of the strip) as excited by 340 and 380 nm u.v.-light was obtained by application of a solution containing 50 μ M ionomycin, 20 mM MnCl_2 , 110 mM KCl and 10 mM MOPS (pH 4.8) after the experiment. Under these conditions, the background fluorescence intensity was 10–15% of the Fura-2 signals in smooth muscle strips at either excitation wavelength. Cytosolic Ca^{2+} concentrations were calculated by the formula of Grynkiewicz *et al.* (1985) and using *in vitro* calibration (Poenie *et al.*, 1986; Becker *et al.*, 1989). The ratio of the maximum (F_{max}) to the minimum fluorescence (F_{min}) was determined in the calibration solution after subtraction of background excited by either 340 or 380 nm. The signals excited by 380 nm u.v.-light were assumed to decrease by 15% in the cell due to the possible intracellular viscosity effects on the dye (Becker *et al.*, 1989). The K_d value for Fura-2 was estimated to be 200 nM (Becker *et al.*, 1989). This method of calculating Ca^{2+} may not be accurate if Fura-2 binds to some proteins in the cell (Konishi *et al.*, 1988).

Experiments on chemically skinned smooth muscle

Chemically skinned smooth muscle strips were made by use of β -escin (Kobayashi *et al.*, 1989). The methods used to prepare skinned muscle and the composition of the solutions have been described elsewhere (Itoh *et al.*, 1986a; Kobayashi *et al.*, 1989). Ionomycin (1 μ M) was present throughout the experiment to deplete the intracellularly stored Ca^{2+} .

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 as required by replacing NaCl with KCl, isosmotically. The calibration solution for Ca^{2+} -measurement in intact strips contained ethyleneglycol-bis-(β -aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA) 11 mM, KCl 110 mM, MgCl_2 1 mM, Fura-2 2 μ M and N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) (pH 7.1) 20 mM with or without CaCl_2 11 mM.

For experiments on skinned muscle, the composition of the relaxing solution was (mM): potassium methanesulphonate (KMs) 87, piperazine-N,N'-bis-(2-ethanesulphonic acid) (PIPES) 20, $\text{Mg}(\text{Ms})_2$ 5.1, ATP 5.2, phosphocreatine 10, EGTA 4 and ionomycin 1 μ M. Various Ca^{2+} concentrations were prepared by adding appropriate amounts of $\text{Ca}(\text{Ms})_2$ to 4 mM EGTA, based on the calculation given previously (Itoh *et al.*, 1986a). The pH of the solution was adjusted to 7.1 at 25°C with KOH and the ionic strength was standardized at 0.2 by changing the amount of KMs added.

To obtain a Ca^{2+} -independent contraction in the skinned strips, a solution containing ATP γ S and 0.3 μ M Ca^{2+} or a rigor-producing solution was used. The ATP γ S solution was composed of (mM): HEPES 20, $\text{Mg}(\text{Ms})_2$ 2.6, ATP γ S 2 and

EGTA 4. The ionic strength of the solution was adjusted to 0.2 M by adding KMs. The free Ca^{2+} concentration was calculated to be 0.3 μ M. The composition of the rigor-producing solution was (mM): KMs 130, $\text{Mg}(\text{Ms})_2$ 1, EGTA 4 and HEPES 2 and the pH was adjusted to 7.1 using KOH.

Drugs

Drugs used were Fura-2, Fura-2AM, EGTA, PIPES, HEPES and MOPS (Dojin), NA, guanosine triphosphate (GTP), β -escin, 12-O-tetradecanoylphorbol-13-acetate (TPA), guanosine 5'-O-(3-thiotriphosphate) (GTP γ S) and glibenclamide (Sigma), guanethidine (Tokyo Kasei), ATP (Na salt, Kojin), ionomycin (Calbiochem), ATP γ S (Boehringer Mannheim-Yamanouchi) and pinacidil (Shionogi).

Statistics

The values recorded were expressed as mean $\pm$ s.d., and statistical significance was determined by Student's *t* test. Probabilities less than 5% ($P < 0.05$) were considered significant.

