# An ATP-sensitive potassium conductance in rabbit arterial endothelial cells

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1.  Whole-cell patch clamp recording was used to study an ATP-sensitive, sulphonylurea-inhibitable potassium ($K^+$) conductance in freshly dissociated endothelial cells from rabbit arteries.

2.  The ATP-sensitive $K^+$ conductance was activated by micromolar concentrations of the $K^+$ channel opener, levcromakalim, and by metabolic inhibition of endothelial cells using dinitrophenol and iodoacetic acid. The current–voltage ($I$–$V$) relationship obtained in isotonic $K^+$ solutions was linear between $-150$ and $-50$ mV and had a slope conductance of approximately 1 nS.

3.  The permeability of the ATP-sensitive $K^+$ conductance determined from reversal potential measurements exhibited the following ionic selectivity sequence: $Rb^+ > K^+ > Cs^+ \gg Na^+ > NH_4^+ > Li^+$.

4.  Membrane currents activated by either levcromakalim or metabolic inhibition were inhibited by the sulphonylurea drugs, glibenclamide and tolbutamide, with half-maximal inhibitory concentrations of 43 nM and 224 $\mu$M and Hill coefficients of 1·1 and 1·2, respectively. Levcromakalim-induced currents were also inhibited by millimolar concentrations of $Ba^{2+}$ or tetraethylammonium ions in the external solution.

5.  Levcromakalim (3 $\mu$M) and metabolic inhibition hyperpolarized endothelial cells by approximately 10–15 mV in normal physiological salt solutions. The hyperpolarization induced by levcromakalim or metabolic inhibition was inhibited by bath application of 10 $\mu$M glibenclamide.

6.  Internal perfusion of the cytosol of whole-cell voltage-clamped endothelial cells with an ATP-free pipette solution activated a membrane current which was reversibly inhibited by internal perfusion with a 3 mM MgATP pipette solution. This current was insensitive to other adenine and guanine nucleotides in the pipette solution. The inward current evoked in a nominally ATP-free internal solution was further increased by bath application of levcromakalim.

7.  Levcromakalim (25 $\mu$M) did not induce a change in the intracellular $Ca^{2+}$ concentration of fura-2-loaded endothelial cells, whereas metabolic inhibition caused a slow and sustained increase in intracellular $Ca^{2+}$ concentration, which was attenuated by 10 $\mu$M glibenclamide applied externally.

8.  ATP-sensitive $K^+$ channel activation in arterial endothelial cells may contribute to endothelium-dependent vascular changes in response to ischaemia-induced hypoxia producing membrane hyperpolarization.

ATP-sensitive $K^+$ ($K_{ATP}$) channels, identified in numerous cell types (pancreatic $\beta$-cells, cardiac, skeletal and vascular smooth muscle cells, renal epithelial cells and cerebral cortical neurons; see reviews, Ashcroft, 1988; Ashcroft & Ashcroft, 1990), serve to transduce changes in cell metabolism into changes in membrane potential. $K_{ATP}$ channels are closed in the presence of millimolar concentrations of cytoplasmic ATP and are open when the ATP concentration decreases below a threshold. These channels are identified pharmacologically by their

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sensitivity to the $K^+$ channel openers, levcromakalim, pinacidil and diazoxide, and to the sulphonylurea drugs, glibenclamide and tolbutamide. In vascular smooth muscle cells, activation of the channel has been shown to influence the resting membrane potential and to mediate relaxation (Clapp & Gurney, 1992; Clapp, Davey & Gurney, 1993; Xu & Lee, 1994). There is recent evidence for the presence of $K_{ATP}$ channels in vascular endothelial cells (Lückhoff & Busse, 1990*a*; Arnould, Michiels, Alexandre & Remacle, 1992; Jangiro, West, Gordon & Winn, 1993); however, the biophysical and pharmacological properties of these channels in endothelial cells remain ill-defined. The capacity of these channels to regulate resting membrane potential makes them an important component in signal transduction.

The arterial endothelium, a physical barrier between circulating blood and vascular smooth muscle cells, plays an important role in the regulation of vascular tone by releasing relaxing and contracting factors. The synthesis and/or release of endothelium-derived relaxing factors (EDRF) in response to stimulation by vasoactive agents is triggered by an increase in the cytoplasmic free calcium ion concentration ([$Ca^{2+}$]$_i$; Lückhoff, Pohl, Mülsch & Busse, 1988). Agonist-induced increases in [$Ca^{2+}$]$_i$ are biphasic, composed of a rapid, transient $Ca^{2+}$ release from intracellular stores and a slow, sustained $Ca^{2+}$ influx across the plasma membrane (see reviews: Adams, Barakeh, Laskey & van Breemen, 1989; Adams, Rusko & Van Slooten, 1993; Himmel, Whorton & Strauss, 1993). The sustained transmembrane influx phase of the $Ca^{2+}$ response has been found to be a prerequisite for the maintained secretion of EDRF. Resting and agonist-stimulated $Ca^{2+}$ influx in cultured endothelial cells is attenuated by membrane depolarization (Johns, Lategan, Lodge, Ryan, van Breemen & Adams, 1987; Cannell & Sage, 1989; Schilling, 1989; Laskey, Adams, Johns, Rubanyi & van Breemen, 1990; Lückhoff & Busse, 1990*b*) suggesting that membrane potential provides an electrochemical gradient for $Ca^{2+}$ entry which leads to synthesis/release of endothelium-derived factors.

Our experiments demonstrate the presence of an ATP-sensitive $K^+$ ($K_{ATP}$) conductance in freshly dissociated rabbit endothelial cells because a membrane current is activated by external application of the $K^+$ channel opener, levcromakalim, by metabolic inhibition and by internal perfusion of the cell with ATP-free solutions. A pharmacological profile of the $K_{ATP}$ conductance is generated by examining the sensitivity of levcromakalim-induced currents to external application of the sulphonylurea drugs, glibenclamide and tolbutamide, and the $K^+$ channel blockers, tetraethylammonium and barium ions. Characterization of the biophysical and pharmacological properties of the $K_{ATP}$ channel in arterial endothelial cells may allow determination of its relative contribution in the transduction of hypoxic conditions in the circulating blood to alterations of vascular smooth muscle tone.

A preliminary report of some of these results has been presented (Katnik & Adams, 1994).

## METHODS

### Endothelial cell preparation

Endothelial cells from rabbit arterial vessels were isolated using an enzymatic dissociation procedure described previously (Rusko, Tanzi, van Breemen & Adams, 1992). Briefly, male New Zealand White rabbits (2–3 kg) were killed by inhalation of 100% $CO_2$ followed by exsanguination. The thoracic aorta and pulmonary arteries were dissected out and placed in physiological salt solution (PSS) with the following composition (mM): 125·4 NaCl, 5·9 KCl, 1·5 $CaCl_2$, 1·2 $MgCl_2$, 11·5 glucose, 10 Hepes, adjusted to pH 7·3 with NaOH. Connective and adipose tissue were removed, the vessels were cut longitudinally, thin sheets of interior endothelium were carefully peeled off, transferred to PSS containing 0·9 mg ml$^{-1}$ papain (14 U mg$^{-1}$) and 0·8 mg ml$^{-1}$ dithiothreitol, and incubated at 37 °C for approximately 40 min. The tissue was then washed with PSS containing 20% fetal calf serum and gently triturated. The resulting suspension was filtered (62 $\mu$m nylon mesh filter) and centrifuged at 2500 r.p.m. for 10 min in PSS. The pellet of endothelial cells was resuspended in PSS, plated on glass coverslips and maintained at 4 °C for at least 2 h, allowing the cells to adhere to the glass. The coverslips were placed in a recording chamber (0·5 ml in volume) mounted on the stage of a microscope and visualized at × 640 magnification with Hoffman modulation contrast optics. The chamber was continuously perfused at the rate of 5 ml min$^{-1}$. All experiments were performed at room temperature (21 ± 2 °C) and within 7 h of the dissociation procedure.

