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Effects of lemakalim on changes in Ca^{2+} concentration and mechanical activity induced by noradrenaline in the rabbit mesenteric artery

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1 Effects of (−)-cromakalim (lemakalim) on tension and Ca^{2+} mobilization induced by noradrenaline (NA) were investigated by measuring intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), isometric tension and production of inositol-1,4,5-trisphosphate (IP_3) in smooth muscle strips of the rabbit mesenteric artery.

2 In thin smooth muscle strips, 10 μM NA produced a large phasic, followed by a small tonic increase in $[Ca^{2+}]_i$, which correlated well with the evoked phasic and tonic contractions, respectively. Lemakalim (0.1–10 μM) lowered the resting $[Ca^{2+}]_i$ without a decrease in the resting tension, and also inhibited the increased $[Ca^{2+}]_i$ and tension induced by 10 μM NA, all in a concentration-dependent manner. Glibenclamide (1 μM) inhibited these actions of lemakalim.

3 In Ca^{2+}-free solution containing 2 mM EGTA, NA (10 μM) transiently increased $[Ca^{2+}]_i$, tension and synthesis of IP_3. Lemakalim (over 0.01 μM) inhibited these actions of NA in Ca^{2+}-free solution containing 5.9 mM K^+, but not in Ca^{2+}-free solution containing 128 mM K^+. These actions of lemakalim were prevented by glibenclamide (1 μM). Lemakalim (1 μM) did not modify the increases in $[Ca^{2+}]_i$ and tension induced by 10 mM caffeine.

4 In β-escin-skinned strips, 10 μM NA increased $[Ca^{2+}]_i$ in Ca^{2+}-free solution containing 50 μM EGTA, 3 μM guanosine triphosphate (GTP) and 2 μM Fura 2 after the storage sites were loaded by application of 0.3 μM Ca^{2+} for 2 min, suggesting that Ca^{2+} is released from intracellular storage sites following activation of the α-adrenoceptor. Lemakalim (1 μM) did not inhibit the Ca^{2+} release from storage sites induced by NA.

5 We conclude that lemakalim inhibits NA-induced Ca^{2+} release due to inhibition of NA-induced IP_3 production in a manner dependent on the membrane potential and causes inhibition of the phasic contraction induced by NA.

Keywords: Cromakalim; noradrenaline; inositol-1,4,5-trisphosphate; cellular Ca^{2+} concentration; vascular smooth muscle

Introduction

Cromakalim ((±)-6-cyano-3,4-dihydro-2,2-dimethyl-*trans*-4-(2-oxo-1-pyrrolidyl)-2H-benzo-[b]pyran-3-ol) represents a new class of vasodilator which hyperpolarizes the membrane by opening K^+ channels in vascular smooth muscle cells (for reviews, see Hamilton & Weston, 1989; Quast & Cook, 1989). The effects of cromakalim are stereospecific and confined to the (−)-enantiomer (lemakalim) (Buckingham *et al.*, 1986; Hof *et al.*, 1988). It has been suggested that cromakalim inhibits the activation of the voltage-dependent Ca^{2+} channel as a result of its hyperpolarization of the membrane (Hamilton *et al.*, 1986; Nakao *et al.*, 1988; Standen *et al.*, 1989). In addition, it has also been reported that this agent inhibits refilling of the depleted store sites, as suggested by its reduction of agonist-induced $^{45}Ca^{2+}$ influx (Chiu *et al.*, 1988), possibly through its membrane hyperpolarizing action. These effects may account for the cromakalin-induced inhibition of the tonic phase of contraction that is induced by NA in some vascular smooth muscle tissues and which results from Ca^{2+}-influx (Hamilton & Weston, 1989; Quast & Cook, 1989).

In the rabbit mesenteric artery, noradrenaline (NA) produces a large phasic and a subsequent small tonic contraction, the phasic contraction being mainly due to NA-induced Ca^{2+} release from intracellular storage sites (Itoh *et al.*, 1983). NA causes production of iositol-1,4,5-trisphosphate (IP_3) through hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2), and the IP_3 releases Ca^{2+} from its storage sites in the rabbit mesenteric artery (Hashimoto *et al.*, 1986). Thus, NA may generate its phasic contraction in the smooth muscle of the rabbit mesenteric artery via the action of IP_3. However, the mechanism underlying the cromakalim-induced inhibition of the phasic contraction induced by NA, which may lead to vasodilatation, has not been clearly identified.

In the present study, we have attempted to clarify this mechanism. For this purpose, we used lemakalim ((−)-cromakalim) instead of (±)-cromakalim, and studied its effects on the NA-induced phasic increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and tension in Ca^{2+}-containing, or Ca^{2+}-free solution under membrane-polarized and -depolarized conditions. In addition, we have studied the action of this drug on NA-induced Ca^{2+} release in β-escin-skinned smooth muscle strips.

Methods

Male albino rabbits, weighing 1.9–2.5 kg, were anaesthetized with pentobarbitone sodium (40 mg kg^{-1}, i.v.), and then exsanguinated. The third branch of the mesenteric artery was excised immediately and cleaned by removal of connective tissue in Krebs solution at room temperature.