Results

Effects of pinacidil (10 μ M) on changes in cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and tension induced by 128 mM K^+ were observed in smooth muscle strips of the rabbit mesenteric artery. Resting $[\text{Ca}^{2+}]_i$ was 135 ± 25 nM ($n = 5$) and no spontaneous fluctuations were observed. When 128 mM K^+ was applied to the strip, $[\text{Ca}^{2+}]_i$ immediately increased to a peak within 4–5 s (phasic phase) and then decayed to a steady level (tonic phase). Following the increase in $[\text{Ca}^{2+}]_i$, tension developed with a phasic and a subsequent tonic phase (Figure 1). The levels of $[\text{Ca}^{2+}]_i$ at the peak and in the tonic phase in the presence of 128 mM K^+ were 1055 ± 134 nM and 347 ± 79 nM, respectively ($n = 4$). Pinacidil (1 or 10 μ M) did not significantly modify these $[\text{Ca}^{2+}]_i$ increases induced by 128 mM K^+ , although the rate of the decay of $[\text{Ca}^{2+}]_i$ following the phasic contraction was slightly accelerated (the levels of $[\text{Ca}^{2+}]_i$ at the peak and in the tonic phase in the presence of 128 mM K^+ were 1028 ± 128 nM and 360 ± 81 nM in the presence of 1 μ M pinacidil and 991 ± 126 nM and 311 ± 70 nM in the presence of 10 μ M pinacidil, respectively, $P > 0.05$, $n = 4$). However, pinacidil at 10 μ M but not at 1 μ M, significantly reduced both the phasic and tonic contractions induced

Figure 1 Effects of 10 μ M pinacidil on changes in $[\text{Ca}^{2+}]_i$ and tension induced by 128 mM K^+ in smooth muscle strip of rabbit mesenteric artery. High K^+ was applied at the arrow. $[\text{Ca}^{2+}]_i$ is indicated by the continuous lines and tension by the dotted lines. Pinacidil was applied 10 min before the application of 128 mM K^+ and was present throughout the experiment. The same effect was observed in 3 other sets of experiments.

by 128 mM K^+ (the values of absolute tension at the peak and in the tonic phase in the presence of 128 mM K^+ were 11.2 ± 0.6 mg and 6.4 ± 1.1 mg in control, respectively and these were 10.9 ± 0.5 mg and 6.0 ± 2.7 mg in the presence of $1 \mu\text{M}$ pinacidil, $P > 0.05$, $n = 4$ and 9.4 ± 0.7 mg and 2.4 ± 0.4 mg in the presence of $10 \mu\text{M}$ pinacidil, respectively, $P < 0.01$, $n = 4$). The pinacidil-induced inhibition of the contraction induced by 128 mM K^+ recovered slowly after washout of this drug (42 ± 15 min was required for 80% recovery of the tonic contraction induced by 128 mM K^+ , $n = 3$). Glibenclamide (1 or $10 \mu\text{M}$) did not modify this action of pinacidil. These results suggest that pinacidil ($10 \mu\text{M}$) may inhibit the tonic contraction induced by 128 mM K^+ via its direct inhibitory action on the contractile apparatus in smooth muscle.

To investigate the direct action of pinacidil on the contractile proteins, its effect was observed on Ca^{2+} -induced contraction in the presence of $1 \mu\text{M}$ ionomycin in β -escin-treated, skinned smooth muscle strips. In the skinned strip, the minimum concentration of Ca^{2+} that produced contraction was $0.1 \mu\text{M}$ and the maximum contraction was obtained at $10 \mu\text{M}$, as previously observed in saponin-skinned smooth muscle (Itoh *et al.*, 1983). Figure 2 shows the effects of pinacidil on contraction induced by various concentrations of Ca^{2+} (0.1 – $10 \mu\text{M}$) in the skinned strips. Pinacidil ($1 \mu\text{M}$) produced no detectable effects on the pCa-tension relationship but, at $10 \mu\text{M}$ it shifted the curve to the right and reduced the maximum contraction induced by $10 \mu\text{M}$ Ca^{2+} . The concentrations of Ca^{2+} required for half maximal tension were $0.94 \pm 0.4 \mu\text{M}$ in control and $1.49 \pm 0.38 \mu\text{M}$ in the presence of $10 \mu\text{M}$ pinacidil ($n = 7$) and these were significantly different ($P < 0.05$). Glibenclamide ($10 \mu\text{M}$) did not affect the inhibitory action of pinacidil on the contraction induced by $0.3 \mu\text{M}$ Ca^{2+} (Figure 4a). Neomycin (0.5 mM), an inhibitor of phospholipase C, did not modify the pCa-tension relationship observed under control conditions or in the presence of $10 \mu\text{M}$ pinacidil (Figure 3).