Positive identification of the cells as endothelial was based on the following criteria: (1) morphological characteristics, (2) specific uptake of low-density lipoprotein visualized by immunofluorescence of rhodamine-labelled 1,1-dioctadecyl-3,3,3,3′-tetramethyl-indocarbocyanine (DiI-Ac-LDL, Biomedical Technologies, Stoughton, MA, USA) as previously described (Rusko *et al.* 1992), and (3) positive cell-surface immunoreactivity for factor VIII antigen using mouse monoclonal antibodies to human factor VIII (Calbiochem Corp., La Jolla, CA, USA) and fluorescein isothiocyanate-conjugated anti-mouse IgG (Sigma ImmunoChemicals, St Louis, MO, USA).

### Electrical recording

Membrane currents and potentials were studied using the whole-cell recording configuration of the patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Patch electrodes (2–5 M$\Omega$) were fabricated from borosilicate glass (GC150F and GC150TF; Clark Electromedical Instruments, Reading, UK) and mounted on the head stage of a List L/M-EPC 7 patch clamp amplifier (List Electronic, Darmstadt, Germany). Pipette resistance and gigaseal formation were determined by the current response to a brief −5 mV voltage step. The cell capacitance and series resistance ($R_s$) were determined from the compensation of the capacity transient by the List patch clamp amplifier. The reduction in $R_s$ of cell-attached patches induced by amphotericin-B insertion into the membrane (Rae, Cooper, Gates & Watsky, 1991) began within seconds of seal formation and experiments were initiated when $R_s$ was less than 20 M$\Omega$, which generally occurred within 10 min. Subsequent changes in $R_s$ were adjusted for during the course of the experiment.

Current–voltage relationships were measured by means of a voltage ramp protocol generated by pClamp programs (Axon Instruments, Foster City, CA, USA) that consisted of holding the endothelial cell at −60 mV, applying five voltage ramps (−150 to +50 mV in 2 s) and averaging the current responses. Membrane current and voltage signals were filtered at 2·5 kHz (−3 dB, 4-pole Bessel filter). Membrane current (or voltage in current clamp mode) was continuously monitored on a digital oscilloscope and on a chart recorder. The signals were simultaneously recorded on video cassette using a digital VCR adapter (PCM-2B, Medical Systems Corp., Greenvale, NY, USA) and VHS recorder and on a 386 PC computer, using an A/D converter (TL-1 DMA interface; Axon Instruments). Measurement of reversal (zero current) potentials were corrected for liquid junction potentials between the bath solution and indifferent electrode with respect to a reference electrode (saturated KCl, reverse sleeve junction; Corning X-EL 47619).

The actions of $K_{ATP}$ channel antagonists were determined by measuring the steady-state current amplitude ($I$) in the presence of the antagonist at each concentration which was normalized against the amplitude of the current obtained in the absence of the antagonist ($I_{max}$). Dose–response data were fitted according to the following equation:

$$\frac{I}{I_{max}} = \frac{1}{1 + (K/[A])^{n_H}},$$ (1)

where $I/I_{max}$ is the fraction of activated current, $A$ is the antagonist concentration, $K$ is the antagonist concentration that produces 50 % inhibition of the maximum response ($IC_{50}$), and $n_H$ is the Hill coefficient. The fitting routine set $K$ and $n_H$ as variables and the origin and end-point of the curve were constrained. Dose–response curves for the $K_{ATP}$ channel activator, levcromakalim, were normalized to the maximum response, and fitted to eqn (1) where $A$ is the agonist concentration, and $K$ is the agonist concentration that evoked the half-maximal response ($EC_{50}$).

All numerical data are represented as the mean ± one standard error of the mean (s.e.m.).

## Microfluorometric measurements

Fluorescence measurements of cytoplasmic free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) in single, non-confluent, endothelial cells were carried out using the fluorescent $Ca^{2+}$ indicator dye, fura-2. Freshly dissociated endothelial cells were incubated in PSS containing 1 $\mu$M of the acetoxymethyl ester of fura-2 (fura-2 AM) at room temperature (23 °C) for 30 min and then washed with buffered PPS. Endothelial cells on glass coverslips were mounted on the stage of an inverted phase contrast microscope (Nikon Diaphot) and continuously perfused at 2 ml min⁻¹ with PSS. Cells were alternately excited (60 Hz) at 340 and 380 nm wavelengths by an optical chopper (OC-4000; Photon Technology International Inc. (PTI), South Brunswick, NJ, USA) in the path of the UV light provided by a 75 W xenon arc lamp. Fura-2 fluorescence was measured at 510 nm wavelength with a photon counter (Hamamatsu R928). Signals were digitized by an A/D converter and analysed using Deltascan software (PTI) and a 486 PC computer. Single cells were viewed with a × 40 Fluor objective (Nikon, 1·3 numerical aperture) and only cells which had an even dye distribution within the cytoplasm were used. The signal ratio from the two excitation wavelengths ($F_{340}/F_{380}$) was determined after subtraction of autofluorescence. $[Ca^{2+}]_i$ was determined following the method of Grynkiewicz, Poenie & Tsien (1985) and rates of change of $[Ca^{2+}]_i$ were determined by linear fits to the data.

## Solutions

Endothelial cells were superfused with normal PSS. In a series of experiments, the external solution was changed to a high-K⁺ solution (HiK) composed of (mM): 126 KCl, 5·3 NaCl, 1·5 $CaCl_2$, 1·2 $MgCl_2$, 11·5 glucose, 10 Hepes, adjusted to pH 7·3 with KOH. In perforated patch experiments, where $[Ca^{2+}]_i$ could not be buffered, 5 mM tetraethylammonium-Cl (TEA-Cl) was added to the external bath solution to block the $Ca^{2+}$-activated K⁺ conductance. Sucrose was substituted for glucose (HiK-S) in metabolic inhibition experiments. In ion substitution experiments, histidine was substituted for Hepes and the chloride salts of K⁺, Cs⁺, Li⁺, Rb⁺ and $NH_4^+$ were substituted for Na⁺ isotonically.

The patch pipette electrode solution used for standard whole-cell recording experiments contained (mM): 126 KCl, 5 NaCl, 10 Hepes, 10 glucose, 0·8 ethyleneglycol-bis-($\beta$-aminoethylether) $N,N,N',N'$-tetraacetic acid (EGTA), 1·2 $MgCl_2$, adjusted to pH 7·2 with KOH. In metabolic inhibition experiments, 2-deoxyglucose was substituted for glucose internally. Internal adenosine 5'-triphosphate (ATP) concentrations were varied by internally perfusing the pipette with solutions containing either $Na_2ATP$ or MgATP at various concentrations. ATP containing internal solutions were freshly prepared and kept on ice during the experiments.

