British Journal of Pharmacology (1999) 128, 1491–1496
http://www.stockton-press.co.uk/bjp

Levcromakalim causes indirect endothelial hyperpolarization *via* a myo-endothelial pathway

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1 Effects of K^+ channel opener, levcromakalim, on vascular endothelial cells were examined. Under voltage- and current-clamp conditions, application of acetylcholine to dispersed endothelial cells isolated from rabbit superior mesenteric artery (dispersed RMAECs) produced hyperpolarization and outward currents. On the other hand, dispersed RMAECs did not respond to levcromakalim.

2 When membrane potential was recorded from endothelium in a mesenteric arterial segment, exposure to levcromakalim in a concentration range of 0.1 to 3 μM caused concentration-dependent hyperpolarization. The hyperpolarization was observed in the absence of external Ca^{2+} and was inhibited by 10 μM glibenclamide.

3 The presence of 1 mM heptanol did not affect the levcromakalin-induced hyperpolarization, whereas treatment of the mesenteric arterial segment with 20 μM 18 β-glycyrrhetinic acid significantly reduced the hyperpolarization. The response to acetylcholine of RMAECs in an arterial segment with 18 β-glycyrrhetinic acid was, however, similar to that without 18 β-glycyrrhetinic acid.

4 These suggest that although RMAECs themselves are functionally insensitive to levcromakalim, those in an arterial segment are hyperpolarized by levcromakalim *via* myo-endothelial electrical communication.

Keywords: Levcromakalim; ATP-dependent K^+ current; rabbit mesenteric artery; endothelial cells; myo-endothelial communication; 18 β-glycyrrhetinic acid

Abbreviations: ACh, acetylcholine; dispersed RMAECs, dispersed endothelial cells isolated from rabbit superior mesenteric artery; DMSO, dimethylsulphoxide; K_{ATP} channel, ATP-dependent K^+ channel; KCOs, K^+ channel openers; NO, nitric oxide; PBS, phosphate-buffered solution; PSS, physiological salt solution; RMASCs, mesenteric arterial smooth muscle cells; seg-RMAECs, endothelial cells in a mesenteric arterial segment

Introduction

K^+ channel openers (KCOs) such as cromakalim, pinacidil and diazoxide effectively dilate smooth muscles in large and small vasculature and the mechanisms have been extensively studied (Kuriyama *et al*., 1995; Quayle *et al*., 1997). In the presence of KCOs, potassium channels which are susceptible to glibenclamide and intracellular ATP (ATP-dependent K^+ channel, K_{ATP} channel), are activated in vascular smooth muscles (Kuriyama *et al*., 1995; Quayle *et al*., 1997). On the other hand, the effects of KCOs on vascular endothelial cells are not conclusive because only a few studies using aortic and capillary endothelial cells have been reported (Luckhoff & Busse, 1990a; Janigro *et al*., 1993; Katnik & Adams, 1995) and furthermore, the responses of endothelial cells to KCOs are controversial depending on cell-conditions (Mehrke *et al*., 1991; Langheinrich & Daut, 1997).

Membrane potential of endothelial cells is an important factor to regulate the cell-functions such as cell-growth/differentation and the activity of nitric oxide synthase. Depending on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$,) they are affected: removal of external Ca^{2+} critically influences these functions (Faury *et al*., 1998; Lantoine *et al*., 1998). In endothelial cells, a large amount of Ca^{2+} involved in these physiological functions is supplied from outside of the cell *via* Ca^{2+} permeable channels (cation channels, Nilius *et al*., 1997b). It is likely that hyperpolarization of endothelial cells increases $[Ca^{2+}]_i$ due to larger driving force for Ca^{2+} entry through the cation channels (Nilius *et al*., 1997b). Therefore, hyperpolarization of endothelial cells by the activation of Ca^{2+}-dependent K^+ channel followed by receptor stimulation, at least in part, contributes to the maintenance of higher $[Ca^{2+}]_i$ (Luckhoff & Busse, 1990b; Nilius *et al*., 1997b). Accordingly, when endothelial cells are hyperpolarized in the presence of KCOs, it is possible that endothelial $[Ca^{2+}]_i$ is increased and the production of nitric oxide (NO) is facilitated (Luckhoff & Busse, 1990a; Lantoine *et al*., 1998).