Calmodulin shifts the pCa-tension relationship to the left

Figure 2 Effects of $10 \mu\text{M}$ pinacidil on pCa-tension relationship in β -escin treated skinned smooth muscle strips of rabbit mesenteric artery. (a) Actual records under control conditions (i) and in the presence of $10 \mu\text{M}$ pinacidil (ii); (i) and (ii) were contiguous records. Ca^{2+} was cumulatively applied in a step-wise manner. Pinacidil was applied 10 min before the application of Ca^{2+} and was present throughout the experiment. (b) Effects of pinacidil on pCa-tension relationship: (●) control; (○) in the presence of $1 \mu\text{M}$ pinacidil; (△) in the presence of $10 \mu\text{M}$ pinacidil. The amplitude of contraction induced by $10 \mu\text{M}$ Ca^{2+} was normalized as 1.0. Results shown are means of 4–6 observations with s.d. shown by vertical bars. * indicates significant difference from the corresponding results in the absence of $10 \mu\text{M}$ pinacidil ($P < 0.05$).

Figure 3 Effects of 0.5 mM neomycin on pCa-tension relationship in the presence or absence of $10 \mu\text{M}$ pinacidil: (●) control; (▲) in the presence of 0.5 mM neomycin; (○) in the presence of $10 \mu\text{M}$ pinacidil; (△) in the presence of $10 \mu\text{M}$ pinacidil and 0.5 mM neomycin. Continuous lines and dotted lines indicate results obtained in the absence or presence of 0.5 mM neomycin, respectively. This experiment was done using a single strip. The amplitude of contraction induced by $10 \mu\text{M}$ Ca^{2+} in the control experiment was normalized as 1.0. Similar results were obtained in another 2 strips.

(Itoh *et al.*, 1986a). To investigate the effects of calmodulin on the pinacidil-induced inhibition of the contraction induced by Ca^{2+} , the effects of $10 \mu\text{M}$ pinacidil on the contraction induced by $0.3 \mu\text{M}$ or $10 \mu\text{M}$ Ca^{2+} were observed in the presence of $2 \mu\text{M}$ calmodulin. Calmodulin ($2 \mu\text{M}$) increased the contraction induced by $0.3 \mu\text{M}$ Ca^{2+} (1.5 times control, see Figure 4a) but had no effect on the contraction induced by $10 \mu\text{M}$ Ca^{2+} (1.06 ± 0.04 times the control, $n = 4$). Pinacidil ($10 \mu\text{M}$) inhibited the contraction induced by either $0.3 \mu\text{M}$ or $10 \mu\text{M}$ Ca^{2+} in the presence of $2 \mu\text{M}$ calmodulin (Figure 4). The extent of the pinacidil-induced inhibition of the contraction induced by $10 \mu\text{M}$ Ca^{2+} was identical whether or not $2 \mu\text{M}$ calmodulin was present although the same concentration of calmodulin did slightly inhibit the action of pinacidil on the contraction