Perforated patch whole-cell recordings were performed using patch pipettes filled with a solution containing (mM): 75 $K_2SO_4$, 55 KCl, 5 $MgSO_4$, 10 Hepes, adjusted to pH 7·2 with KOH. Pipettes were dipped briefly in the pipette solution and back-filled with the same solution containing 180 $\mu$g ml⁻¹ Amphotericin-B (120 mg ml⁻¹ stock solution in DMSO). Amphotericin-containing solutions were prepared daily and kept on ice and light protected. The osmotic pressures of intra- and extracellular solutions were measured with a vapour pressure osmometer (Wescor 5500).

## Reagents

All chemical reagents used were of analytical grade. The following drugs were used: levcromakalim (BRL 38227, Smith Kline Beecham Pharmaceuticals, UK), adenosine 5'-triphosphate ($Na_2ATP$ and MgATP), amphotericin-B, charybdotoxin, dimethyl-sulphoxide (DMSO), dinitrophenol, dithiothreitol, glibenclamide, iodoacetic acid, papain, tetraethylammonium chloride and tolbutamide (Sigma).

# RESULTS

Experiments were performed on isolated endothelial cells enzymatically dissociated from rabbit aorta and pulmonary arteries. Under resting conditions in the presence of PSS and a pipette solution containing 140 mM K⁺, the arterial endothelial cells studied with the perforated patch configuration had resting membrane potentials ranging from −20 to −50 mV and mean cell capacitances of 25·6 ± 0·2 pF ($n = 370$).

## Levcromakalim activates a glibenclamide-sensitive current

Whole-cell patch clamp experiments were carried out to investigate the activation of $K_{ATP}$ channels in freshly dissociated endothelial cells by levcromakalim and metabolic inhibition. Figure 1$A$ shows perforated patch, whole-cell current recorded at a holding potential of −60 mV during
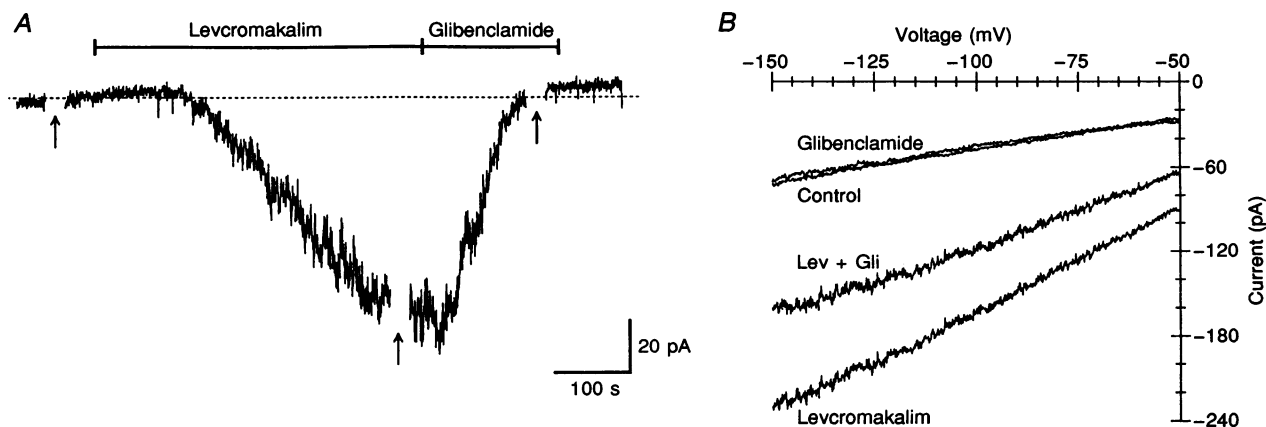
**Figure 1. Glibenclamide-sensitive membrane current evoked by levcromakalim in a rabbit aortic endothelial cell**

Perforated patch whole-cell current recorded from an endothelial cell bathed in HiK solution containing 5 mM TEA. *A*, membrane current obtained with 3 $\mu$M levcromakalim added to the bath solution followed by 10 $\mu$M glibenclamide. The holding current ($-35$ pA, dashed line) began to increase 2 min following bath application of levcromakalim to a maximum of $-80$ pA within 6 min. The levcromakalim-induced current was completely inhibited within 1 min of bath application of glibenclamide. Discontinuities in the current trace indicate times at which voltage ramps were applied (arrows). *B*, whole-cell currents obtained in response to voltage ramps from $-150$ to $-50$ mV applied before (Control) and following application of 3 $\mu$M levcromakalim and upon inhibition of the levcromakalim-induced current by 10 $\mu$M glibenclamide. The net-activated current (Lev + Gli) was obtained by subtracting the *I*–*V* curve obtained following inhibition from that obtained during activation.
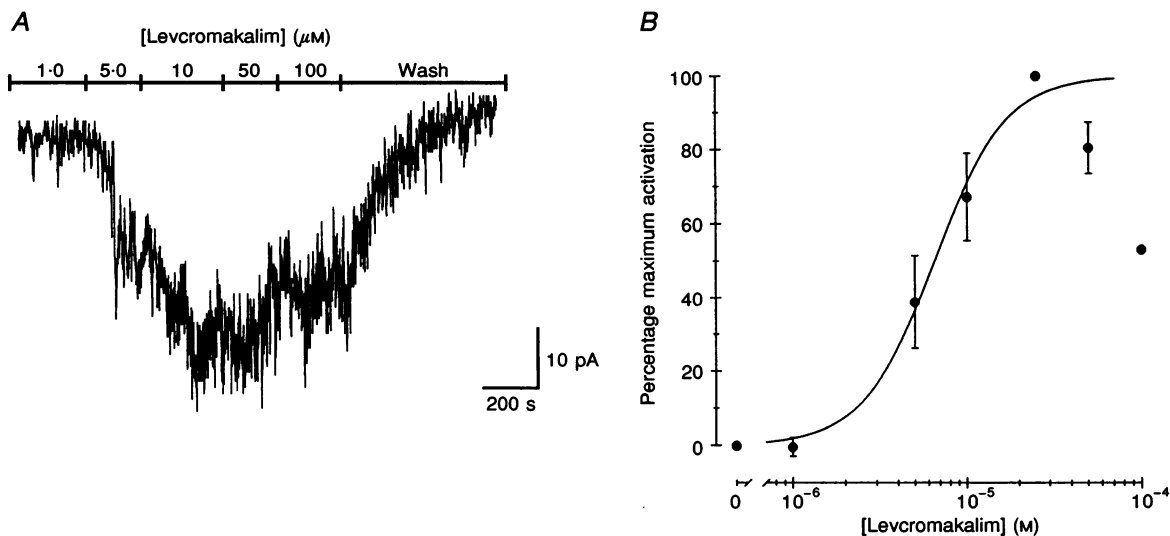


**Figure 2. Dose–response relationship for levcromakalim activation of currents in rabbit arterial endothelial cells**

Membrane currents were recorded using the perforated patch, whole-cell configuration with symmetrical K⁺ solutions (140 mM K⁺, 5 mM TEA external) before and during bath application of levcromakalim. *A*, holding current recorded at $-60$ mV in response to increasing concentrations of bath-applied levcromakalim. *B*, dose–response relationship obtained for levcromakalim-activated currents. Current amplitudes were measured after steady state levels were reached within 100–120 s. The levcromakalim-induced increase in membrane current for each cell ($n = 5$) was normalized with respect to the maximum increase (Percentage maximum activation). The curve is a least-squares fit of the initial five data points to eqn (1) giving a calculated half-maximal current activation at 6·5 $\mu$M levcromakalim and a Hill coefficient of 2·1. Standard errors of the mean are indicated by the bars where they are larger than the symbols.

