British Journal of Pharmacology (1996) 118, 635-642

Activation by levcromakalim and metabolic inhibition of glibenclamide-sensitive K channels in smooth muscle cells of pig proximal urethra

[1]Noriyoshi Teramoto & Alison F. Brading

University Department of Pharmacology, Mansfield Road, Oxford OX1 3QT

1 The effects of levcromakalim (BRL 38227) on ionic currents recorded from pig proximal urethra were investigated by use of tension measurement and patch clamp techniques (conventional whole-cell configuration, nystatin perforated patch, and cell-attached configuration).

2 Levcromakalim (1 μM) caused a relaxation in the resting tone. This levcromakalim-induced relaxation was inhibited by the pretreatment with 1 μM glibenclamide.

3 The resting membrane potential recorded from single cells in current-clamp mode was -36.1 ± 4.4 mV ($n=5$).

4 Levcromakalim induced a concentration-dependent hyperpolarization with a maximum (at $\geqslant 10$ μM) close to the theoretical equilibrium potential of potassium (E_K). The membrane hyperpolarization caused by 1 μM levcromakalim (24.7 ± 5.8 mV, $n=4$) was abolished by 1 μM glibenclamide.

5 Levcromakalim (100 μM) caused an outward K current in whole-cell recordings which was unaffected by iberiotoxin (300 nM) but abolished by glibenclamide (10 μM).

6 In cell-attached patches, levcromakalim activated a 43 pS K channel which was inhibited by the application of glibenclamide.

7 The metabolic poison, cyanide (CN), also activated a 43 pS K channel which was suppressed by the application of 10 μM glibenclamide.

8 These results indicate that levcromakalim and metabolic inhibition activate the same 43 pS K channel in pig proximal urethra. The resultant urethral hyperpolarization might reduce the usefulness of K channel openers in the treatment of detrusor instability, but be of value in treating outflow obstruction.

Keywords: Glibenclamide; levcromakalim; potassium channels; iberiotoxin; cyanide; potassium channel opener; resting membrane potential; urethra; urethral resting tone

Introduction

A potassium channel inhibited by intracellular ATP (referred to as an ATP-sensitive K channel, K_{ATP}) was first observed in cardiac muscle (Noma, 1983). K_{ATP} opened when inside-out patches excised from cells with normal intracellular ATP concentrations ($[ATP]_i$) were exposed to ATP-free conditions. Subsequently, channels with similar characteristics were identified in pancreatic β-cells (Cook & Hales, 1984), skeletal muscle (Spruce *et al.*, 1985) and neurones (Ashford *et al.*, 1988).

In smooth muscles, a wide variety of K channels with many different characteristics have been reported to be targets for activation by several kinds of K channel openers (KCOs). More recently a general agreement seems to have been reached regarding the characteristics of KCO-activated K channels. The channel is (i) of a relatively small conductance (about 20 pS under quasi-physiological conditions, approximately 30-50 pS channel in symmetrical 140 mM K^+ conditions), (ii) Ca-insensitive and (iii) inhibited by intracellular ATP. Moreover, this channel has a potent sulphonylurea-sensitivity at a submicromolar concentration (reviewed by Edwards & Weston, 1993). KCOs have been investigated for clinical treatment of bladder instability. This is a common sequelae of bladder outlet obstruction caused by benign prostatic hyperplasia in adult males and gives rise to significant bladder dysfunction such as frequency and urgency of micturition. The unstable detrusor contractions may also lead to urge incontinence (reviewed by Andersson, 1993; Brading & Turner, 1994). Micturition in the pig closely resembles that in the human subject (Melick *et al.*, 1961), and experimentally-induced bladder instability can be abolished by KCOs (Foster *et al.*, 1989b). To be effective in the treatment of bladder instability, KCOs would need to be bladder-selective and not relax urethral smooth muscle. We have therefore studied pig urethral smooth muscle and revealed the presence of tolbutamide- and glibenclamide-sensitive K channels (a 43 pS K channel) in proximal urethra (Teramoto & Brading, 1995). In the present experiments, we have further investigated the effects of levcromakalim and glibenclamide on both the resting tone and the resting membrane potential and also studied the effects of the metabolic poison, cyanide, on this channel.