Figure 4 (a) Effects of $2 \mu\text{M}$ calmodulin on Ca^{2+} -induced contraction in the presence or absence of $10 \mu\text{M}$ pinacidil: (i) effects of $10 \mu\text{M}$ pinacidil and $10 \mu\text{M}$ glibenclamide on contraction induced by $0.3 \mu\text{M}$ Ca^{2+} ; (ii) effect of $10 \mu\text{M}$ pinacidil on contraction induced by $2 \mu\text{M}$ calmodulin in the presence of $0.3 \mu\text{M}$ Ca^{2+} ; (i) and (ii) were contiguous records. (b) Effects of $10 \mu\text{M}$ pinacidil on contraction induced by $0.3 \mu\text{M}$ or $10 \mu\text{M}$ Ca^{2+} in the presence or absence of $2 \mu\text{M}$ calmodulin. Pinacidil ($10 \mu\text{M}$) was applied on the steady level of the Ca^{2+} -induced contraction. Calmodulin ($2 \mu\text{M}$) was applied 10 min before application of the Ca^{2+} and was present throughout the experiment. The amplitude of contraction induced by $10 \mu\text{M}$ Ca^{2+} was normalized as 1.0. Results represented are means of 3–4 observations with s.d. shown by vertical bars. * indicates significant difference from the results obtained in the absence of $10 \mu\text{M}$ pinacidil ($P < 0.05$).

Figure 5 (a) Effects of $10 \mu\text{M}$ pinacidil on Ca^{2+} -independent contraction: (i) effects of $10 \mu\text{M}$ pinacidil on contraction induced by $10 \mu\text{M Ca}^{2+}$. In (ii), rigor solution (Ca^{2+} -free, no ATP containing solution) was applied for 1.5 min. After application of 2 mM adenosine-5'-O-(3-thiotriphosphate) ($\text{ATP}\gamma\text{S}$) with $0.3 \mu\text{M Ca}^{2+}$ for 10 min in rigor solution, the strip was rinsed with rigor solution for 1.5 min and the relaxing solution (containing 4 mM MgATP and no Ca^{2+} with 4 mM EGTA) was applied. When the tension reached a steady level $10 \mu\text{M}$ pinacidil was applied. (b) Effects of $10 \mu\text{M}$ pinacidil on the contraction induced by $10 \mu\text{M Ca}^{2+}$ and on the Ca^{2+} -independent contraction. The amplitude of contraction was normalized relative to the contraction induced by $10 \mu\text{M Ca}^{2+}$ in the absence of $10 \mu\text{M}$ pinacidil. Results shown are means of 3–4 observations with s.d. shown by vertical bars. * indicates significant difference from the results obtained in the absence of $10 \mu\text{M}$ pinacidil ($P < 0.05$).

induced by $0.3 \mu\text{M Ca}^{2+}$ (Figure 4b). These results suggest that pinacidil inhibited the Ca^{2+} -induced contraction through a mechanism not dependent upon calmodulin.

To investigate further the mechanism of action of pinacidil on the contractile proteins, the effect of this drug on Ca^{2+} -independent contraction was observed. The experimental procedure is indicated in Figure 5. After the maximum contraction had been induced by $10 \mu\text{M Ca}^{2+}$, the strip was rinsed with the relaxing solution until it was completely relaxed. Following application of the rigor solution (Ca^{2+} -free solution containing no ATP) for 1.5 min, only a small contraction was evoked ('rigor contraction'). When 2 mM $\text{ATP}\gamma\text{S}$ with $0.3 \mu\text{M Ca}^{2+}$ was applied for 10 min, tension developed slowly. Subsequent application of the Ca^{2+} -free solution containing no ATP halted the contraction at the level reached at the termination of the application of $\text{ATP}\gamma\text{S}$ with Ca^{2+} . Then, when the relaxing solution (a Ca^{2+} -free solution containing 4 mM EGTA, 4 mM MgATP and 10 mM phosphocreatine) was applied to the strip, additional tension rapidly developed and the contraction was sustained at the same amplitude for over 30 min (' Ca^{2+} -independent contraction'). The amplitude of this Ca^{2+} -independent contraction was almost the same as that induced by $10 \mu\text{M Ca}^{2+}$. Pinacidil ($10 \mu\text{M}$) did not modify the Ca^{2+} -independent contraction (Figure 5).

The effect of pinacidil on the rigor contraction was also observed. Following application of $10 \mu\text{M Ca}^{2+}$ to the strips, the solution was rinsed off with Ca^{2+} -free solution containing 4 mM EGTA and no ATP and thus a rigor contraction was produced, as described previously (Itoh *et al.*, 1986a). Pinacidil ($10 \mu\text{M}$) had no effect on this rigor contraction (not shown) suggesting that pinacidil did not inhibit actin-myosin interactions directly.