Ca^{2+} and tension measurement

To enable recording of $[Ca^{2+}]_i$ and isometric tension simultaneously, fine circularly-cut strips (0.3–0.5 mm length, 0.04–0.05 mm width, 0.02–0.03 mm thickness) were prepared as previously described (Itoh *et al.*, 1983). Endothelial cells were removed by gentle rubbing of the internal surface of the vessels with small knives. The absence of endothelial cells was confirmed by the inability of acetylcholine (1 μM) to cause relaxation during contractions induced by NA, as described previously (Nishiye *et al.*, 1989). The strip was transferred into a chamber of 0.3 ml volume and mounted horizontally on a

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invert-microscope (Diaphoto TMD with special optics for epi-fluorescence, Nikon). The resting tension was adjusted to obtain a maximal contraction in 128 mM K^+.

To enable loading of Fura 2 into smooth muscle cells of the strip, 1 μM acetoxy methyl ester of Fura 2 (Fura 2AM) dissolved in dry dimethyl sulphoxide (1 mM stock solution) was applied for 1 h in Krebs solution at room temperature (20–23°C). The position of the strip was adjusted to the centre of the field with a mask placed in an intermediate image plane to reduce fluorescence from the background (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 passed through the lens (20 times 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, N.J., U.S.A.) and the data analysed with 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. Background fluorescence (including the autofluorescence of the strip) as excited by 340 and 380 nm u.v.-light was measured following application of a solution containing 50 μM ionomycin, 20 mM $MnCl_2$, 110 mM KCl and 10 mM 3-(N-morpholino) propanesulphonic acid (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 with the formula described by Grynkiewicz *et al.* (1985) and using *in vitro* calibration (Poenie *et al.*, 1986; Becker *et al.*, 1989). The ratio of maximum (F_{max}) to minimum fluorescence (F_{min}) was determined in the calibration solution after subtraction of background excited by either 340 or 380 nm and the 380 signals of Fura 2 were assumed to decrease by 15% in the cell due to the possible intracellular viscosity effects of the dye (Becker et al., 1989). The K_d value for Fura 2 was estimated to be 200 nM (Becker *et al.*, 1989).

We also tried, unsuccessfully, to estimate $[Ca^{2+}]_i$ in some strips by the internal calibration method described by Himpens *et al.* (1989). However, since the value of F_{max} was not consistently bigger than that of 128 mM K^+ in Krebs solution even in very thin strips, we could not use this method in the present experiments.

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 make skinned muscles and the compositions of the solution have been described elsewhere (Itoh *et al.*, 1986; Kobayashi *et al.*, 1989). To enable measurement of Ca^{2+} release from the store sites, 0.3 μM Ca^{2+} buffered with 4 mM EGTA was applied for 2 min (to load Ca^{2+} into the store sites) and Ca^{2+} removed from the solution by application of Ca^{2+}-free solution containing 4 mM EGTA for 0.5 min. Then, a solution containing 50 μM EGTA, 3 μM GTP and 2 μM Fura 2 was applied for 2 min. Finally, 10 μM NA with 3 μM GTP was applied for 2 min in a solution containing 50 μM EGTA and 2 μM Fura 2.

Measurement of inositol-1,4,5-trisphospate

Endothelium-denuded strips were equilibrated for over 2 h at 32°C in Krebs solution. After this, the strips were transferred to Ca^{2+}-free Krebs solution containing 2 mM EGTA for 2 min and then 10 μM NA was applied for various times. Lemakalim or glibenclamide was given as pretreatment for 3 min in Krebs solution, for 2 min in Ca^{2+}-free solution and during application of NA. The reaction was stopped by addition of a large amount of ice-cold trichloroacetic acid (final concentration 8%) and the strips were homogenized. The homogenate was centrifuged and the supernatant fraction treated with ether three times and assayed with a radioimmunoassay kit from Amersham International plc. Care was taken to maintain the pH of the homogenate at 9.0–9.5 to optimize the binding properties of the binding protein to IP_3. To minimize the loss of IP_3, teflon tubes were used instead of glassware after homogenization.

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 replacing NaCl with KCl, isosmotically. To prevent both NA outflow from sympathetic nerve terminals and β-adrenoceptor stimulation by exogenously-applied NA, 3 μM guanethidine and 0.3 μM propranolol were added to the Krebs solution throughout the experiment. 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 O_2 and 5% CO_2, and their pH maintained at 7.3–7.4.

The calibration solution for Ca^{2+} measurement in intact strips 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 muscle, the composition of the relaxing solution was: 87 mM potassium methanesulphonate (KMS), 20 mM piperazine-N-N'-*bis*-(2-ethanesulphonic acid) (PIPES), 5.1 mM $Mg(MS)_2$, 5.2 mM ATP, 10 mM phosphocreatine and 4 mM ethyleneglycol-*bis*-(β-aminoethyl)-N,N,N'N'-tetraacetic acid (EGTA). To enable measurement of Ca^{2+} release from skinned strips, the concentration of EGTA was reduced to 50 μM and 2 μM Fura 2 added. Various Ca^{2+} concentrations were prepared by adding appropriate amounts of $Ca(MS)_2$ to 4 mM EGTA, based on the calculation reported 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 2AM, EGTA, PIPES, HEPES and MOPS (Dojin, Japan), NA, IP_3, GTP, β-escin and glibenclamide (Sigma), guanethidine (Tokyo Kasei, Japan), ATP (Na salt; Kojin, Japan), propranolol (Nacalai, Japan), A23187 and ionomycin (free acid; Calbiochem), and lemakalim (Beecham).

Statistics

The values recorded were expressed as mean ± s.d., and statistical significance determined by a paired or unpaired Student's *t* test. Probabilities less then 5% ($P < 0.05$) were considered significant.