Methods

Fresh female pig urethras were obtained from a local slaughter house and transported in a cold saline solution (mM): Na^+ 137, K^+ 5.9, Mg^{2+} 0.5, Ca^{2+} 0.5, Cl^- 128.3, HCO_3^- 15.4, $H_2PO_4^-$ 1.2 and glucose 11.5, bubbled with 97% O_2 and 3% CO_2 (pH 7.25-7.3). A segment of proximal of urethra was excised from a region 1-2 cm from the bladder neck. Connective tissue and the mucosa were removed by dissection.

Tension measurement

Fine strips (1.0-1.2 mm length, 0.4-0.5 mm width, 0.3-0.4 mm thickness) were prepared as previously described (Fujii *et al.*, 1990), transferred to a small chamber (0.2 ml volume) and attached to a Dynamometer UFI transducer (Harvard Apparatus Ltd., Kent, U.K.) and isometric tension measurement was performed at 37°C. The strips were tensioned by

[1] Author for correspondence.

applying a 1 g weight, and allowed to equilibrate for 1 h before the start of the experiments. To prevent noradrenaline outflow from sympathetic nerve terminals and β-adrenoceptor stimulation, 3 μM guanethidine and 0.3 μM propranolol were added to all solutions. Drugs were administered in the desired concentrations in the superfusing solution (1.4 ml min^{-1}). The data were recorded on a DAT tape recorder (48 kHz, SONY, DTC-1000ES, Tokyo, Japan) and analysed on a computer (Macintosh Quadra 610, U.S.) using the commercial software 'MacLab 3.4.2' (ADInstruments Pty Ltd, Castle Hill, Australia).

Cell dispersion

We employed a method described by Clapp & Gurney (1991) with minor modification. The cell preparation was essentially the same as described previously (Teramoto & Brading, 1995). Thin strips of smooth muscle (10–15 mm × 2–4 mm) were dissected from the fresh proximal urethral wall and stored in nominally Ca^{2+}-free solution (mM): Na^+ 140, K^+ 5, Mg^{2+} 0.5, Cl^- 146, HEPES 10/Tris, titrated to pH 7.35–7.4, containing papain (Sigma, 17 unit mg^{-1} protein, 0.2–0.3 mg ml^{-1}) bubbled with 97% O_2 and 3% CO_2, at 6–8°C for 12–14 h. The digested strips were washed in Ca^{2+}-free solution complemented with 1 mg ml^{-1} bovine serum albumin (BSA), and preincubated in Ca^{2+}-free solution at 35°C for 5–6 min. The strips were then incubated in Ca^{2+}-free solution containing 0.3–0.4 mg ml^{-1} collagenase (Type IA) at 35°C for 10–15 min. Finally, after washing the strips with Ca^{2+}-free solution, the digested strips were kept in a test-tube and then the test-tube was tapped until sufficient cells were yielded. They were normally used within 5 h for experiments and were stored at 4°C.