NA with GTP enhances the Ca^{2+} -induced contraction in α -toxin treated, chemically skinned smooth muscle strips (Nishimura & van Breemen, 1988; Kitazawa *et al.*, 1989;

Figure 6 (a) Effects of $10 \mu\text{M}$ pinacidil on the contraction induced by 0.3 or $10 \mu\text{M Ca}^{2+}$ in the presence of $10 \mu\text{M}$ noradrenaline (NA) with $3 \mu\text{M}$ guanosine triphosphate (GTP) (i, ii) or $3 \mu\text{M}$ guanosine 5'-O-(3-thiotriphosphate) ($\text{GTP}\gamma\text{S}$) (iii). Typical examples are shown of the effect of $10 \mu\text{M}$ pinacidil on the contraction induced by $0.3 \mu\text{M Ca}^{2+}$ (i), $10 \mu\text{M Ca}^{2+}$ (ii) and $0.3 \mu\text{M Ca}^{2+}$ with $3 \mu\text{M GTP}\gamma\text{S}$ (iii); (i–iii) were contiguous records. NA ($10 \mu\text{M}$) with GTP ($3 \mu\text{M}$) or $\text{GTP}\gamma\text{S}$ ($3 \mu\text{M}$) were applied for 5 min before and during the application of Ca^{2+} . (b) Effects of $10 \mu\text{M}$ pinacidil on the contraction induced by $0.3 \mu\text{M}$ or $10 \mu\text{M Ca}^{2+}$ in the presence or absence of $10 \mu\text{M NA}$ with $3 \mu\text{M GTP}$ or $3 \mu\text{M GTP}\gamma\text{S}$. The amplitude of contraction was normalized relative to the contraction induced by $10 \mu\text{M Ca}^{2+}$. Results shown are means of 3–4 observations with s.d. shown by vertical bars. * indicates significant difference from the results obtained in the absence of $10 \mu\text{M}$ pinacidil ($P < 0.05$). † indicates significant difference from the results obtained in the presence of $10 \mu\text{M Ca}^{2+}$ with $10 \mu\text{M}$ pinacidil.

Kobayashi *et al.*, 1989). To investigate the mechanism of action of pinacidil on the Ca^{2+} -induced contraction further, its effect on the Ca^{2+} -induced contraction was observed in the presence of NA with GTP. NA ($10 \mu\text{M}$) with GTP ($3 \mu\text{M}$) enhanced the contraction induced by $0.3 \mu\text{M Ca}^{2+}$ (Figure 6). Pinacidil ($10 \mu\text{M}$) inhibited the contraction induced by $0.3 \mu\text{M Ca}^{2+}$ more strongly in the presence of $10 \mu\text{M NA}$ with $3 \mu\text{M GTP}$ than in its absence. Thus, the pinacidil-inhibited contractions were 0.40 ± 0.13 times and 0.27 ± 0.33 times control in the absence or presence of NA with GTP, respectively ($n = 4$). $\text{GTP}\gamma\text{S}$ ($3 \mu\text{M}$) also enhanced the contraction induced by $0.3 \mu\text{M Ca}^{2+}$, as found previously for saponin-treated skinned muscle (Fujiwara *et al.*, 1989). Pinacidil ($10 \mu\text{M}$) inhibited the contraction induced by $0.3 \mu\text{M Ca}^{2+}$ with $3 \mu\text{M GTP}\gamma\text{S}$ more strongly than it did the control (Figure 6b).

A phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA), activates protein kinase C and enhances the Ca^{2+} -induced contraction in skinned smooth muscle strips (Itoh *et al.*, 1986b; Fujiwara *et al.*, 1988). As shown in Figure 7, TPA ($0.1 \mu\text{M}$) enhanced the contraction induced by $0.3 \mu\text{M Ca}^{2+}$ by a factor of 3.74, the enhanced contraction being 0.83 ± 0.02 times the contraction induced by $10 \mu\text{M Ca}^{2+}$ ($n = 4$). Pinacidil ($10 \mu\text{M}$) inhibited the contraction induced by $0.3 \mu\text{M Ca}^{2+}$ with $0.1 \mu\text{M TPA}$ more strongly than that induced by $10 \mu\text{M Ca}^{2+}$. Thus, pinacidil reduced the former to 0.46 ± 0.10 times control and the latter to 0.73 ± 0.09 times control ($n = 3$, $P < 0.05$, Figure 7b).