Results

Effects of lemakalim on changes in $[Ca^{2+}]_i$ *and tension induced by noradrenaline in* Ca^{2+}*-containing solution*

Figure 1 shows traces of the effects of lemakalim on $[Ca^{2+}]_i$ and tension induced by 10 μM NA in a thin smooth muscle strip of the rabbit mesenteric artery. NA was applied for 2 min at 30 min intervals to get reproducible responses and then lemakalim applied for 5 min before, and during subsequent applications of NA. NA (10 μM) produced a transient phasic, followed by a small tonic increase in $[Ca^{2+}]_i$ and in tension. The resting $[Ca^{2+}]_i$ and tension were 151 ± 21 nM and 1.8 ± 0.5 mg, respectively ($n = 8$). Changes in $[Ca^{2+}]_i$ always preceded tension development. Lemakalim (0.01–10 μM) lowered the resting $[Ca^{2+}]_i$ and inhibited the increases in

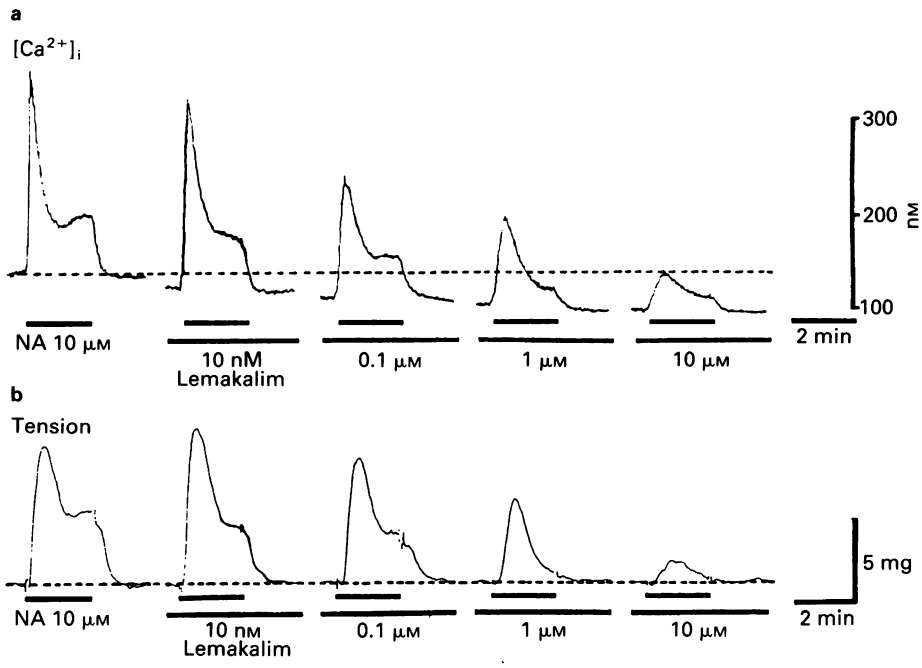

Figure 1 Effects of lemakalim on changes in $[Ca^{2+}]_i$ (a) and tension (b) induced by 10 μM noradrenaline (NA) in a smooth muscle strip of rabbit mesenteric artery. Broken lines indicate resting $[Ca^{2+}]_i$ (a) and tension (b) levels under control conditions. NA (10 μM) was applied for 2 min at 30 min intervals in Krebs solution. Guanethidine (3 μM) and propranolol (0.3 μM) were present throughout the experiment. Lemakalim was given as pretreatment for 5 min and was present during the application of NA. The results illustrated were obtained from a single smooth muscle strip and were reproducible in another 3 strips.

$[Ca^{2+}]_i$ (Figure 1a) and tension (Figure 1b) induced by 10 μM NA, in a concentration-dependent manner. Figure 2 summarizes the effects of lemakalim on the resting $[Ca^{2+}]_i$ and the increase in $[Ca^{2+}]_i$ induced by 10 μM NA ($n = 3$–5). NA (10 μM) increased the $[Ca^{2+}]_i$ to 436 ± 63 nM in the muscle strips ($n = 8$). Lemakalim (0.1–10 μM) lowered the resting $[Ca^{2+}]_i$ and inhibited the evoked increase in $[Ca^{2+}]_i$, both in a concentration-dependent manner (Figure 2). Glibenclamide

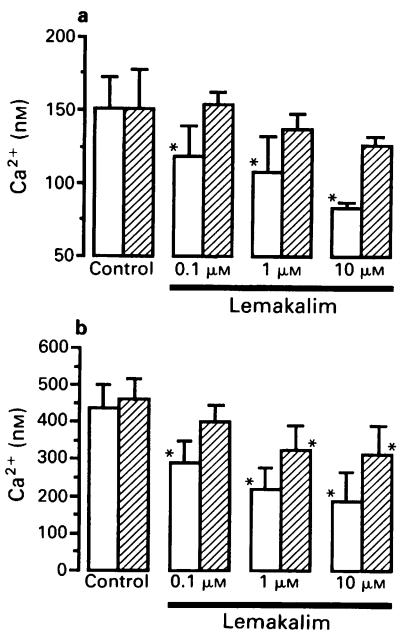

Figure 2 Effects of lemakalim (a) on resting $[Ca^{2+}]_i$ and (b) on active $[Ca^{2+}]_i$ induced by 10 μM noradrenaline (NA) in the presence or absence of 1 μM glibenclamide in smooth muscle strips of rabbit mesenteric artery. The experimental protocol was similar to that described in Figure 1. Glibenclamide (1 μM) was applied for 10 min before, and throughout application of lemakalim. Open columns: control, hatched colums: in the presence of 1 μM glibenclamide. Each column represents mean ($n = 3$–5) with s.d. shown by vertical bars. * Represents statistically significant difference from the control.