Solutions

For tension measurement, a modified Krebs solution was used (mM): Na^+ 137, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.0, Cl^- 132.7, HCO_3^- 15.4, $H_2PO_4^-$ 1.2 and glucose 11.5, bubbled with 97% O_2 and 3% CO_2 (pH 7.35–7.40 at 37°C). The following solutions were used for whole-cell recordings (conventional whole-cell and nystatin-perforated patch). The physiological salt solution (PSS) had the following composition (mM): Na^+ 140, K^+ 5, Mg^{2+} 1.2, Ca^{2+} 2.0, Cl^- 151.4, HEPES 10 and was titrated to pH 7.35–7.40 with Tris base; 60 mM K^+ and 140 mM K^+ PSS were obtained by replacing 55 mM Na^+ and 135 mM Na^+ with equimolar K^+. The pipette solution contained (mM): K^+ 140, Cl^- 140, ethylene glycol-*bis* (β-aminoethylether) *N,N,N,N'*-tetraacetic acid (EGTA) 5, HEPES 10/Tris (pH 7.35–7.40). For the single-channel recordings (cell-attached configuration), the composition of both pipette and bath solution was the same (mM): K^+ 140, Cl^- 140, EGTA 5, HEPES 10/Tris (pH 7.35–7.40) i.e. symmetrical conditions. In some experiments, a proportion of external Cl^- was replaced with equimolar CN^-. Cells were allowed to settle in the small experimental chamber (80 μl in volume). The bath solution was superfused by gravity throughout the experiments at a rate of 2 ml min^{-1}. For rapid drug application we used the flowing tube system (concentration-jump technique) described by Yellen (1982). The following chemicals were used: BSA, EGTA, HEPES, iberiotoxin, collagenase, glibenclamide, guanethidine, propranolol, nystatin and papain (Sigma, Dorset, U.K.), KCN and Tris (BDH Chemicals Ltd., Dorset, U.K.). Levcromakalim (kindly provided by the SmithKline Beecham, U.K.) was prepared daily as a 100 mM stock solution in dimethysulphoxide (DMSO). Glibenclamide was also prepared as a 100 mM stock solution in DMSO. Both drugs were diluted just before use. The final concentration of DMSO was less than 0.1% and this concentration did not affect either the membrane currents or the potassium channels. The perforated-patch technique with nystatin (Horn & Marty, 1988) was also occasionally used to record whole-cell currents. Nystatin was freshly dissolved in acidified methanol (1 N HCl to about pH 2). After dissolving nystatin, the pH was adjusted to 7.4 with Tris base. This stock solution (10 mg ml^{-1}) was diluted in the pipette solution at a final concentration of 50 μg ml^{-1} just before use. Whole-cell recording was performed with a pipette which was first dipped in normal pipette solution (nystatin-free) and then back-filled with nystatin-containing pipette solution, which resulted in chemical perforation of the membrane.

Recording procedure

The experimental system used was essentially the same as described previously (Inoue & Brading, 1990). Briefly, patch electrodes were prepared with a double-step electrode puller (PP-83, Narisige Co. Ltd., Tokyo, Japan) and a home-made heat polisher. A high resistance seal (>10 GΩ) was obtained by application of a negative pressure to the suction electrode as described by Hamill *et al.* (1981). Current-clamp and voltage-clamp recordings were obtained with the conventional whole-cell configuration. Generation of voltage pulses was performed using a L/M-EPC 7 patch-clamp amplifier (List-Medical-Electronic, Darmstadt, Germany) in conjunction with a circuit board which has both an analog to digital and a digital to analog conversion function (AD/DA converter, DT2801A, Data Translation, U.K.), using a 'VGEN' software package (kindly provided by Dr Dempster, the University of Strathclyde, U.K.). The sampled current data were filtered at 10 kHz and stored together with potential records on videotape using a pulse code modulation unit (16 bit resolution, SONY PCM-701, Tokyo, Japan) coupled to a video recorder (Panasonic AG-6200, Osaka, Japan) for subsequent off-line analysis. Junction potentials between bath and pipette solutions were measured using a 3 M KCl reference electrode and were <2 mV, so that correction for these potentials was not made. Capacitance noise was kept to a minimum by maintaining the test solution in the electrode as low as possible. At the beginning of each experiment, the series resistance was compensated. Experiments were carried out at room temperature (21–23°C).