bath application of an isotonic K$^+$ (140 mM) solution containing 3 $\mu$M levcromakalim. Levcromakalim induced an inward current within 1–2 min of bath application and reached a maximum amplitude of $-45\cdot3 \pm 4\cdot2$ pA corresponding to a current density of $-1\cdot76 \pm 0\cdot15$ pA pF$^{-1}$ ($n = 61$). Currents generated by voltage ramps from $-150$ to $+50$ mV applied under control conditions, during levcromakalim (3 $\mu$M) activation and after addition of 10 $\mu$M glibenclamide (levcromakalim and glibenclamide) inhibition are shown in Fig. 1B. Glibenclamide (10 $\mu$M), an inhibitor of K$_{ATP}$ channels (Ashcroft, 1988; Edwards & Weston, 1993), completely inhibited the membrane current activated by levcromakalim ($n = 36$). The glibenclamide-sensitive current (levcromakalim-induced current) was linear over the voltage range $-150$ to $-50$ mV and had a slope conductance of $0\cdot93 \pm 0\cdot18$ nS which corresponds to a conductance density of $37\cdot1 \pm 8\cdot7$ pS pF$^{-1}$ ($n = 22$). At membrane potentials positive to $-40$ mV, a glibenclamide-insensitive current was observed which was most probably due to activation of a Ca$^{2+}$-dependent K$^+$ current (not shown; Rusko *et al.* 1992; Ling & O'Neill, 1992).

### Concentration dependence of levcromakalim-induced currents

A dose–response relationship obtained for levcromakalim activation of inward currents in rabbit aortic endothelial cells ($n = 5$) is shown in Fig. 2. Voltage-clamped cells were held at $-60$ mV and the holding current was recorded as increasing concentrations of levcromakalim were bath

applied (Fig. 2A). The dose–response relationship was biphasic with half-maximal activation obtained at $6\cdot5$ $\mu$M levcromakalim and maximal activation produced by 25 $\mu$M levcromakalim (Fig. 2B). The magnitude of levcromakalim-induced current decreased with higher levcromakalim concentrations. The curve of best fit to the data was determined for concentrations up to 25 $\mu$M levcromakalim (maximum activation) using eqn (1) (see Methods) and had a Hill coefficient of $2\cdot1$.

### Sulphonylurea inhibition of levcromakalim-induced currents

The dose–response relationship for inhibition of the levcromakalim-induced current by bath application of glibenclamide is shown in Fig. 3. Half-maximal inhibition (IC$_{50}$) of levcromakalim-activated currents was produced by 43 nM glibenclamide and the current was completely inhibited by 10 $\mu$M glibenclamide. Bath application of tolbutamide similarly inhibited the inward K$^+$ currents activated by 3 $\mu$M levcromakalim with half-maximal inhibition occurring at 224 $\mu$M (not shown). The Hill coefficients calculated from fits to the dose–response data for both sulphonylurea drugs were approximately $1\cdot0$ (see Table 1).

### Inhibition of levcromakalim-induced current by Ba$^{2+}$ and tetraethylammonium ions

Bath application of Ba$^{2+}$ or TEA also inhibited the inward currents activated by 3 $\mu$M levcromakalim. Table 1 summarizes the results of dose-dependent inhibition of the
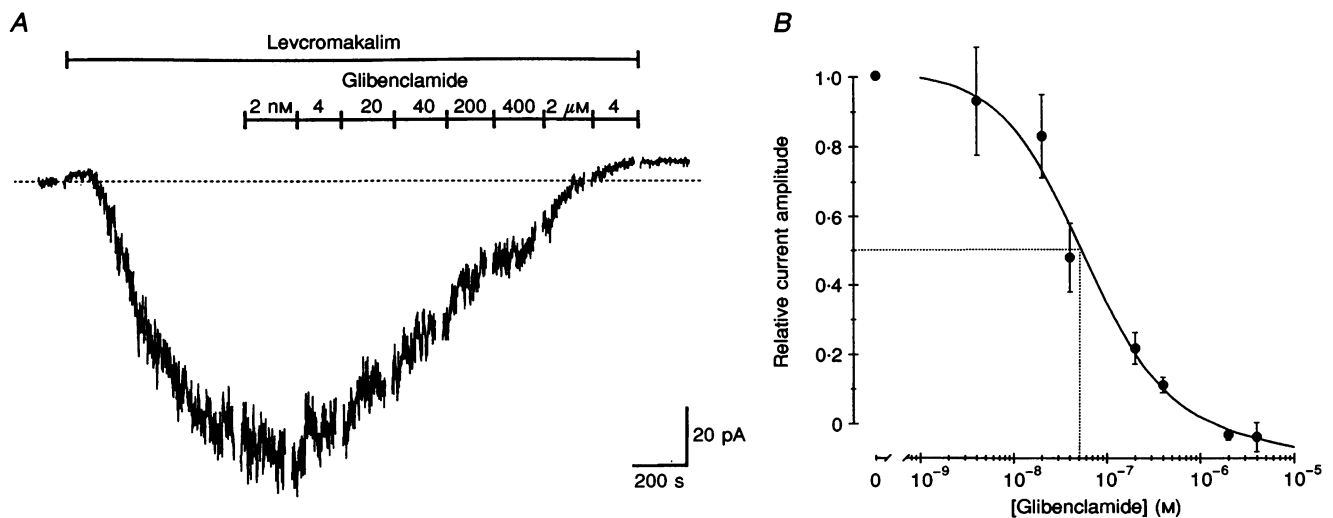


Figure 3. Dose–response relationship for glibenclamide inhibition of levcromakalim-induced currents in rabbit arterial endothelial cells

Membrane currents were recorded using the perforated patch, whole-cell configuration with symmetrical K$^+$ solutions (140 mM K$^+$, 5 mM TEA external). A, holding current recorded before and following activation by 3 $\mu$M levcromakalim and following increasing concentrations of bath-applied glibenclamide. B, dose–response relationship obtained for inhibition of levcromakalim-induced currents by glibenclamide. Currents were measured at $-140$ mV, after steady-state levels were reached within 100–120 s, normalized to currents obtained in the absence of glibenclamide (Relative current amplitude) and plotted as a function of glibenclamide concentration. Data points represent means $\pm$ S.E.M. for three cells. The curve is a least-squares best fit to eqn (1).

Table 1. Half-maximal inhibitory concentrations (IC$_{50}$) and Hill coefficients ($n_H$) for inhibition
of K$_{ATP}$ currents in rabbit arterial endothelial cells

| Inhibitor | IC$_{50}$ | $n_H$ |
|---|---|---|
| Glibenclamide | 43 nM | 1·1 |
| Tolbutamide | 224 $\mu$M | 1·2 |
| Ba$^{2+}$ | 0·6 mM | 0·9 |
| TEA | 5·6 mM | 1·9 |

Dose–response measurements were made in symmetrical K$^+$ solutions (140 mM K$^+$) at a holding potential
of −60 mV. Values were obtained from a least-squares fit of the data to a single site adsorption isotherm
according to eqn (1) (see Methods).

levcromakalim-activated K$_{ATP}$ conductance by Ba$^{2+}$ and TEA. The peak amplitude of levcromakalim-activated currents were measured in symmetrical K$^+$ solutions at −60 mV. The calculated half-maximal inhibitory concentrations (IC$_{50}$) for Ba$^{2+}$ and TEA were 0·6 mM and 5·6 mM, respectively. Membrane currents generated by voltage ramps indicate that both Ba$^{2+}$ and TEA inhibited the levcromakalim-activated currents in a voltage-independent manner (not shown). Although the IC$_{50}$ for TEA inhibition of the levcromakalim-induced current was 5·6 mM, this concentration of TEA completely blocks the Ca$^{2+}$-activated K$^+$ conductance in this preparation (Rusko et al. 1992). Bath application of 100 nM charybdotoxin, which has been shown to block the large conductance Ca$^{2+}$-dependent K$^+$ channel in freshly dissociated endothelial cells (Rusko et al. 1992), failed to change the peak amplitude of the levcromakalim-induced whole-cell current obtained at −60 mV in symmetrical K$^+$ solutions ($n = 4$).