In the present study, effects of levcromakalim on endothelial cells in rabbit superior mesenteric artery were examined. We show that levcromakalim has no effect on dispersed endothelial cells but hyperpolarizes endothelium in an arterial segment *via* myo-endothelial communication which is effectively disrupted by 18 β-glycyrrhetinic acid, an inhibitor of gap junctions (Yamamoto *et al*., 1998).

Methods

Cell-isolation

Male New Zealand white rabbits weighing 1.5–2.0 kg were anaesthetized by i.v. sodium pentobarbital (30 mg kg^{-1}) and killed by exsanguination. All experiments were carried out in

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accordance with guiding principles for the care and use of laboratory animals (the Science and International Affairs Bureau of the Japanese Ministry of Education, Science, Sports and Culture) and also with the approval of the ethics committee in Nagoya City University. For isolation of mesenteric arterial endothelial cells, the isolated superior mesenteric artery was cleaned of fat and connective tissue, and cut open longitudinally. The tissue was pinned in a 60 mm culture dish filled with Ca^{2+}-Mg^{2+} free phosphate-buffered solution (PBS) containing 0.05% collagenase (Amano) and 0.05% dispase (Boehringer Mannheim, Tokyo, Japan). The culture dish was kept in an incubator at 37°C for 60 min and then the enzyme solution containing isolated endothelial cells was centrifuged at 1200 r.p.m. for 10 min. Thereafter, the supernatant was removed and the pellet was resuspended in the culture medium. Endothelial cells were allowed to attach to gelatin-coated glass coverslips over 8 h at 37°C in air with 5% CO_2 and used within 48 h. When the freshly dispersed cells were used, the pellet containing endothelial cells was resuspended in the PBS solution. These cells were settled down on gelatin coated coverslips and used within 8 h.

Electrophysiological experiments

Whole-cell membrane currents and membrane potential were recorded with the amphotericin B-perforated-patch technique by using a CEZ-2300 (Nihon-Koden, Tokyo, Japan) amplifier. The resistance of microelectrodes filled with pipette solution was approximately 3–5 MΩ. Membrane currents and voltage signals were stored and analysed as described previously (Imaizumi *et al*., 1989; Muraki *et al*., 1997; Taki *et al*., 1999). Briefly, membrane currents and voltage signals were monitored on a storage oscilloscope (VC-6041, Hitachi Tokyo, Japan) and stored on videotape after being digitized with a PCM-recording system (modified to acquire a DC signal, PCM 501ES; SONY, Tokyo, Japan). The data on the tape were replayed later and loaded into a computer (IBM-AT compatible) through an A-D converter (Data translation, DT2801A). Membrane currents and potential changes were printed out by using a thermal array recorder (RTA-1200; Nihon-Koden, Tokyo, Japan). When transmembrane potential was recorded from endothelial cells in an intact artery, a vascular segment (2 × 2 mm) was fixed on the luminal side up on the rubber at the bottom of a chamber. Input resistance of endothelial cells in an intact artery was calculated by the following equation (Yamamoto *et al*., 1998);

$$R_{in} = V_p I_s^{-1} - V_p I_i^{-1}$$

where V_p is the amplitude of the voltage step, I_i and I_s are the amplitude of membrane current at the beginning and the end of the voltage step, respectively.

All experiments were carried out at 25±1°C in a physiological salt solution (PSS) containing (mM) NaCl 137, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 2.2, glucose 10, and HEPES 10. The pH was adjusted to 7.4 with 10 N NaOH. PBS contained (mM) NaCl 137, KCl 2.7, Na_2HPO_4 8.1, KH_2PO_4 1.47, $MgCl_2$ 1.0, $CaCl_2$ 1.5. The pipette solution contained (mM) K-aspartate 110, KCl 30, $MgCl_2$ 4, HEPES 10 and 300 μg^{-1} ml amphotericin B. The pH of the pipette solution was adjusted to 7.2 with KOH.