Data analysis

The whole-cell current data were low-pass filtered at 500 Hz (continuous traces) or 5 kHz (ramp currents) (−3 dB) by an 8 pole Bessel filter, sampled at 50 ms (continuous traces) or 0.5 ms (voltage step pulse currents and ramp currents) and analysed on a computer (Macintosh Quadra 610, U.S.) using the commercial software 'Mac Lab 3.4.2' (ADInstruments Pty Ltd, Castle Hill, Australia) and leakage current was not subtracted. For single channel recording, the stored data were low-pass filtered at 1 kHz (−3dB) and sampled into the computer with an interval of 200 μs using 'PAT' programme (kindly provided by Dr Dempster, the University of Strathclyde, U.K.), but events briefer than 200 μs were not included in the evaluation. Data points were fitted by a least-squares fitting. The all-point amplitude histogram was obtained from a continuous recording of 2 min and fitted with the Gaussian equation using a least-squares fitting. Continuous traces in the figures (>10 s) were obtained from records filtered at 500 Hz for presentation. In the present experiments, we did not define the total number of channels present in each patch membrane, so the channel activity was calculated with the following equation from an all-point amplitude histogram and expressed as an NP_o value (number of channels (N) × open state probability (P_o)).

$$NP_O = (\sum_{j=1}^{N} t_j.j)/T$$

where t_j is the time spent at each current level corresponding to j=0, 1,2,...N, T is the duration of the recording (2 min).

Statistics

Statistical analyses were performed with a two paired t test or analysis of variance (ANOVA) test (two-factor with replication). Changes were considered significant at $P<0.01$. Data are expressed as the mean ± s.d.

Results

Effects of levcromakalim and glibenclamide on the resting tone of pig proximal urethra

Figure 1a shows that 1 μM levcromakalim caused a relaxation of the resting tone of urethral smooth muscle. After wash out, it took about 6 min (5 min 45 s ± 1 min 4 s, $n=9$) for the tone to recover to the control level; 30 min later, although application of 1 μM glibenclamide slightly increased resting tone, 6 min pretreatment with 1 μM glibenclamide completely inhibited levcromakalim-induced relaxation ($n=5$). After 30 min washing-out, 1 μM levcromakalim-induced relaxation recovered to 69 ± 6% of the prior control relaxation in 5 strips (Figure 1b). Levcromakalim caused a detectable relaxation from 100 nM (data not shown).