(1 μM) itself did not modify resting $[Ca^{2+}]_i$ or the increase induced by 10 μM NA but it did inhibit the effects of 1 μM lemakalim. Figure 3 shows records of the inhibitory effects of 1 μM glibenclamide on the lemakalim-induced inhibition of both the $[Ca^{2+}]_i$ (Figure 3a(i)) and tension (Figure 3b(i)) induced by 10 μM NA in the same smooth muscle strip. The effects of glibenclamide in concentrations over 1 μM were not examined because the vehicle (dimethyl sulphoxide) used to dissolve glibenclamide itself inhibited the NA-contraction (final concentration of vehicle was 0.001%).

Effects of lemakalim on changes in $[Ca^{2+}]_i$ and tension induced by noradrenaline in Ca^{2+}-free solution

In smooth muscle strips of the rabbit mesenteric artery, the phasic contraction induced by NA persists in Ca^{2+}-free solution (Figure 3b(ii)), and this has been supposed to be due to release of Ca^{2+} from storage sites (Itoh *et al.*, 1983). To study the effects of lemakalim on NA-induced Ca^{2+} release, its effects on the increase in $[Ca^{2+}]_i$ and tension induced by 10 μM NA were studied in Ca^{2+}-free solution containing 2 mM EGTA (see Figure 4). At 30 min intervals, NA (10 μM) was applied for 2 min in Ca^{2+}-free solution containing 2 mM EGTA after a 2 min period of Ca^{2+} removal, the strips being kept in Krebs solution (containing 2.6 mM Ca^{2+}) for the 25 min between tests. In Ca^{2+}-free solution containing 2 mM EGTA, the increase in $[Ca^{2+}]_i$ and tension induced by 128 mM K^+ was completely abolished within 15 s. Following application of Ca^{2+}-free solution containing 2 mM EGTA, the resting $[Ca^{2+}]_i$ rapidly decreased from 151 ± 21 to 86 ± 16 nM ($n = 4$) within 1 min and then remained at a new steady level, but the resting tension was not changed significantly (1.6 ± 0.4 to 1.4 ± 0.3 mg, $n = 4$). NA (10 μM) transiently increased $[Ca^{2+}]_i$ (to 234 ± 42 nM, $n = 4$) and tension (to 6.5 ± 1.2 mg, $n = 4$) in Ca^{2+}-free solution. Lemakalim (over 0.1 μM) lowered the resting $[Ca^{2+}]_i$ and inhibited this evoked increase in $[Ca^{2+}]_i$ and tension, in a concentration-dependent manner (Figure 4 and Figure 5). The resting $[Ca^{2+}]_i$ was 81 ± 12 nM in the presence of 0.1 μM lemakalim and 75 ± 11 nM in 1 μM lemakalim ($n = 4$), and these were both significantly different from control ($P < 0.05$ paired t test). However, the resting tension remained unchanged. The