Discussion

Pinacidil hyperpolarizes the smooth muscle membrane through activation of the K^+ channel and then causes vascular relaxation (Southerton *et al.*, 1988; Standen *et al.*, 1989). Glibenclamide is a selective inhibitor of this channel in vascular smooth muscles (Standen *et al.*, 1989). In the present experiments, however, we observed that pinacidil ($10 \mu\text{M}$)

Figure 7 (a) Effect of pinacidil on the contraction induced by $0.3 \mu\text{M Ca}^{2+}$ in the presence of $0.1 \mu\text{M}$ 12-O-tetradecanoylphorbol-13-acetate (TPA): (i) contraction induced by $10 \mu\text{M Ca}^{2+}$; (ii) effects of $10 \mu\text{M}$ pinacidil on the contraction induced by $0.3 \mu\text{M Ca}^{2+}$ with $0.1 \mu\text{M TPA}$; (i) and (ii) were contiguous records. Pinacidil ($10 \mu\text{M}$) was applied during the steady level of the Ca^{2+} -induced contraction in the presence of $0.1 \mu\text{M TPA}$. (b) Effects of pinacidil on the contraction induced by $0.3 \mu\text{M Ca}^{2+}$ with TPA or $10 \mu\text{M Ca}^{2+}$. The amplitude of contraction was normalized relative to the contraction induced by $10 \mu\text{M Ca}^{2+}$ in the absence of $10 \mu\text{M}$ pinacidil. Results shown are means of 3–4 observations with s.d. shown by vertical bars. * indicates significant difference from the results obtained in the absence of $10 \mu\text{M}$ pinacidil ($P < 0.05$). † indicates significant difference from the results obtained in the presence of $10 \mu\text{M Ca}^{2+}$ with $10 \mu\text{M}$ pinacidil.

inhibited the contraction induced by 128 mM K^+ without any change in $[\text{Ca}^{2+}]_i$. Under these conditions, the membrane hyperpolarizing effect of pinacidil may be negligible because the experiments were done with high concentrations of extracellular K^+ (Nakashima *et al.*, 1990) and glibenclamide did not affect the inhibitory action of pinacidil. The extent of the contraction induced by high K^+ is thought to be closely related to the $[\text{Ca}^{2+}]_i$ (Morgan & Morgan, 1984), yet $10 \mu\text{M}$ pinacidil inhibited the contraction without any decrease in $[\text{Ca}^{2+}]_i$. These results suggest that pinacidil, at higher concentrations, may have a direct inhibitory action on the contractile apparatus in smooth muscle.

In smooth muscle of the rabbit mesenteric artery, myosin phosphorylation has a crucial role in the Ca^{2+} -induced contraction (Kamm & Stull, 1985; Satoh *et al.*, 1987; Fujiwara *et al.*, 1988). In our experiments, pinacidil shifted the pCa-tension relation curve to the right and inhibited the maximum Ca^{2+} -induced contraction in β -escin-treated, skinned smooth muscle. Neomycin, an inhibitor of phospholipase C in skinned smooth muscle (Kitazawa *et al.*, 1989), did not modify this inhibitory action of pinacidil on the pCa-tension relationship. Pinacidil ($10 \mu\text{M}$) inhibited the contraction induced by $10 \mu\text{M Ca}^{2+}$ in the presence or absence of $2 \mu\text{M}$ calmodulin and the extent of the inhibition was similar in the two conditions.