### Metabolic inhibition activates a glibenclamide-sensitive current

Inhibition of mitochondrial ATP synthesis with dinitrophenol and the glycolytic pathway with iodoacetic acid, blocks production of intracellular ATP (van Breemen, Wuytack & Casteels, 1975). A glucose-free, isotonic K$^+$ (140 mM) solution containing dinitrophenol (50 $\mu$M) and iodoacetic acid (1 mM) activated an inward current in endothelial cells (Fig. 4A). The inward current induced by metabolic inhibition exhibited a slower onset (3–4 min) than that observed for the levcromakalim-induced current and maximal activation of the current −57·8 ± 20·2 pA (−2·47 ± 0·96 pA pF$^{-1}$; $n = 9$) occurred within 1–3 min following onset. Glibenclamide (10 $\mu$M) completely inhibited the current activated by metabolic inhibition ($n = 9$), as shown in Fig. 4A. Voltage ramps applied under control conditions, during metabolic inhibition and after addition of 10 $\mu$M glibenclamide (MI + Gli) are shown in Fig. 4B. The K$^+$ current activated by metabolic inhibition has a linear I–V relationship in the −150 to −50 mV voltage range and a slope conductance of 1·3 ± 0·2 pS, which corresponds to a conductance density of 47·8 ± 8·1 pS pF$^{-1}$ ($n = 12$).

### Sensitivity of membrane current to internal perfusion of ATP

Inhibition of ATP production is an indirect method of changing intracellular ATP concentrations. To directly manipulate intracellular ATP levels of voltage-clamped endothelial cells, an internal (pipette) perfusion system was implemented (Nelson, Jow & Jow, 1990), allowing for changes of the pipette solution in the whole-cell recording configuration. Figure 5 shows the results of an internal perfusion experiment in which the intracellular ATP concentration was changed. Following depletion of intracellular ATP levels by metabolic inhibition, exchange of the nominally ATP-free (0·1 M) patch pipette solution with the same solution containing 3 mM MgATP decreased the magnitude of the inward current (Fig. 5A). The membrane currents elicited by voltage ramps were linear in the range −150 to −50 mV and had a slope conductance of 0·36 ± 0·09 nS, which corresponds to a conductance density of 12·7 ± 2·7 pS pF$^{-1}$ ($n = 5$; Fig. 5B). In these five cells we were unable to reactivate the ATP-sensitive inward K$^+$ current by internally perfusing the cell with a 0·1 $\mu$M MgATP pipette solution following internal perfusion with 3 mM ATP. However, superfusion of endothelial cells, which did not respond to an ATP-free pipette solution, with 25 $\mu$M levcromakalim produced an inward current of 132 ± 16 pA ($n = 31$). Inclusion of either 0·5 mM ADP and/or 0·5 mM GTP in ATP-free internal solutions had no effect on the K$_{ATP}$ current (not shown).

### Effect of levcromakalim and glibenclamide on the resting membrane potential

The activation of a membrane current by levcromakalim hyperpolarized the resting membrane potential of arterial endothelial cells measured with perforated patch recordings. Figure 6A shows a membrane potential response to bath application of 3 $\mu$M levcromakalim followed by the addition of 10 $\mu$M glibenclamide ($n = 12$). The time courses for levcromakalim-induced hyperpolarization (−10·9 ± 1·9 mV changes in membrane potential) and for glibenclamide inhibition were both similar to that observed for the change in holding current under voltage clamp conditions. A hyperpolarization of similar amplitude (−15 mV) was
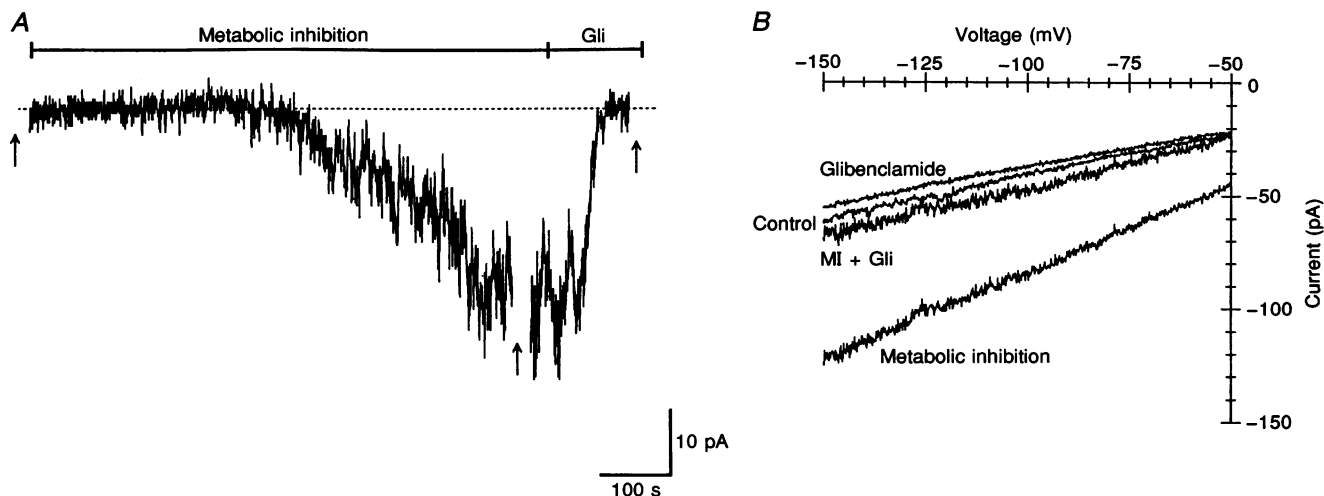
**Figure 4. Glibenclamide-sensitive membrane currents evoked by metabolic inhibition of rabbit aortic endothelial cell**