Measurement of tension development

After connective tissue and adventia were removed carefully, 1 mm lengths of vessel ring were cut out from the superior mesenteric artery. The endothelium was removed by rubbing the inner lumen of the vessel with a cotton pad. The vessel ring was set up in a 4 ml organ bath to measure isometric tension with a force-transducer and perfused with Kreb's solution which was maintained at 37±1°C and gassed with 95% O_2 and 5% CO_2. The composition of Kreb's solution was (mM); NaCl 117, $MgCl_2$ 1.2, $CaCl_2$ 2.2, KCl 4.7, KH_2PO_4 1.2, Glucose 14 and $NaHCO_3$ 25.

Drugs

The following drugs were used: acetylcholine (ACh, Wako, Tokyo, Japan), phenylephrine (Wako), levcromakalim, nicorandil, diazoxide (Sigma, St. Louis, USA), glibenclamide (Sigma), heptanol (Sigma), acetylated low-density lipoprotein labelled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indo-carbocyanine perchlorate (Funakoshi, Tokyo, Japan), 18 β-glycyrrhetinic acid (Sigma) and amphotericin B (Sigma). Each drug except levcromakalim, nicorandil, diazoxide, glibenclamide, 18 β-glycyrrhetinic acid and amphotericin B was dissolved in distilled water to make 10 mM stock solutions. Levcromakalim, nicorandil, diazoxide, glibenclamide, 18 β-glycyrrhetinic acid and amphotericin B were dissolved in dimethylsulphoxide (DMSO, 100 mM as stock). These solvents (distilled water and DMSO) had no effect on membrane currents and potentials when a corresponding amount was applied. Drug concentrations are expressed as the final concentration in PSS and the pH of PSS was readjusted after the addition of drugs. All drugs were applied at a constant flow rate of 0.1 ml s^{-1}. A change of PSS could be achieved within 10 s.

Statistics

Data are expressed as mean±s.e.mean. Statistical significance between two or among multiple groups was examined using Student's *t*- or Scheffé's test, respectively. Statistical significance at *P* values of 0.05 and 0.01 is indicated in figures and text by * and **, respectively.

Results

Effects of levcromakalim on rabbit mesenteric arterial endothelial cells

A large number of cells dispersed in the present study (>80%) were identified as endothelial cells by their specific uptake of acetylated low-density lipoprotein labelled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indo-carbocyanine perchlorate (not shown). Moreover, cells that had hyperpolarizing response to ACh were used in the present study. Averaged resting membrane potential of dispersed RMAECs was −30.8±3.1 mV (n=13). As shown in Figure 1A, when 5 μM ACh was applied to a dispersed RMAEC, oscillatory hyperpolarization was elicited and its peak amplitude was −59.2±4.1 mV (n=13). Addition of 1 μM levcromakalim had a small effect on the membrane potential of the cell; in contrast, re-administration of 5 μM ACh induced the marked hyperpolarization (Figure 2, n=5, the closed triangle).

When a cell was voltage-clamped at a holding potential of −40 mV, application of 5 μM ACh elicited outward currents (Figure 1B) and the averaged peak amplitude was 72.0±14.8 pA (n=6). Subsequent application of levcromakalim up to 3 μM failed to activate any membrane currents (Figure 1B). During the treatment of the cell with levcromakalim, the response to ACh was reproducible

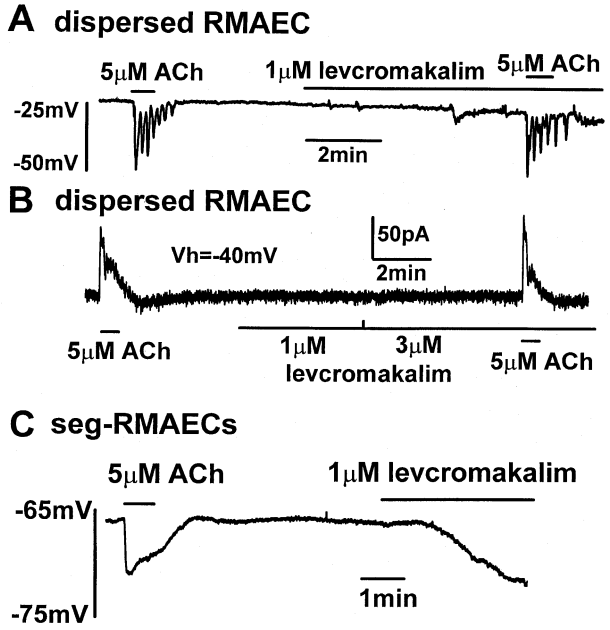

Figure 1 Effects of levcromakalim on RMAECs. (A) A dispersed RMAEC was treated with 5 μM ACh and subsequently 1 μM levcromakalim under current-clamp conditions. (B) Under voltage-clamp conditions at -40 mV, a dispersed RMAEC was superfused with 5 μM ACh, 1 and 3 μM levcromakalim. (C) Effects of 5 μM ACh and 1 μM levcromakalim on seg-RMAECs.