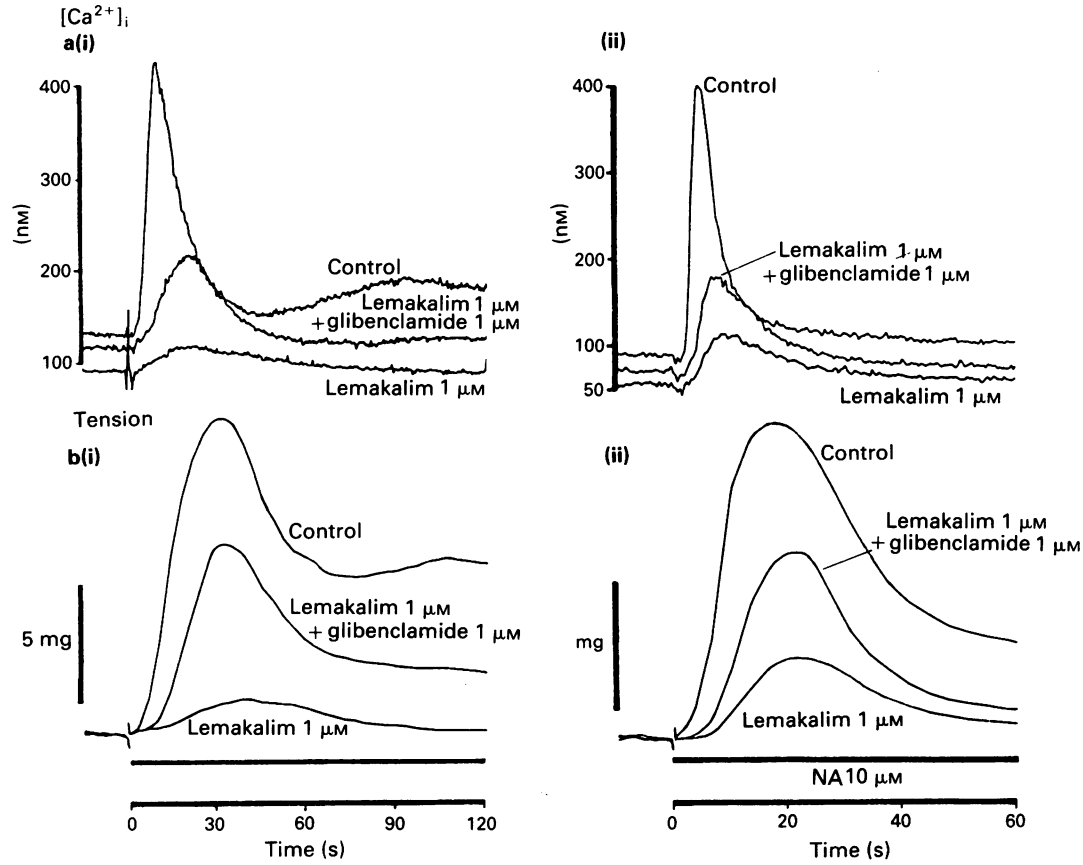

Figure 3 Effects of 1 μM lemakalim, with or without 1 μM glibenclamide, on changes in $[Ca^{2+}]_i$ (a) and tension (b) induced by 10 μM noradrenaline (NA) in Ca^{2+} containing (a(i) and b(i)) and Ca^{2+}-free solution containing 2 mM EGTA (a(ii) and b(ii)) in a smooth muscle strip of rabbit mesenteric artery. NA (10 μM) was applied for 2 min at 30 min intervals in Krebs solution. Guanethidine (3 μM) and propranolol (0.3 μM) were present throughout the experiment. Lemakalin (1 μM) was given as pretreatment for 5 min and was present during application of 10 μM NA. Glibenclamide (1 μM) was applied for 10 min before and throughout application of lemakalim. The results shown were obtained from a single smooth muscle strip and were reproducible in another 3 strips.

maximum $[Ca^{2+}]_i$ induced by 10 μM NA in Ca^{2+}-free solution was 197 $\pm$ 35 nM or 153 $\pm$ 33 nM in the presence of 0.1 μM or 1 μM lemakalim, respectively (Figure 5, $n = 4$; $P < 0.05$, paired t test). The tension induced by 10 μM NA in Ca^{2+}-free solution was inhibited to 81% of control by 0.1 μM lemakalim and to 47% of control by 1 μM lemakalim ($n = 4$). Glibenclamide (1 μM) significantly inhibited the effects of 1 μM lemakalim both on the resting $[Ca^{2+}]_i$, and on the changes in $[Ca^{2+}]_i$ and tension induced by 10 μM NA (Figure 3a(ii) and 3b(ii)). In Ca^{2+}-free solution containing 2 mM EGTA, the

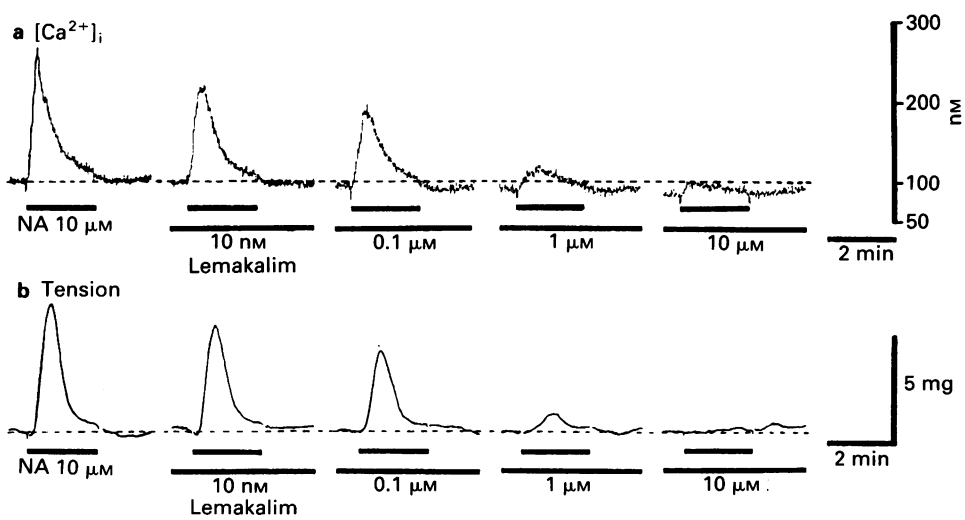

Figure 4 Effects of lemakalim on changes in $[Ca^{2+}]_i$ (a) and tension (b) induced by 10 μM noradrenaline (NA) in Ca^{2+}-free solution containing 2 mM EGTA in a smooth muscle strip of rabbit mesenteric artery. NA (10 μM) was applied for 2 min after a 2 min period of Ca^{2+} removal, then Ca^{2+}-free solution was applied for 1 min to wash out NA. This protocol was repeated at 30 min intervals with the strips being kept in Krebs solution (containing 2.6 mM Ca^{2+}) for the 25 min between tests. Lemakalim was applied for 5 min before and throughout application of NA. Guanethidine (3 μM) and propranolol (0.3 μM) were present throughout the experiment. Broken lines indicate resting $[Ca^{2+}]_i$ (a) and tension (b) level in Ca^{2+}-free solution in the absence of lemakalim. The results illustrated were obtained from a single smooth muscle strip and were reproducible in another 3 strips. N.B. the resting $[Ca^{2+}]_i$ before application of NA was lower in the presence of lemakalim than in control.