Perforated patch, whole-cell current recorded at a holding potential of −60 mV from an endothelial cell bathed in HiK-S solution containing 5 mM TEA. The pipette solution contained 140 mM K+ and 55 mM Cl−. *A*, membrane current activated by metabolic inhibition (MI) of the cell using a HiK-S solution containing 50 μM dinitrophenol and 1 mM iodoacetic acid. Bath application of 10 μM glibenclamide (Gli) inhibited the MI-activated current. Holding current, −25 pA (dashed line), began to increase ∼3 min following bath application of the metabolic inhibitors and increased to a maximum of −32 pA in 9 min. The current was completely inhibited within 2 min of bath application of glibenclamide. Discontinuities in the current trace indicate the times at which voltage ramps were applied (arrows). *B*, whole-cell currents obtained in response to voltage ramps from −150 to −50 mV applied before (Control) and during application of metabolic inhibitors and following inhibition by 10 μM of glibenclamide. Subtraction of the current trace obtained following inhibition from that obtained during activation gives the net-activated current (MI + Gli).
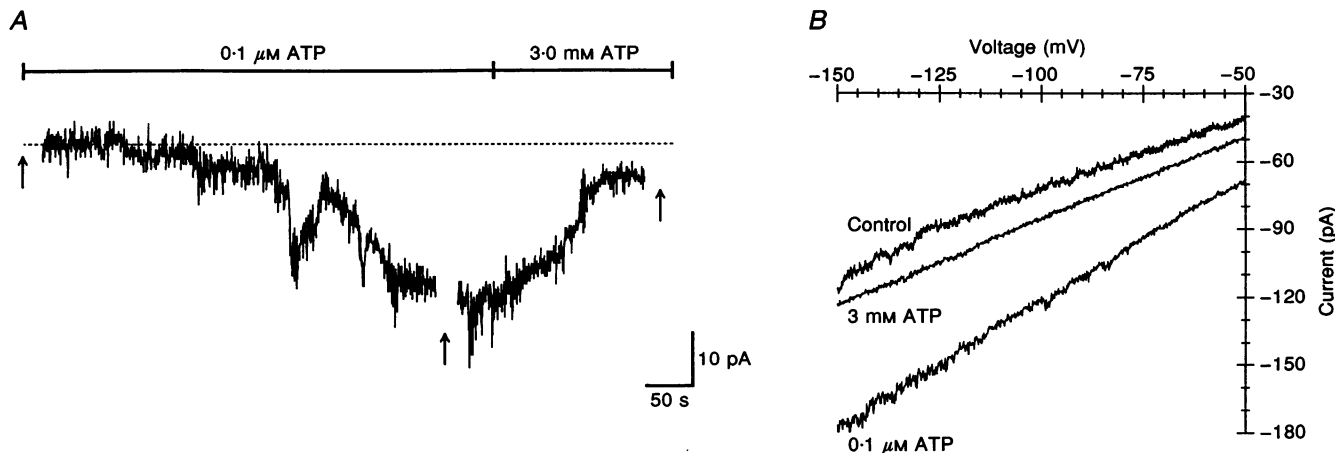


**Figure 5. Sensitivity of membrane current to intracellular [ATP] in rabbit aortic endothelial cell**

Membrane current recorded in symmetrical K+ solutions from a cell held at −60 mV. The cell was metabolically inhibited by pre-incubation in PSS-sucrose solution for 30 min. *A*, response of the holding current to exchange of cytosol with a nominally ATP-free pipette solution, followed by an internal perfusion of the cell with a 3 mM MgATP solution. The dashed line indicates the initial holding current of −45 pA. Discontinuities in the current trace (arrows) indicate the times at which voltage ramps were applied. *B*, whole-cell currents in response to voltage ramps from −150 to −50 mV were applied immediately after rupturing the membrane patch (Control), during dialysis of the cell with a nominally ATP-free (0·1 μM) pipette solution and following internal perfusion of the pipette/cell with a 3 mM MgATP solution.

**Table 2. Reversal potentials ($E_{rev}$) of levcromakalim-induced currents
carried by various external monovalent cations**

| External cation | $E_{rev}$ (mV) | $n$ |
|---|---|---|
| K$^+$ | 6·6 ± 3·2 | 8 |
| Rb$^+$ | 21·9 ± 10·1 | 3 |
| Cs$^+$ | −3·2 ± 16·7 | 3 |
| Na$^+$ | −43·6 ± 22·7 | 3 |
| NH$_4$$^+$ | −60·8 ± 26·4 | 3 |
| Li$^+$ | −77·6 ± 11·5 | 3 |

Values represent the mean ± S.E.M. External cation concentrations were 140 mM and the internal pipette solution contained 140 mM K$^+$. The reversal (zero current) potential was determined from the averaged current response to five voltage ramps from −140 to −40 mV obtained during levcromakalim activation and following tolbutamide inhibition. The averaged currents following tolbutamide inhibition were subtracted from the averaged currents obtained during levcromakalim activation. A least-squares linear fit of the difference current was extrapolated to give the reversal potential (corrected for liquid junction potentials).

induced by metabolic inhibition (50 μM dinitrophenol and 1 mM iodoacetic acid in PSS-sucrose) and was reversed by bath application of 10 μM glibenclamide (not shown). The shift in reversal (zero current) potential of the whole-cell I–V relationship upon channel activation by levcromakalim is also evident from membrane currents generated by voltage ramps. Figure 6B shows membrane currents in response to voltage ramps from −150 to +50 mV obtained before and after levcromakalim activation and following glibenclamide block of the activated current. The reversal potential is shifted to more negative potentials by 36 mV following application of levcromakalim.

In some experiments described above, the addition of glibenclamide (in the presence of levcromakalim) reduced the inward holding current below control levels. In 47% (twenty of forty-five) of cells studied whose levcromakalim-activated currents were inhibited by glibenclamide (10 μM), the holding currents measured following glibenclamide inhibition were smaller than the pre-activation currents (see Figs 1A and 3A). Similarly, following tolbutamide (1 mM) inhibition of levcromakalim-activated currents, in 39% (twelve of thirty-three) of cells studied, the holding current recorded at −60 mV was reduced from the initial, pre-activation, holding current. However, no decrease in
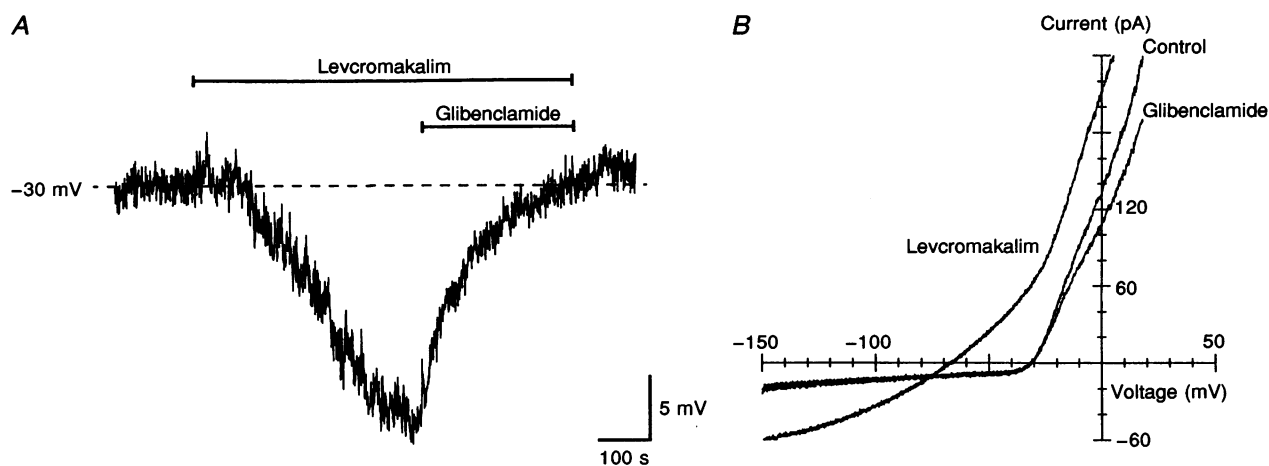


**Figure 6. Levcromakalim-induced hyperpolarization of the resting membrane potential**
*A*, membrane potential recorded from an endothelial cell using the perforated patch, whole-cell recording configuration in current clamp mode. The levcromakalim (3 μM)-induced hyperpolarization was reversed by bath application of 10 μM glibenclamide. The bathing solution was normal PSS containing 5 mM TEA. The pipette solution contained 75 mM K$_2$SO$_4$, 55 mM KCl and 180 μg ml$^{-1}$ amphotericin-B. *B*, whole-cell membrane currents measured in response to voltage ramps from −150 to 50 mV. Levcromakalim (25 μM) activated an ionic current shifting the zero current potential in the hyperpolarized direction. Bath application of 10 μM glibenclamide completely blocked the levcromakalim-induced current and partly reduced the outward current. The bathing solution was normal PSS and the pipette solution contained 75 mM K$_2$SO$_4$ and 55 mM KCl.

holding current was observed in endothelial cells exposed to glibenclamide alone ($n = 18$).