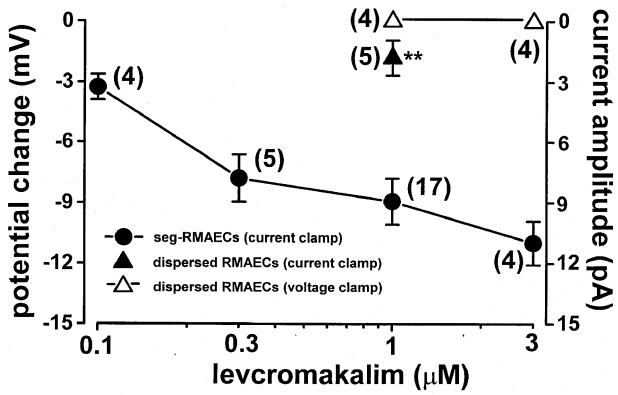

Figure 2 Summarized data describing effects of levcromakalim on dispersed and seg-RMAECs under current and voltage-clamp conditions. Number in the parentheses indicates the number of cells employed. $^{**}P<0.01$ vs seg-RMAECs.

(87.2$\pm$18.0 pA, $P>0.05$. vs 72.0$\pm$14.8 pA). Morover, when cells were superfused with 140 mM K^+, application of 10 μM levcromakalim had no effects on membrane currents at a holding potential of -60 mV ($n=3$). The resting membrane potential of freshly dispersed RMAECs was -30.4 ± 2.4 mV ($n=8$), which was not significantly different from that of dispersed RMAECs kept in culture medium over 8 h (-30.8 ± 3.1 mV ($n=13$)). Application of 3 μM levcromakalim had no effects on the resting membrane potential and the holding current of -40 mV recorded from the freshly dispersed RMAECs, whereas 5 μM ACh induced the hyperpolarization (-37.7 ± 1.5 mV, $n=3$) and the outward current at -40 mV (81.3$\pm$23 pA, $n=4$). These results suggest that levcromakalim does not have any effects on electrophysiological responses of dispersed RMAECs.

Figure 1C shows effects of ACh and levcromakalim on membrane potential recorded from endothelial cells in a mesenteric arterial segment (seg-RMAECs). After transmembrane potential was stably recorded, seg-RMAECs were treated with 5 μM ACh to confirm that electrical signals came from endothelial cells. The averaged resting membrane potential of the seg-RMAECs and the hyperpolarized by 5 μM ACh were -61.1 ± 1.2 mV ($n=68$) and -6.8 ± 0.8 mV ($n=17$), respectively. Surprisingly, subsequent application of 1 μM levcromakalim as well as ACh hyperpolarized seg-RMAECs by -8.9 ± 1.1 mV ($n=17$). Onset of hyperpolarization by levcromakalim was significantly slower than that by ACh (62.7$\pm$6.0 vs 11.9$\pm$1.5 s, respectively, $n=17$, $P<0.01$). Moreover, it took longer for the hyperpolarization elicited by levcromakalim to reach a maximum compared with that elicited by ACh (224.1$\pm$22.5 vs 5.1$\pm$0.5 s, respectively, $n=17$, $P<0.01$). The effects of levcromakalim on RMAECs that were dispersed or existed in the arterial segment are summarized in Figure 2. Responses of dispersed RMAECs to levcromakalim under current-clamp (a closed triangle) and voltage-clamp conditions (open triangles) were substantially small in comparison with that of seg-RMAECs (closed circles). Levcromakalim hyperpolarized seg-RMAECs in a concentration-dependent manner and the concentration required for 50% of the maximum response was approximately 0.3 μM. Moreover, neither 300 μM diazoxide nor 100 μM nicorandil had effects on the dispersed RMAECs ($n=2$ and $n=3$, respectively). A marked hyperpolarization was, however, observed when 100 μM nicorandil was applied to the seg-RMAECs (-11.5 ± 2.3 mV, $n=3$). Levcromakalim possibly affects not RMAECs but mesenteric arterial smooth muscle cells (RMASCs).