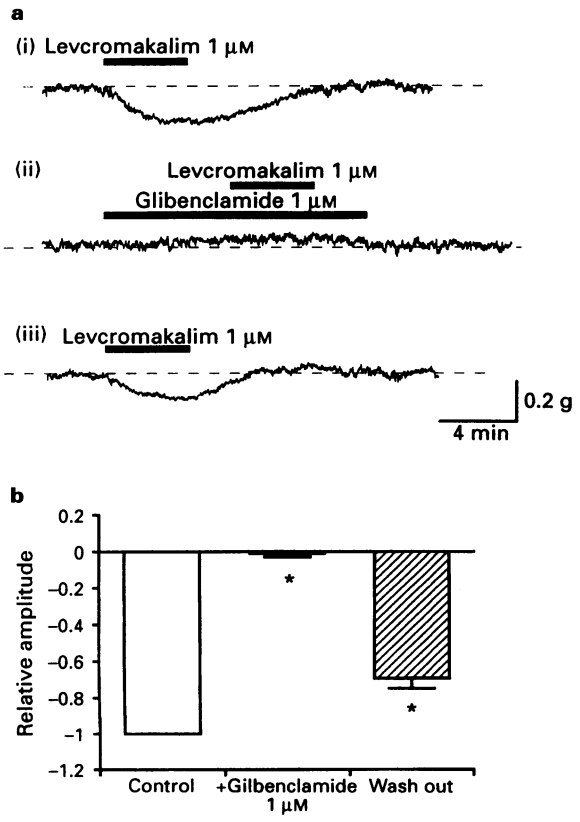

Figure 1 Effects of both levcromakalim and glibenclamide on the resting tone of pig proximal urethra. Guanethidine (3 μM) and propanolol (0.3 μM) were present throughout the experiments in the modified Krebs solution. (a) (i) Levcromakalim 1 μM (4 min application) caused a relaxation on the resting tone in pig urethra. (ii) In the presence of 1 μM glibenclamide, the levcromakalim-induced relaxation was suppressed. (iii) After the removal of 1 μM glibenclamide, the levcromakalim-induced relaxation recovered. Similar results were obtained from 4 other strips. The results illustrated were obtained from a single smooth muscle strip. The dashed line indicates the resting urethral tone. (b) Relative amplitude of 1 μM levcromakalim-induced relaxation: just before applying 1 μM levcromakalim, the average resting tone for 2 min was measured. The amplitude of relaxation was obtained during the last 30 s of levcromakalim application as the mean value after subtracting the average resting tone. The amplitude of 1 μM levcromakalim-induced relaxation was normalized as 1.0 (Control, open column). The solid column indicates the relative amplitude of 1 μM levcromakalim-induced relaxation in the presence of 1 μM glibenclamide (0.01 ± 0.02). The hatched column represents the relative amplitude of 1 μM levcromakalim-induced relaxation after wash out for 30 min (0.69 ± 0.06). *Significantly different from the control ($P<0.01$). Each column represents the relative mean value with s.d. ($n=5$).

Characteristics of the membrane potential of pig urethral smooth muscle and the effects of levcromakalim

Single smooth muscle cells had an input resistance of 1.1 ± 0.4 GΩ ($n=65$) and a capacitance of 62.2 ± 19.2 pF ($n=65$) under quasi-physiological conditions (pipette solution; 140 mM KCl containing 5 mM EGTA, the bath solution; PSS). Under current-clamp recording conditions, the resting membrane potential ranged between −32 mV and −42 mV (−36.1 ± 4.4 mV, $n=5$) over a 5 min recording period. Figure 2a (i) shows a typical recording. The membrane potential was reasonably stable and neither spontaneous action potentials nor appreciable hyperpolarizing spikes were generated under these experimental conditions. Figure 2a (ii) shows the events histogram which was obtained from the trace in Figure 2a (i) fitted with the Gaussian equation. The mean membrane potential for this cell was −36.2 ± 1.9 mV. Using the same cell,

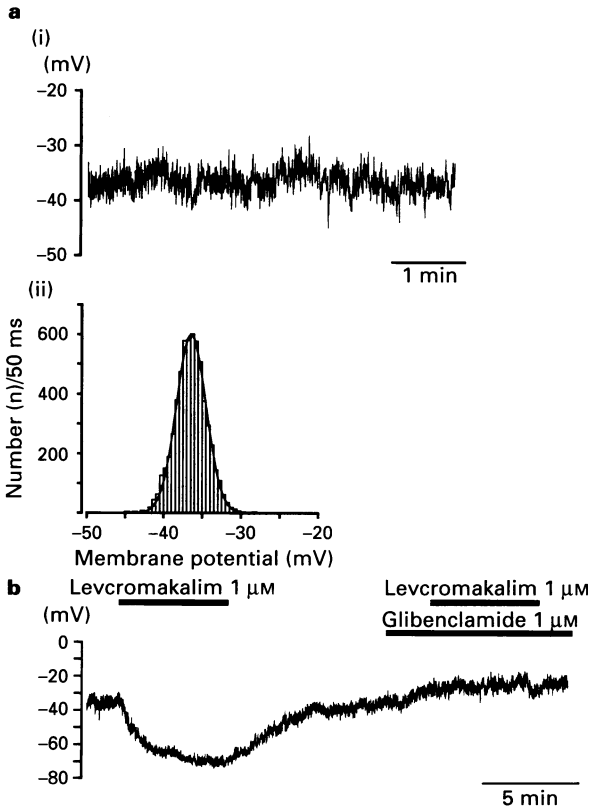

Figure 2 Current-clamp recordings from single isolated smooth muscle cells of pig proximal urethra and the effects of 1 μM levcromakalim and 1 μM glibenclamide on the membrane potential. The bathing solution was PSS (2 mM Ca^{2+}) and the pipette solution contained 140 mM KCl and 5 mM EGTA. (a) (i) Five min recording of the resting membrane potential for pig urethra. (ii) The events histogram obtained from the trace in (i), with a Gaussian distribution fitted using the following equation,

$$f(X,M)=A \exp[(-(X-M)^2)/(2\,S)]$$

where A, X, M and S are the maximum number of observations, the amplitude of membrane potential, mean membrane potential and variance, respectively. A = 601, M = 36.2 mV, S = 3.71 mV^2. (b) Hyperpolarizing effect on the resting membrane potential of 1 μM levcromakalim and its antagonism by 1 μM glibenclamide.