References

- BECKER, P.L., SINGER, J.J., WALSH, J.V. & FAY, F.S. (1989). Regulation of calcium concentration in voltage-clamped smooth muscle cells. *Science*, **244**, 211–214.
- CASSIDY, P.S., HOAR, P.E. & KERRICK, W.G.L. (1979). Irreversible thiophosphorylation and activation of tension in functionally skinned rabbit ileum strips by (^{35}S)ATP γS . *J. Biol. Chem.*, **254**, 11148–11153.
- COCKCROFT, S. & STUTCHFIELD, J. (1988). G-proteins, the inositol lipid signalling pathway, and secretion. *Phil. Trans. R. Soc.*, **B320**, 247–265.
- FUJIWARA, T., ITOH, T., KUBOTA, Y. & KURIYAMA, H. (1988). Actions of a phorbol ester on factors regulating contraction in rabbit mesenteric artery. *Circ. Res.*, **63**, 893–902.
- FUJIWARA, T., ITOH, T., KUBOTA, Y. & KURIYAMA, H. (1989). Effects of guanosine nucleotides on skinned smooth muscle tissue of the rabbit mesenteric artery. *J. Physiol.*, **408**, 535–547.
- GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260**, 3440–3450.
- ITO, T., KANMURA, Y. & KURIYAMA, H. (1986a). Inorganic phosphate regulates the contraction-relaxation cycle in skinned muscles of the rabbit mesenteric artery. *J. Physiol.*, **376**, 231–252.
- ITO, T., KANMURA, Y., KURIYAMA, H. & SUMIMOTO, K. (1986b). A phorbol ester has dual actions on the mechanical response in the rabbit mesenteric and porcine coronary arteries. *J. Physiol.*, **375**, 515–534.
- However, pinacidil had no effect on the contraction induced by the Ca^{2+} -free, 4 mM MgATP -containing solution following application of ATP γS with Ca^{2+} (Ca^{2+} -independent contraction). This Ca^{2+} -independent contraction is due to the irreversible thio-phosphorylation of myosin light chain by myosin light chain kinase in the presence of ATP γS with Ca^{2+} , because the thio-phosphorylated myosin light chain is resistant to phosphatase (Cassidy *et al.*, 1979). Furthermore, pinacidil ($10 \mu\text{M}$) also had no effect on the rigor contraction induced by application of the solution containing no ATP. These results suggest that in concentrations over $10 \mu\text{M}$, pinacidil probably acts on the process between Ca^{2+} -calmodulin complex formation and myosin light chain phosphorylation and thus inhibits the contraction of the smooth muscle.
- NA with GTP or GTP γS enhanced the contraction induced by $0.3 \mu\text{M Ca}^{2+}$ and this was inhibited by guanosine 5'-O-(β -thiodiphosphate) (GDP βS) in α -toxin-treated, skinned smooth muscle (Fujiwara *et al.*, 1989; Kitazawa *et al.*, 1989). These workers suggested that an action of NA with GTP on the contractile proteins of smooth muscle may underly the agonist-induced tonic contraction at low $[\text{Ca}^{2+}]_i$ levels. Recently, Nishimura & van Breemen (1988) reported that 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H-7), an inhibitor of protein kinase C, inhibits the enhancement of the Ca^{2+} -induced contraction induced by NA with GTP in skinned muscle. These results suggest that GTP-binding protein activated by NA may be implicated in the increase in the Ca^{2+} -induced contraction via activation of protein kinase C. In the present experiment, pinacidil ($10 \mu\text{M}$) inhibited the Ca^{2+} -induced contraction more strongly in the presence of NA with GTP or TPA than under control conditions. These results suggest that pinacidil inhibits the action of protein kinase C, though the mechanism is unknown.
- In the present experiments, $10 \mu\text{M}$ pinacidil inhibited the tonic contraction induced by 128 mM K^+ more than the phasic ones. Raising $[\text{Ca}^{2+}]_i$ induced by 128 mM K^+ may provoke the hydrolysis of phosphatidylinositol-4,5-bisphosphate and lead to the production of inositol-1,4,5-trisphosphate and diacylglycerol (DG), as reported in non-muscle cells (Cockcroft & Stutchfield, 1988). DG or its analogue slowly enhances Ca^{2+} -induced contraction in skinned smooth muscles through an activation of protein kinase C (Nishizuka, 1984; Itoh *et al.*, 1986b). The inhibition of protein kinase C (described above) by pinacidil may relate to the much stronger inhibition of the tonic contraction induced by 128 mM K^+ than the phasic ones.
- We conclude that at high concentrations ($> 10 \mu\text{M}$), pinacidil acts directly on the contractile proteins in smooth muscle of the rabbit mesenteric artery and inhibits the Ca^{2+} -induced contraction in high K^+ conditions in intact as well as in skinned muscle strips. The sites of action of pinacidil have not been identified, but a modification of myosin phosphorylation after Ca^{2+} -calmodulin complex formation is postulated.