Membrane current component involved in levcromakalim-induced hyperpolarization in seg-RMAECs

In Figure 3, membrane current components involved in levcromakalim-induced hyperpolarization in seg-RMAECs were examined using potassium channel inhibitors. After seg-RMAECs were treated with 5 μM ACh, exposure to 1 μM levcromakalim elicited marked hyperpolarization. Addition of 10 μM glibenclamide, an ATP-dependent K^+ channel (K_{ATP} channel) inhibitor, gradually reduced the hyperpolarization (Figure 3B). On the other hand, 10 μM glibenclamide had no effect on the membrane potential of seg-RMAECs without levcromakalim (Figure 3A). As shown in Figure 3B, 100 μM Ba^{2+} further inhibited the hyperpolarization ($n=4$). Hyperpolarization induced by 1 μM levcromakalim was suppressed by 49.5$\pm$8.3% ($n=5$) 10 min after addition of 10 μM glibenclamide. In the absence of external Ca^{2+}, 1 μM levcromakalim induced -16.5 mV ($n=2$) hyperpolarization (Figure 3C) and subsequent application of 10 μM glibenclamide suppressed this hyperpolarization. These results give evidence that ATP-dependent K^+ current but neither inwardly rectified K^+ nor Ca^{2+}-dependent K^+ current plays an obligatory role in hyperpolarization induced by levcromakalim in seg-RMAECs.

Effects of gap junction channel inhibitors on levcromakalim-induced hyperpolarization in seg-RMAECs

To confirm that the hyperpolarization in seg-RMAECs induced by levcromakalim is derived from that in RMASCs *via* myo-endothelial electrical communications, effects of hepatanol and 18 β-glycyrrhetinic acid, inhibitors of gap

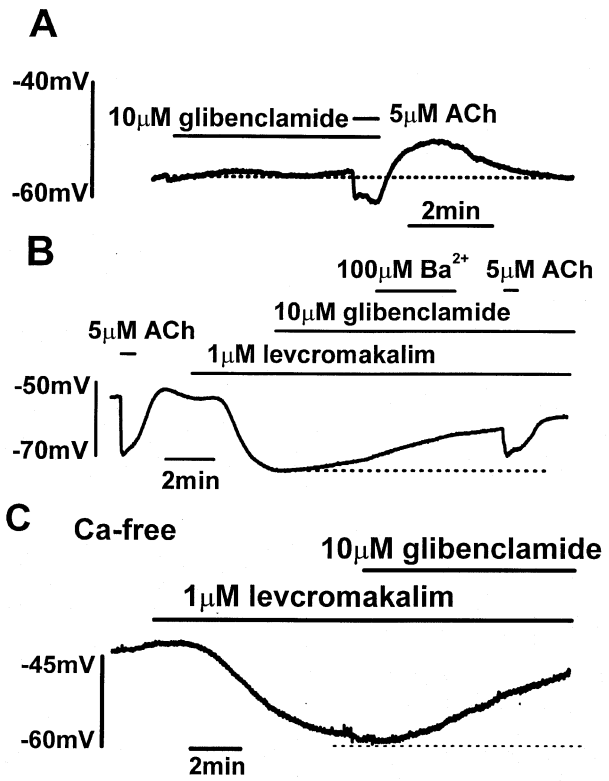

Figure 3 Effects of potassium channel inhibitors and removal of external Ca^{2+} on levcromakalim-induced hyperpolarization in seg-RMAECs. (A) Membrane potential was recorded from seg-RMAECs. In the absence of levcromakalim, 10 μM glibenclamide and subsequently 5 μM ACh were applied. (B) After seg-RMAECs were hyperpolarization in the presence of 1 μM levcromakalim, seg-RMAECs were exposed to 10 μM glibenclamide and 100 μM Ba^{2+}. (C) Hyperpolarization of seg-RMAECs induced by 1 μM levcromakalim in the absence of external Ca^{2+}.