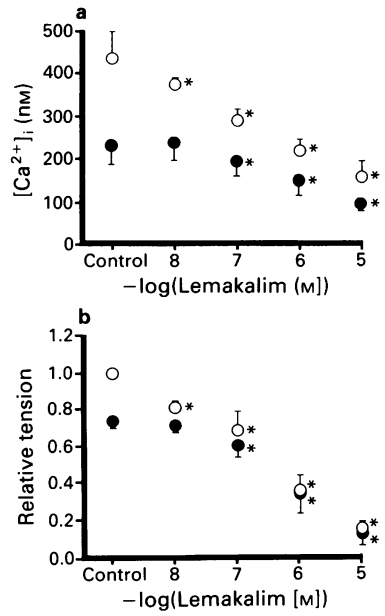

Figure 5 Concentration-dependent effects of lemakalim on increase in $[Ca^{2+}]_i$ (a) and tension (b) induced by 10 μM noradrenaline (NA) in Ca^{2+}-containing (○) or Ca^{2+}-free solution containing 2 mM EGTA (●) in smooth muscle strips of rabbit mesenteric artery. The experimental protocol was similar to that described in Figures 1 and 3. In (b), the maximum amplitude of contraction induced by 10 μM NA in Ca^{2+}-containing solution was normalized as 1.0 for each muscle strip. Each symbol represents mean ($n = 4$) with s.d. shown by vertical bars. * represents statistically significant difference from the value obtained in the absence of lemakalim in either Ca^{2+}-containing or Ca^{2+}-free solution (control).

resting $[Ca^{2+}]_i$ in the presence of 1 μM glibenclamide was 88 ± 12 nM, 88 + 13 nM in 0.1 μM lemakalim with 1 μM glibenclamide and 83 ± 13 nM in 1 μM lemakalim with 1 μM glibenclamide ($n = 3$; $P > 0.05$ when any of these is compared with the resting $[Ca^{2+}]_i$ in the absence of glibenclamide and lemakalim). The maximum $[Ca^{2+}]_i$ induced by 10 μM NA in the presence of 1 μM glibenclamide was 234 ± 26 nM, 201 ± 37 nM in 0.1 μM lemakalim with 1 μM glibenclamide and 169 ± 39 nM in 1 μM lemakalim with 1 μM glibenclamide. These values were not significantly different from the maximum $[Ca^{2+}]_i$ induced by 10 μM NA in the absence of glibenclamide and lemakalim (Figure 5, $n = 3$; $P > 0.05$, paired t test).

Figure 6 shows the effects of 1 μM lemakalim on the increase in $[Ca^{2+}]_i$ and tension induced by 10 μM NA in Ca^{2+}-free solution containing 128 mM K^+. Lemakalim (1 μM) marginally inhibited the maximum increase in $[Ca^{2+}]_i$ and tension induced by 10 μM NA and slightly accelerated decay of both responses. Glibenclamide (1 μM) had no effect on this inhibition.

Lemakalim (0.01–1 μM) had no effect on the increase in $[Ca^{2+}]_i$ and tension induced by 10 mM caffeine in Ca^{2+}-free solution (not shown).

Effects of lemakalim on inositol-1,4,5-trisphosphate production induced by noradrenaline

It has been shown that IP_3 releases Ca^{2+} from intracellular storage sites as a second messenger in smooth muscle cells of the rabbit mesenteric artery (Hashimoto *et al.*, 1986). To investigate further the mechanisms of the inhibitory effects induced by lemakalim during the NA-induced contraction, the effects of lemakalim (0.1 and 1 μM) on the synthesis of IP_3 induced by 10 μM NA were observed in Ca^{2+}-free solution containing 2 mM EGTA. NA transiently increased the amount of IP_3 within 10 s and the effect gradually decayed. The effects of lemakalim (0.1 and 1 μM) on this response were measured 10 s after NA-application and it was found that it inhibited the

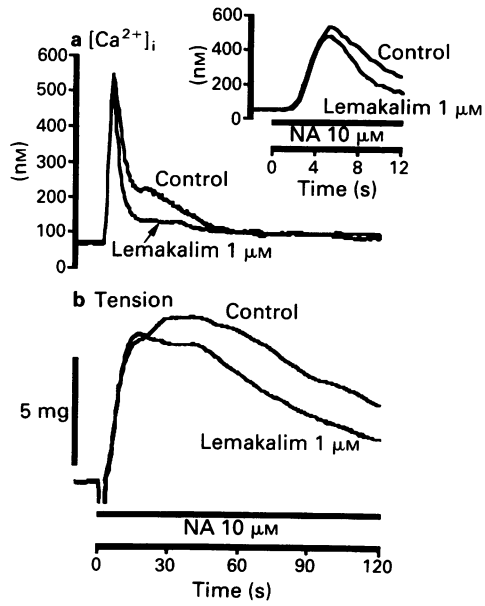

Figure 6 Effects of lemakalim on changes in $[Ca^{2+}]_i$ (a) and tension (b) induced by 10 μM noradrenaline (NA) in Ca^{2+}-free solution containing 2 mM EGTA and 128 mM K^+ in a smooth muscle strip of rabbit mesenteric artery. NA (10 μM) was applied for 2 min after a 2 min period of Ca^{2+} removal by Ca^{2+}-free solution containing 2 mM EGTA with 128 mM K^+, then the Ca^{2+}-free solution was applied for 1 min to wash out NA. This protocol was repeated at 30 min intervals with the strips in Krebs solution (containing 2.6 mM Ca^{2+} with 5.9 mM K^+) for the 25 min between tests. Lamakalim was applied for 5 min before and throughout application of NA. Guanethidine (3 μM) and propranolol (0.3 μM) were present throughout the experiment. In (a) the inset illustrates the initial changes in $[Ca^{2+}]_i$ on an expanded time scale. The results shown were obtained from a single smooth muscle strip and were reproducible in another 3 strips.

synthesis of IP_3 induced by 10 μM NA, in a concentration-dependent manner (Figure 7). Glibenclamide (1 μM) itself did not significantly modify the IP_3 production induced by 10 μM NA but it prevented the inhibitory actions of 0.1 μM lemakalim (Figure 7).