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- ITOH, T., KURIYAMA, H. & SUZUKI, H. (1983). Differences and similarities in noradrenaline- and caffeine-induced mechanical responses in the rabbit mesenteric artery. *J. Physiol.*, **337**, 609–629.
- KAMM, K.E. & STULL, J.T. (1985). The function of myosin and myosin light chain kinase phosphorylation in smooth muscle. *Annu. Rev. Pharmacol. Toxicol.*, **25**, 593–620.
- KITAZAWA, T., KOBAYASHI, S., HORIUTI, K., SOMLYO, A.V. & SOMLYO, A.P. (1989). Receptor-coupled, permeabilized smooth muscle. *J. Biol. Chem.*, **264**, 5339–5342.
- KOBAYASHI, S., KITAZAWA, T., SOMLYO, A.V. & SOMLYO, A.P. (1989). Cytosolic heparin inhibits muscarinic and α -adrenergic Ca^{2+} release in smooth muscle. *J. Biol. Chem.*, **264**, 17997–18004.
- KONISHI, M., OLSON, A., HOLLINGWORTH, S. & BAYLOR, S.M. (1988). Myoplasmic binding of fura-2 investigated by steady state fluorescence and absorbance measurements. *Biophys. J.*, **54**, 1089–1104.
- MORGAN, J.P. & MORGAN, K.G. (1984). Stimulus-specific patterns of intracellular calcium levels in smooth muscle of ferret portal vein. *J. Physiol.*, **351**, 155–167.
- NAKASHIMA, M., LI, Y., SEKI, N. & KURIYAMA, H. (1990). Pinacidil inhibits neuromuscular transmission indirectly in the guinea-pig and rabbit mesenteric arteries. *Br. J. Pharmacol.*, **101**, 581–586.
- NISHIZUKA, Y. (1984). The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature*, **308**, 693–698.
- NISHIMURA, J. & VAN BREEMEN, C. (1989). Direct regulation of smooth muscle contractile elements. *Biochem. Biophys. Res. Commun.*, **163**, 929–935.
- POENIE, M., ALDERTON, J., STEINHART, R.A. & TSIEN, R.Y. (1986). Calcium rises abruptly and briefly throughout the cell at the onset of anaphase. *Science*, **233**, 886–889.
- QUAST, U. & COOK, N.S. (1989). Moving together: K^+ channel openers and ATP-sensitive K^+ channels. *Trends Pharmacol. Sci.*, **10**, 431–435.
- SATOH, S., KUBOTA, Y., ITOH, T. & KURIYAMA, H. (1987). Mechanisms of the Ba^{2+} -induced contraction in smooth muscle cells of the rabbit mesenteric artery. *J. Gen. Physiol.*, **89**, 215–237.
- SOUTHERTON, J.S., WESTON, A.H., BRAY, K.M., NEWGREEN, D.T. & TAYLOR, S.G. (1988). The potassium channel opening action of pinacidil; studies using biochemical, ion flux and microelectrode techniques. *Naunyn-Schmiedeberg Arch. Pharmacol.*, **338**, 310–318.
- STANDEN, N.B., QUAYLE, J.M., DAVIES, N.W., BRAYDEN, J.E., HUANG, Y. & NELSON, M.T. (1989). Hyperpolarizing vasodilators activate ATP-sensitive K^+ channels in arterial smooth muscle. *Science*, **245**, 177–180.
- VIDEBAEK, L.M., AALKJAERM, C. & MULVANY, M.J. (1988). Pinacidil opens K^+ -selective channels causing hyperpolarization and relaxation of noradrenaline contractions in rat mesenteric resistance vessels. *Br. J. Pharmacol.*, **95**, 103–108.

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