junction channels, on the levcromakalim-induced hyperpolarization were examined in Figure 4. In the presence of 1 mM heptanol, the resting membrane potential of seg-RMAECs was -58.4 ± 4.0 mV ($n=5$, $P>0.05$) vs control (-61.1 ± 1.2 mV, $n=68$). Addition of 5 μM ACh and 1 μM levcromakalim hyperpolarized seg-RMAECs by -11.0 ± 4.2 and -8.3 ± 1.2 mV ($n=3$), which were not significantly different from those without heptanol (Figure 4Bb, the open columns vs the hatched columns). In contrast, when 20 μM 18 β-glycyrrhetinic acid was present in the bathing solution, seg-RMAECs were depolarized to -48.4 ± 2.4 mV ($n=27$) and the input resistance was markedly increased to 0.58 ± 0.24 GΩ ($n=5$) vs 5.6 ± 1.5 MΩ ($n=6$) in the absence of 18 β-glycyrrhetinic acid. Under these conditions, changes of membrane potential from a holding potential of -50 mV in 10 mV increments mainly produced a leak current shown in Figure 4Aa, Ab. Depolarization to more positive potentials than 0 mV, however, elicited small fluctuatory currents (Figure 4Aa). As shown in Figure 4Ba, in the presence of 20 μM 18 β-glycyrrhetinic acid, levcromakalim-induced hyperpolarization was abolished ($+2.2\pm1.8$ mV ($n=8$, $P<0.01$) vs control (-8.9 ± 1.1 mV, $n=17$), Figure 4Bb); nevertheless, the seg-RMAECs were hyperpolarized by application of 5 μM ACh (-12.8 ± 3.4 mV, $n=8$, vs control (-6.8 ± 0.9 mV, $n=17$), $P>0.05$, Figure 4Bb).

It was possible that 18 β-glycyrrhetinic acid directly blocked K_{ATP} channels in RMASCs. This was examined by measuring muscle tension from a rabbit mesenteric arterial ring in which endothelium was denuded. In the absence and presence of 20 μM 18 β-glycyrrhetinic acid, the muscle ring was contracted by 1 μM phenylephrine. Relaxation of the muscle ring in response to 1 μM levcromakalim was not significantly affected by incubation with 20 μM 18 β-glycyrrhetinic acid for 20 min (82.4 ± 10.5 and 81.4 ± 4.8% relaxation in the absence and presence of 18 β-glycyrrhetinic acid, $P>0.05$, $n=4$).

Discussion

The present study clearly demonstrated that although dispersed RMAECs were functionally insensitive to levcromakalim, RMAECs in an arterial segment were significantly hyperpolarized by levcromakalim *via* myo-endothelial communication which is effectively disrupted by 18 β-glycyrrhetinic acid.

Physiological and pharmacological characteristics of RMAECs were first clarified in the present study. Dispersed RMAECs have: (1) a resting membrane potential of approximately -30 mV, (2) a response to ACh that involves $I_{K\text{-}Ca}$ and hyperpolarization, (3) lack of response to levcromakalim. Low resting membrane potential has been reported in various types of endothelial cells (ECs); rabbit aortic ECs: Katnik & Adams, 1995; human umbilical vein ECs: Nilius *et al*., 1997a. Since the resting membrane potential in macrovascular ECs is generally more positive than that in microvascular ECs, the resting membrane potential of RMAECs (~-30 mV) may not be exceptional. In contrast, a lack of response of RMAECs to levcromakalim was distinct from that reported previously (Janigro *et al*., 1993; Katnik & Adams, 1995). In rabbit, porcine and rat aortic ECs, KCOs elicited hyperpolarization and/or currents sensitive to glibenclamide. The reason of this discrepancy is not clear. In guinea-pig coronary capillaries, K_{ATP} channels are absent or inactivated under cell-culture conditions (Langheinrich *et al*., 1998). On the other hand, the response of cultured rabbit mesenteric arterial smooth muscle cells to KCOs was not affected by cell-culture within 7 days (Kleppisch *et al*., 1996). Since diazoxide and nicorandil as well as levcromakalim did not affect dispersed RMAECs, it is most likely that the RMAECs are functionally insensitive to KCOs under the present experimental conditions.