on application of 1 μM levcromakalim, the membrane was hyperpolarized to approximately -66 mV (Figure 2b). The membrane potential slowly recovered to its previous level after washout of levcromakalim. Application of 1 μM glibenclamide depolarized the membrane slightly beyond the pre-drug level ($+7.1 \pm 4.0$ mV, $n=5$). In the presence of glibenclamide, the hyperpolarizing action of levcromakalim was markedly inhibited.

In Figure 3a, the effects of increasing concentrations of levcromakalim are shown in one cell and the mean effect on 4–5 cells is shown in Figure 3b; 100 nM caused a small membrane hyperpolarization (to -41.2 ± 4.5 mV, $n=4$) and the hyperpolarization increased in a concentration-dependent manner. The onset time for these responses became quicker in a concentration-dependent manner, but the time course of recovery was slower than that of the onset at all concentrations of levcromakalim. The levcromakalim-induced membrane hyperpolarization was stable in the presence of all concentration of levcromakalim and at high concentrations (≥ 10 μM) was close to the theoretical potassium equilibrium potential (E_K; -84.2 mV). These results indicate that levcromakalim-induced hyperpolarization is due to an increased permeability to K^+ in pig proximal urethra.

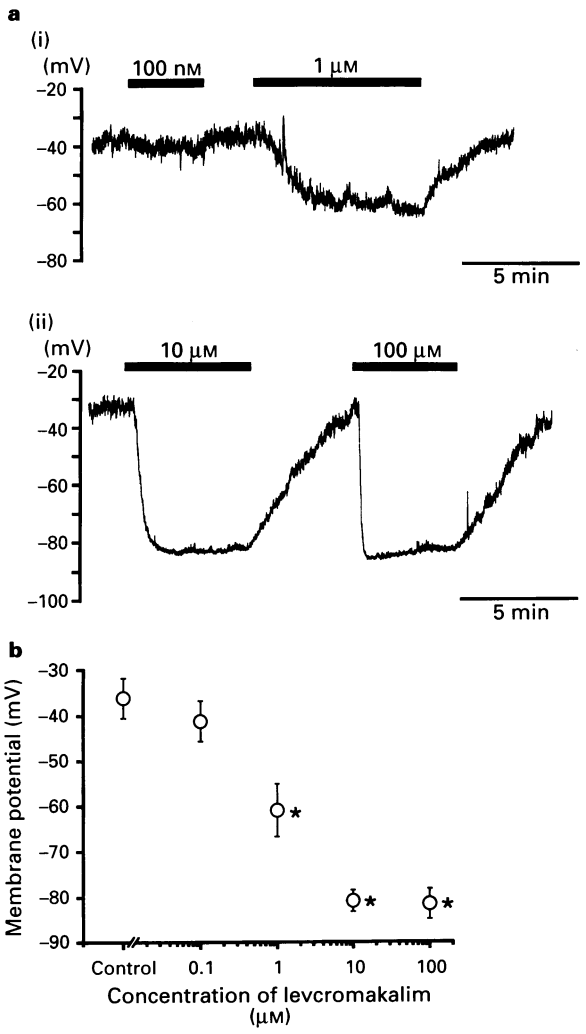

Figure 3 Concentration-dependent effects of levcromakalim on membrane potential using current-clamp mode. The bath contained PSS and the pipette solution was 140 mM KCl containing 5 mM EGTA. (a) (i), (ii), Effects of levcromakalim (from 100 nM to 100 μM) on membrane potential using the concentration-jump method. Data were obtained from the same cell. (b) Dose-response relationships for the effects of levcromakalim on membrane potential. Control indicates the condition just before the application of levcromakalim. Each symbol shows the mean of 4–5 observations with s.d. *Significantly different from the control ($P<0.01$).