## Ionic selectivity of the sulphonylurea-sensitive conductance

To determine the ionic selectivity of the $K_{ATP}$ conductance, the levcromakalim-induced, tolbutamide-inhibited current was measured with various monovalent cations substituted for external $Na^+$. The levcromakalim-induced current was $K^+$-selective whereby a 50 mV shift to less negative potentials was observed when normal PSS (140 mM $Na^+$) was replaced with a 140 mM $K^+$ solution. In the presence of 140 mM $K^+$ bath solution, the reversal potential measured was $6.6 \pm 3.2$ mV ($n = 8$). Using the Goldman–Hodgkin–Katz voltage equation, the calculated permeability ratio of $Na^+$ to $K^+$ was 0.13. Table 2 summarizes the reversal (zero current) potentials measured in several cells upon replacement of external $Na^+$ with various monovalent cations. The reversal potentials were calculated from extrapolations of linear fits of the $I–V$ relationships

obtained in response to voltage ramps applied from −150 to −50 mV and corrected for junction potentials. The maximum amplitude and reversal potential of levcromakalim-induced inward currents were unchanged upon replacement of external $Cl^-$ by $SO_4^{2-}$, suggesting that the levcromakalim-induced currents are insensitive to changes in external $Cl^-$ concentration (not shown).

## Response of cytoplasmic [Ca²⁺] to levcromakalim and metabolic inhibition

Intracellular $Ca^{2+}$ levels in freshly dissociated endothelial cells were monitored using the fluorescent $Ca^{2+}$ indicator dye, fura-2. Fura-2-loaded endothelial cells (see Methods), with resting $[Ca^{2+}]_i$ of approximately 100 nM, were exposed (3 min) to bath application of either 25 $\mu$M levcromakalim, or 50 $\mu$M dinitrophenol and 1 mM iodoacetic acid. In all endothelial cells studied ($n = 13$), levcromakalim failed to produce a significant change in resting $[Ca^{2+}]_i$, whereas metabolic inhibition produced a slow and sustained increase in $[Ca^{2+}]_i$ ($n = 4$) as shown in Fig. 6. The magnitude of the
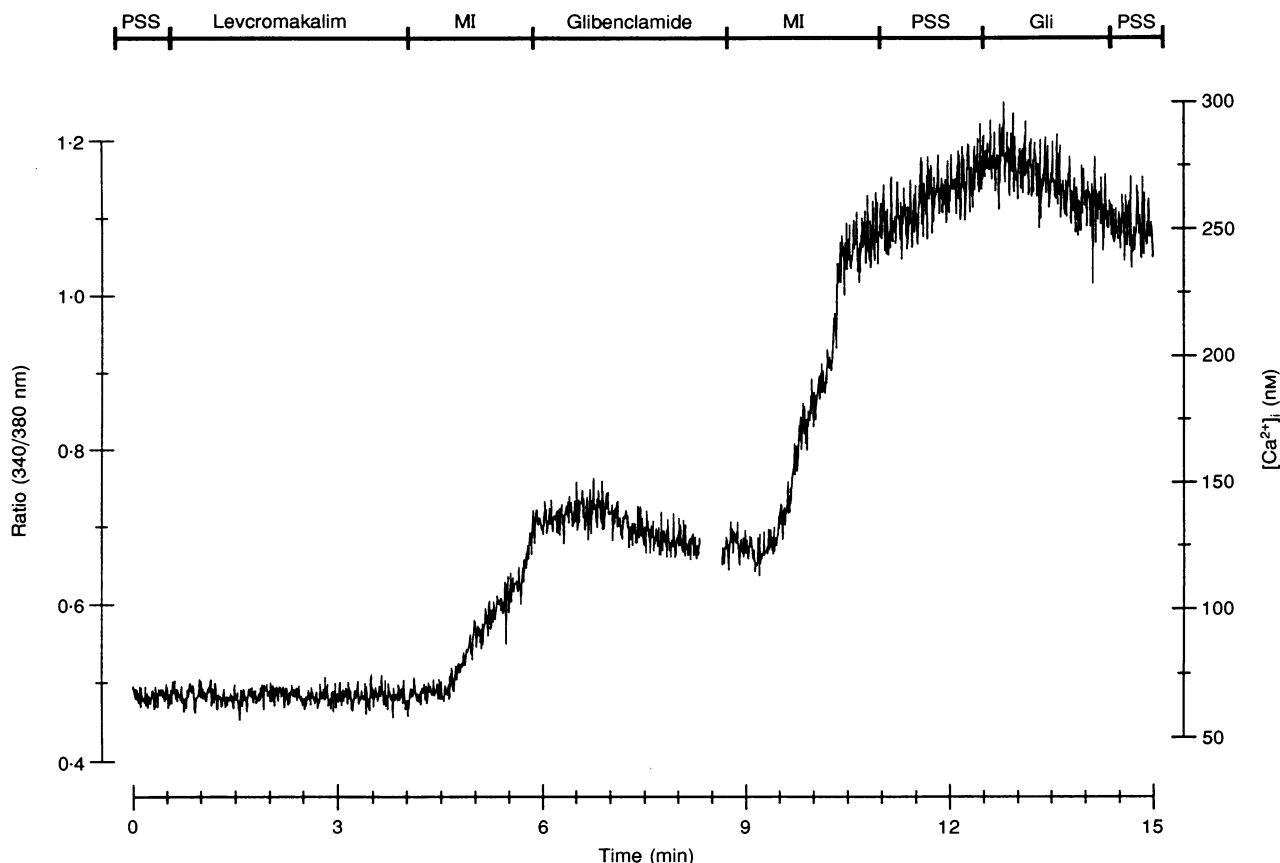


**Figure 7. Endothelial cell cytoplasmic Ca²⁺ levels in response to levcromakalim and metabolic inhibition (MI)**

Bath application of 25 $\mu$M levcromakalim in PSS (physiological salt solution) to fura-2-loaded endothelial cells produced no change in resting $[Ca^{2+}]_i$ as measured by the ratio of fluorescence intensities from excitation by 340 and 380 nm wavelength light. The addition of 50 $\mu$M dinitrophenol and 1 mM iodoacetic acid to the bath solution produced a slow increase in $[Ca^{2+}]_i$ which was reduced by external application of 10 $\mu$M glibenclamide, but not by washing with PSS. Experimental data are plotted as the ratio of emission intensities and as the calculated $[Ca^{2+}]_i$ (see Methods).

increase in $[Ca^{2+}]_i$ produced by metabolic inhibition was attenuated by bath application of 10 $\mu$M glibenclamide, whereas superfusing the cell with PSS following metabolic inhibition did not decrease $[Ca^{2+}]_i$. Metabolic inhibition increased $[Ca^{2+}]_i$ at a rate of $47\cdot2 \pm 14\cdot8$ nM min$^{-1}$ ($n = 5$) and subsequent perfusion with 10 $\mu$M glibenclamide decreased $[Ca^{2+}]_i$ at a rate of $10\cdot3 \pm 2\cdot2$ nM min$^{-1}$ ($n = 5$). Superfusion of endothelial cells with PSS only slowed the rate of increase of $[Ca^{2+}]_i$ to $2\cdot5 \pm 7\cdot8$ nM min$^{-1}$ ($n = 3$).