Electrical response of seg-RMAECs to levcromakalim was distinct from that of dispersed RMAECs. Seg-RMAECs were effectively hyperpolarized by application of levcromakalim: the EC_{50} was ~0.3 μM. It has been reported that cromakalim in a concentration range between 0.1 and 10 μM produced 10 to 20 mV hyperpolarization in several types of vascular smooth muscles: in addition, the treatment with glibenclamide decreased the hyperpolarization; rabbit mesenteric artery (McHarg *et al*., 1990; Muraki *et al*., unpublished observation) and guinea-pig basilar artery (Plane & Garland, 1993) (as reviews: Kuriyama *et al*., 1995; Quayle *et al*., 1997). In dispersed RMASCs, KCOs such as cromakalim, pinacidil and diazoxide activate glibenclamide-sensitive membrane currents, indicating that K_{ATP} channels are abundant in rabbit mesenteric artery (Quayle *et al*., 1995; Muraki *et al*., unpublished observation). Our finding that levcromakalim induces glibenclamide-sensitive hyperpolarization in seg-RMAECs but not in dispersed RMAECs strongly suggests that activation of K_{ATP} channels in smooth muscle predominantly affects the membrane potential of endothelium.

Application of 18 β-glycyrrhetinic acid inhibited the levcromakalim-induced hyperpolarization in seg-RMAECs but heptanol did not. Although inhibitors of gap junctions, such as heptanol, octanol and halotane, disrupt gap junctional