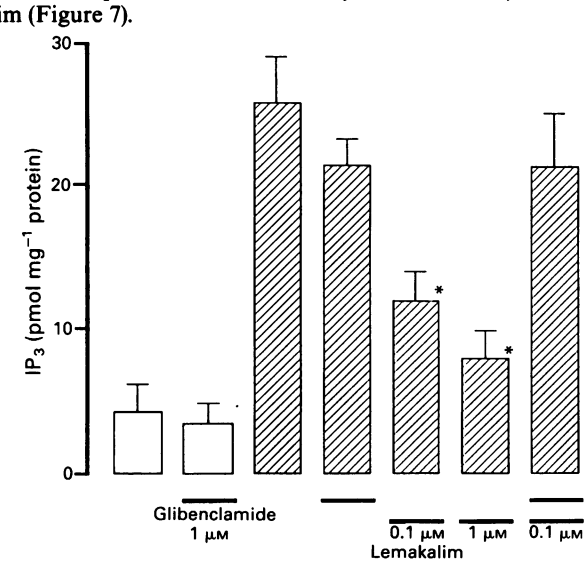

Figure 7 Effects of lemakalim on inositol-1,4,5-trisphosphate (IP_3)-synthesis induced by 10 μM noradrenaline (NA) in Ca^{2+}-free solution in smooth muscle strips of rabbit mesenteric artery. NA (10 μM) was applied for 10 s in Ca^{2+}-free solution containing 2 mM EGTA after a 2 min period of Ca^{2+} removal. Lemakalin (0.1 or 1 μM) was applied for 5 min before, and throughout application of NA. Glibenclamide (1 μM) was applied for 5 min before and throughout application of 10 μM NA or 0.1 μM lemakalim. Results represented are means of 4–8 observations with s.d. shown by vertical bars. * indicates statistically significant difference from response to NA alone ($P < 0.05$). Open columns: strips not stimulated by NA; hatched columns: NA-treated strips.

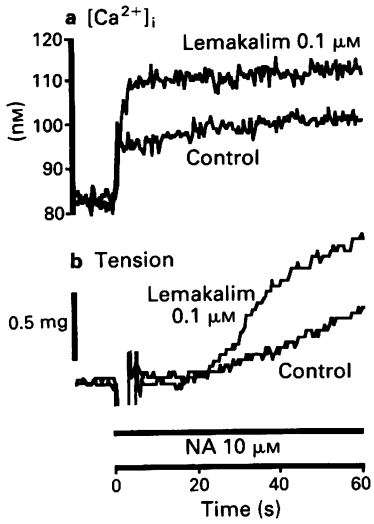

Figure 8 Effects of 0.1 μM lemakalim on increase in Ca^{2+} and tension induced by 10 μM noradrenaline (NA) in β-escin-skinned smooth muscle strips of rabbit mesenteric artery. After strips were skinned by application of 20 μM β-escin for 25 min, 0.3 μM Ca^{2+} buffered with 4 mM EGTA was applied for 2 min, and Ca^{2+}-free solution containing 4 mM EGTA then applied for 0.5 min to remove Ca^{2+} from the solution. Subsequently, 10 μM NA was applied for 2 min in Ca^{2+}-free solution containing 50 μM EGTA, 3 μM GTP and 2 μM Fura 2 following application of Ca^{2+}-free solution containing 50 μM EGTA, 3 μM GTP and 2 μM Fura 2 for 2 min. Lemakalim (0.1 μM) was applied for 2 min before, and throughout application of NA. These results were reproducible in another 2 strips.

Effects of lemakalim on noradrenaline-induced Ca^{2+}-release in chemically skinned smooth muscle strips

To investigate the site of action of lemakalim, the effects of 1 μM lemakalim on NA-induced Ca^{2+} release were observed in smooth muscle strips skinned by a 25 min application of β-escin (20 μM). NA increased $[Ca^{2+}]_i$ and tension in Ca^{2+}-free solution containing 50 μM EGTA. 3 μM GTP and 2 μM Fura 2 application of 0.3 μM Ca^{2+} for 2 min, possibly due to release of Ca^{2+} from its storage sites (Figure 8). Lemakalim (1 μM) did not inhibit, but rather enhanced this evoked increase in Ca^{2+} and tension (Figure 8).