## DISCUSSION

Our results indicate that native rabbit aortic endothelial cells exhibit an $K_{ATP}$ conductance which contributes to the resting membrane potential. This $K_{ATP}$ conductance in endothelial cells is inhibited by millimolar concentrations of intracellular ATP and is activated either by the $K^+$ channel opener, levcromakalim, or by metabolic inhibition. The $K_{ATP}$ current is reversibly inhibited by the sulphonylurea drugs, glibenclamide and tolbutamide, and by external TEA or $Ba^{2+}$ but not by charybdotoxin. The pharmacological properties of the $K_{ATP}$ conductance in endothelial cells are characteristic of the family of $K_{ATP}$ channels (Ashcroft, 1988; Edwards & Weston, 1993). Endothelial cells freshly dissociated from rabbit pulmonary artery also responded to levcromakalim, metabolic inhibition and sulphonylurea drugs in a similar manner (not shown).

Under physiological conditions, the ionic channels underlying the ATP-sensitive conductance are $K^+$-selective, exhibiting the following ionic selectivity sequence: $Rb^+ > K^+ > Cs^+ \gg Na^+ > NH_4^+ > Li^+$. The ATP-sensitive inward $K^+$ current is insensitive to changes in external $Cl^-$ concentration. Activation of the $K_{ATP}$ conductance by levcromakalim hyperpolarized the endothelial cell by $-10\cdot9 \pm 1\cdot9$ mV ($n = 12$) in the presence of physiological saline solutions.

An increase in $[Ca^{2+}]_i$ in cultured bovine aortic endothelial cells was observed when cromakalim (BRL 34915) was externally applied to cultured porcine aortic endothelial cells prestimulated with thimerosal (Lückhoff & Busse, 1990a). In the present study, microfluorometric measurements of fura-2-loaded endothelial cells freshly dissociated from rabbit aorta indicate that activation of the $K_{ATP}$ conductance by levcromakalim is insufficient to produce a change in $[Ca^{2+}]_i$. Therefore, it is unlikely that under hypoxic conditions vascular tone is modulated by activation of $K_{ATP}$ channels through $Ca^{2+}$-dependent release of EDRF (see Richards, Gibson & Martin, 1991). However, the endothelium may influence smooth muscle tone by increasing extracellular $K^+$, which is itself a potent vasodilator. Efflux of $K^+$ through open $K_{ATP}$ channels rather than activation of a $Ca^{2+}$-dependent release of EDRF may regulate vascular tone. Bioassay studies of EDRF release, for example, have shown that elevation of extracellular $K^+$ produces relaxation of smooth muscle cells (Rubanyi & Vanhoutte, 1988).

Sulphonylurea drugs applied following levcromakalim activation reduced whole-cell currents below initial levels in 44% of the endothelial cells studied. This change in the holding current of $> 5$ pA obtained at $-60$ mV in symmetrical $K^+$ solutions suggests that the $K_{ATP}$ conductance may be partly activated in freshly isolated endothelial cells, possibly due to ATP depletion during the isolation procedure. It is also possible that the sulphonylureas may be inhibiting another type of ionic channel other than the $K_{ATP}$ channel. For example, sulphonylureas have been found to block a $Cl^-$ conductance in fibroblasts infected with a retrovirus expressing human cystic fibrosis transmembrane conductance regulator (Sheppard & Welsh, 1992). The lack of effect of glibenclamide alone on whole-cell currents, however, suggests that the decrease in membrane current observed in the presence of glibenclamide following stimulation by levcromakalim may be due to a time-dependent change in the holding current.

The magnitude of $K_{ATP}$ currents measured in other tissues using the excised patch or whole-cell configurations typically exhibit 'rundown' during the course of an experiment. A proposed mechanism for current rundown is dephosphorylation of the channel protein by endogenous phosphatases which can be reversed by exposure to micromolar concentrations of MgATP (Findlay & Dunne, 1986). In whole-cell patched endothelial cells, we were unable to reactivate the ATP-blocked inward current by internally perfusing the cell with a $0\cdot1$ $\mu$M MgATP pipette solution; however, external application of levcromakalim was able to evoke an inward current in these cells that was inhibited by glibenclamide. These data suggest that the inability to reactivate the $K_{ATP}$ conductance may be due to a higher local ATP concentration adjacent to the $K_{ATP}$ channel than is present in the bulk pipette solution, rather than due to rundown of the $K_{ATP}$ conductance exclusively.

In cultured endothelial cells, the predominant resting conductance is an inwardly rectifying $K^+$ channel (Adams et al. 1989); however, recent studies of native endothelial cells have not found evidence for the existence of an inwardly rectifying $K^+$ conductance (Rusko et al. 1992; Katnik & Adams, 1994). Interestingly, an inwardly rectifying $K^+$ conductance in cultured bovine aortic endothelial cells has recently been shown to exhibit an ATP dependence opposite to that of the $K_{ATP}$ conductance observed in the present study (Olesen & Bundgaard, 1993). Exposure of excised patches from cultured endothelial cells to ATP-free solutions or solutions containing metabolic inhibitors decreased the open probabilities of the inward rectifying $K^+$ channel. In contrast, our data from freshly dissociated endothelial cells show that a $K^+$ current is blocked by 3 mM internal ATP and is activated by both metabolic inhibition and levcromakalim resulting in membrane hyperpolarization.

It is interesting to speculate as to what role endothelial $K_{ATP}$ channels play in vascular physiology. $K^+$-selective

conductances are responsible for establishing the resting membrane potential of aortic endothelial cells (see review by Adams *et al.* 1989). Therefore, the existence of K$_{ATP}$ channels in these cells suggests that the membrane potential may be regulated in response to altered cellular metabolism, presumably due to a change in the relative concentrations of intracellular ADP and ATP. The vascular endothelium, serving as an interface between the circulating blood and the smooth muscle cells, is capable of transducing changes in blood composition and rheology into changes in smooth muscle tone. K$_{ATP}$ channels have been shown to mediate both K$^+$ channel activator-induced vasodilatation in rabbit pulmonary artery strips (Clapp *et al.* 1993) and hypoxia-induced vasodilatation in guinea-pig coronary arteries (Daut, Maier-Rudolph, von Beckerath, Mehrke, Günther & Goedel-Meinen, 1990). Hypoxic conditions caused a minor change in membrane potential and increased the input resistance of monolayers of cultured bovine aortic and guinea-pig coronary endothelium whereas the K$^+$ channel openers, cromakalim and levcromakalim, at a concentration of 1 $\mu$M had no effect on the resting membrane potential (Mehrke, Pohl & Daut, 1991). However, glibenclamide-sensitive release of EDRF in response to increased shear stress has recently been reported in rabbit aortic preparations (Hutcheson & Griffith, 1994). The presence of K$_{ATP}$ channels in endothelial cells suggests that a potential site of action of sulphonylurea drugs and ATP-depletion on vascular tone may be the endothelium.

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