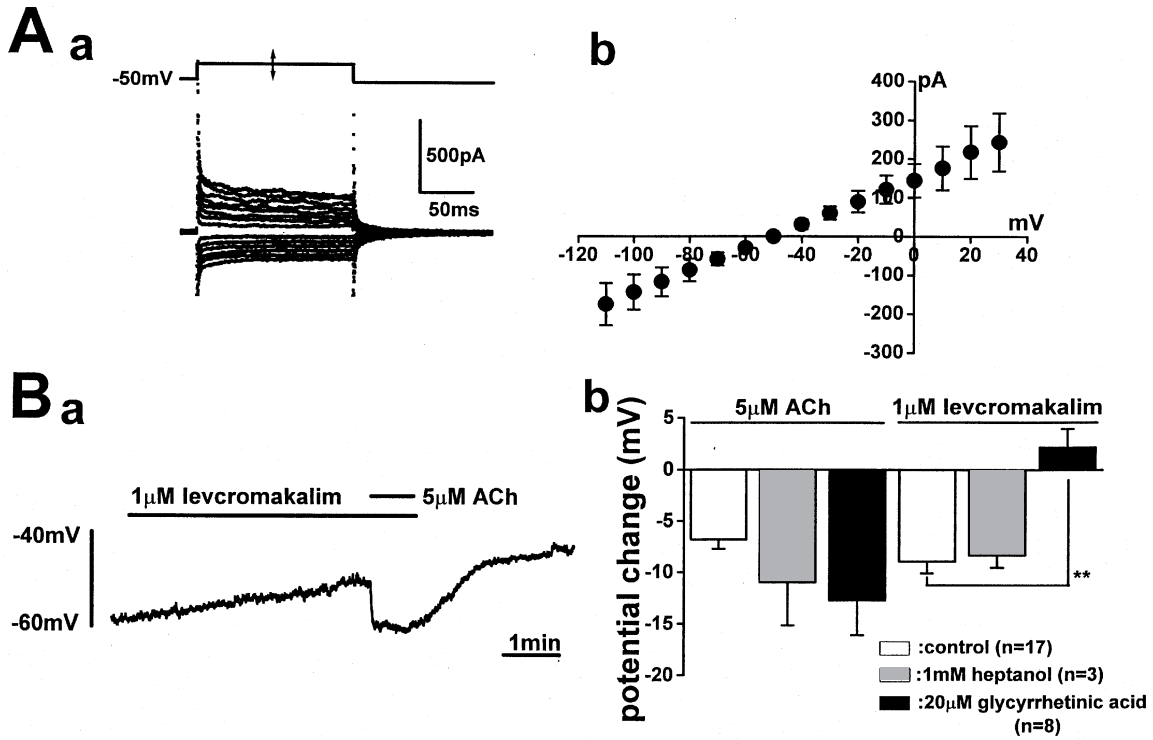

Figure 4 Effects of gap junction channel inhibitors on levcromakalim-induced hyperpolarization in seg-RMAECs. (Aa) Gap junctions were disrupted by incubation of seg-RMAECs with 20 μM 18 β-glycyrrhetinic acid for 20 min. The seg-RMAECs were de- and hyperpolarized every 10 s from a holding potential of -50 mV to potentials between -110 and $+30$ mV at 10 mV step. The input resistance of this cell was 0.26 GΩ. (Ab) Summary of the current and voltage relationship obtained from five separate seg-RMAECs which were treated with 20 μM 18 β-glycyrrhetinic acid. (Ba) Effects of 1 μM levcromakalim and 5 μM ACh on seg-RMAECs in which gap junctions were disrupted by 20 μM 18 β-glycyrrhetinic acid. The input resistance of this cell was 0.46 GΩ. (Bb) Summarized data describing the effects of heptanol and 18 β-glycyrrhetinic acid on ACh and levcromakalim-induced hyperpolarizations in seg-RMAECs. $^{**}P<0.01$ vs control.

communication in several types of cells, these inhibitors may have non-specific actions and their effectiveness in smooth muscle has not been clear (Spray & Burt, 1990; Hashitani & Suzuki, 1997; Chaytor *et al*., 1998). On the other hand, glycyrrhetinic acid is a lipophilic aglycone, which can exert gap junctional communication in several types of cells (Davidson & Baumgarten, 1988; Guan *et al*., 1996; Guo *et al*., 1999). Recent findings demonstrate that glycyrrhetinic acid disrupts myo-endothelial communication (Taylor *et al*., 1998; Yamamoto *et al*., 1998). Although the β isoform of glycyrrhetinic acid shows non-specific effects, the present results may exclude this possibility: the response to ACh of seg-RMAECs with 18 β-glycyrrhetinic acid was not significantly different from that without 18 β-glycyrrhetinic acid and the incubation of the muscle ring with 18 β-glycyrrhetinic acid did not affect the levcromakalim-induced relaxation. Consistently, endothelial electrical responses to ACh of guinea-pig mesenteric arterioles in the presence of 20–40 μM 18 β-glycyrrhetinic acid were similar to those in the absence (Yamamoto *et al*., 1998; 1999). Our finding, therefore, gives strong evidence that glycyrrhetinic acid-sensitive gap junctions conduct the hyperpolarization in RMASCs to seg-RMAECs, resulting in a negative offset of the membrane potential of seg-RMAECs.

The influence of the endothelium/NO on vasorelaxation to KCOs has not been concluded. In the rat mesenteric arterial bed, removal of basal NO significantly augmented the responses to KCOs *via* cyclic GMP dependent mechanisms (McCulloch & Randall, 1996). On the other hand, the vasorelaxing effect of a KCO in endothelium-intact rat aorta was more potent than in an endothelium-denuded one (Tanaka *et al*., 1999). The amount of NO released from endothelium in small arterioles is substantially greater than that in large arteries, possibly being involved in the difference of effects of NO on vasorelaxation to KCOs. Although the present results raises the possibility that KCOs facilitate release of NO from seg-RMAECs by the endothelial hyperpolarization *via* myo-endothelial communication, it is not clear that the facilitation of NO release increases or decreases the relaxation of RMASCs to KCOs. Further investigation is required to clarify the influence of NO on the vasorelaxation to KCOs.

In conclusion, levcromakalim causes hyperpolarization of seg-RMAECs *via* myo-endothelial communication as well as the relaxation and the hyperpolarization of RMASCs. When vascular smooth muscle cells are hyperpolarized by certain vasoactive factors, endothelial cells, which are even electrically insensitive to these factors, can be hyperpolarized *via* the myo-endothelial communication.

We thank Dr W. Giles (University of Calgary, Canada) for supplying the data acquisition (AQ) and analysis (Cellsoft) software. This study was supported by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Science, Sports and Culture to K. Muraki.

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(Received September 9, 1999
Revised September 20, 1999
Accepted September 24, 1999)