Discussion

(±)-Cromakalim hyperpolarizes the membrane in vascular smooth muscle through activation of the K^+-channel and causes vascular relaxation (Hamilton *et al.*, 1986; Quast, 1987; Nakao *et al.*, 1988; Quast & Baumlin, 1988; Standen *et al.*, 1989). A sulphonylurea, glibenclamide, inhibits the membrane hyperpolarization induced by (±)-cromakalim in smooth muscle cells of the rabbit mesenteric artery (Standen *et al.*, 1989). The effects of cromakalim are stereospecific, the (−)-enantiomer (lemakalim) having a more potent vasodilator action than its racemic form (Buckingham *et al.*, 1986; Hof *et al.*, 1988). In the present experiments, we found that lemakalim lowered the resting $[Ca^{2+}]_i$ in both Ca^{2+}-containing and Ca^{2+}-free solutions and these actions were prevented by glibenclamide. In Ca^{2+}-free solution containing 2 mM EGTA, the extracellular Ca^{2+} has been calculated to be below 10 nM (Itoh *et al.*, 1986) and intracellular Ca^{2+} concentration estimated to be 50–90 nM. Under the above conditions, while influx of Ca^{2+} may be minimized, lemakalim still reduced the $[Ca^{2+}]_i$. Thus, the membrane hyperpolarization may in some way negatively control the resting $[Ca^{2+}]_i$.

In smooth muscle cells of the rabbit mesenteric artery, NA (10 μM) depolarized the membrane and induced oscillatory potential changes (Itoh *et al.*, 1983). NA produced a transient phasic increase in $[Ca^{2+}]_i$ and tension, possibly induced by release of Ca^{2+} from its storage sites, and a subsequent tonic increase which may be due to activation of Ca^{2+}-influx, as suggested by tension measurements in presence or absence of Ca^{2+} (Itoh *et al.*, 1983). Lemakalim (0.01–10 μM) inhibited the increase in $[Ca^{2+}]_i$ and tension induced by 10 μM NA in a concentration-dependent manner. No tonic increases in $[Ca^{2+}]_i$ or tension were induced by NA in Ca^{2+}-free solution containing EGTA. Lemakalim (10 μM) almost abolished the tonic increases in $[Ca^{2+}]_i$ and tension induced by 10 μM NA and glibenclamide prevented these inhibitory actions of lemakalim. These results suggest that lemakalim inhibits the increases in $[Ca^{2+}]_i$ and tension induced by NA through an inhibition of Ca^{2+}-influx.

It has been proposed that NA binds to the α-adrenoceptor and synthesizes IP_3 which releases Ca^{2+} from its store sites in smooth muscle of the rabbit mesenteric artery (Hashimoto *et al.*, 1986). Lemakalim inhibited the Ca^{2+} increase induced by NA, but not by caffeine, in intact smooth muscle strips in Ca^{2+}-free solution containing 5.9 mM K^+. This inhibitory action of lemakalim was prevented by glibenclamide and greatly diminished in Ca^{2+}-free solution containing 128 mM K^+. Lemakalim inhibited the synthesis of IP_3 induced by NA in a concentration-dependent manner and these actions were inhibited by glibenclamide. In Ca^{2+}-free solution containing 2 mM EGTA, nifedipine (a calcium antagonist) inhibited neither the contraction nor the hydrolysis of PIP_2 induced by NA (Kanmura *et al.*, 1983; Itoh *et al.*, 1987). Thus, the present results suggest that lemakalim selectively inhibits the NA-induced Ca^{2+} release, in a manner dependent on the membrane potential, through the inhibition of NA-induced IP_3 synthesis.

It has been reported that smooth muscle skinned with β-escin retains the α receptor-GTP binding protein-phospolipase C coupling mechanism (Kobayashi *et al.*, 1989). In the present experiments, we confirmed this with the finding that NA increased Ca^{2+} and tension in β-escin-treated skinned smooth muscle strips in the presence of GTP and a low concentration of EGTA. Lemakalim (1 μM) did not inhibit, but rather enhanced this response and this absence of an inhibitory action in β-escin skinned smooth muscle (which cannot generate a membrane potential) supports the notion that membrane hyperpolarization may play an essential role in the inhibition of NA-induced synthesis of IP_3 in smooth muscle cells.

In dog brain microsomes, K^+-channel blockers (such as tetraethylammonium, quinine, 4-aminopyridine and barium) inhibit the rate or extent of IP_3-induced Ca^{2+} release (Palade *et al.*, 1989). Thus, it may be that lemakalim increases the amount of stored Ca^{2+} or activates an IP_3-induced Ca^{2+} releasing mechanism through activation of K^+-channels on the storage sites. This remains to be clarified.

In Ca^{2+}-free solution containing 128 mM K^+, lemakalim (1 μM) slightly inhibited the increase in $[Ca^{2+}]_i$ and tension induced by 10 μM NA and slightly accelerated the decay of each response. Glibenclamide (1 μM) did not alter the effects of lemakalim in high K^+ containing solution. Since (±)-cromakalim did not hyperpolarize the membrane of smooth muscle cells of the guinea-pig mesenteric artery in a solution containing 128 mM K^+ (Nakao *et al.*, 1988), the membrane hyperpolarizing action of lemakalim may have been negligible in our experiments under these conditions. These results suggest that some effects of lemakalim other than its membrane hyperpolarizing action may underlie its inhibition of NA-responses in Ca^{2+}-free solution containing 128 mM K^+.

In conclusion, in smooth muscle of the rabbit mesenteric artery, lemakalim lowered the resting $[Ca^{2+}]_i$ and inhibited the active increase in $[Ca^{2+}]_i$ and tension and the synthesis of IP_3 induced by NA. Since glibenclamide consistently inhibited the change in the $[Ca^{2+}]_i$, IP_3 synthesis and tension induced by lemakalim, it is suggested that the membrane hyperpolarization induced by lemakalim may play a role in the inhibition of NA-induced IP_3 synthesis.

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