Effects of levcromakalim on the membrane current evoked by the application of rectangular voltage pulses in pig urethra

To investigate the levcromakalim-induced hyperpolarization, whole-cell voltage-clamp experiments were performed. Current-voltage relationships were determined before and during the application of 100 μM levcromakalim by applying voltage steps (10 mV increment from -120 mV to $+40$ mV for 2 s duration) every 10 s with the membrane holding potential kept at -50 mV. The pipette solution was 140 mM KCl containing 5 mM EGTA and the bath solution was PSS. As shown in Figure 4b (i), in the absence of levcromakalim (control), time-independent sustained currents were evoked by voltage step pulses from -120 mV to -40 mV and time-dependent outward currents were evoked by steps from -30 mV to $+40$ mV. When 100 μM levcromakalim was applied, an outward current was evoked (about 60 pA, Figure 4a); 4 min later, the same pulse protocol was performed in the presence of the drug (Figure 4b (ii)). The current-voltage relationships obtained from measurements of the peak amplitude during the initial 500 ms before, and during the application of levcro-

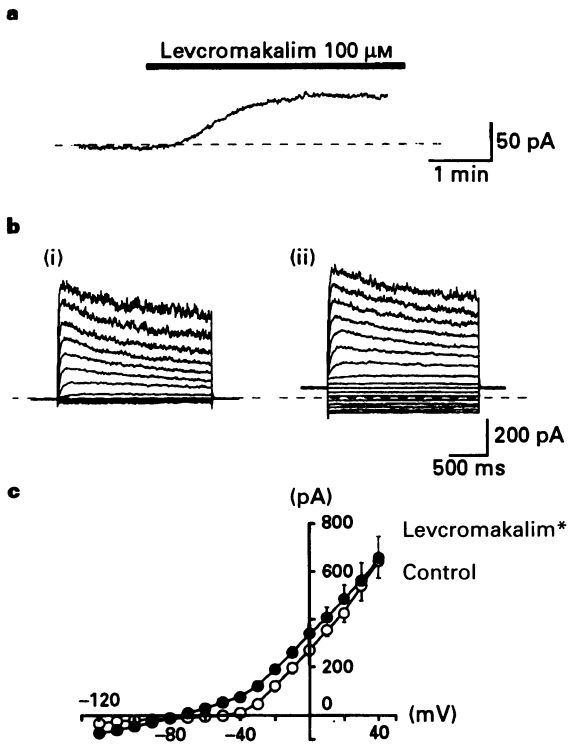

Figure 4 Effects of 100 μM levcromakalim on the membrane current recorded from dispersed smooth muscle cells at -50 mV using conventional whole-cell recording. Levcromakalim was applied in the bath solution. The dashed line indicates the zero current level. (a) Effects of 100 μM levcromakalim on the membrane current. (b) Effects of levcromakalim (100 μM) on the membrane current evoked by application of rectangular pulses (duration 2 s) at 10 s intervals. Traces of membrane currents generated by application of various command pulses from -120 mV to $+40$ mV before (control) (i) and during (ii) the application of 100 μM levcromakalim. (c) Current-voltage relationships obtained in the absence (○) or presence (●) of 100 μM levcromakalim. The membrane current amplitude was measured as the peak amplitude within the initial 500 ms of the command pulses ($n=4$). Each symbol shows the mean of 4 observations with s.d. *Significantly different from the control (ANOVA, $P<0.01$). The lines were drawn by eye.

makalim are expressed as the mean (Figure 4c, $n=4$). The current-voltage curves in the presence and absence of 100 μM levcromakalim intersected at about -80 mV. Levcromakalim 100 μM caused a clear increase both in the inward currents over the potential range from -120 mV to -90 mV and the membrane currents from -70 mV to $+40$ mV (ANOVA, $P<0.01$).

The membrane current induced by levcromakalim

Voltage ramps were used to make a rough estimation of which ions carry the levcromakalim-induced current and to obtain both the current-voltage relationships and the reversal potential. Firstly, eight 600 ms ramps from -120 mV to 0 mV following a 300 ms conditioning pulse (-120 mV) were applied at 10 s intervals before and during application of 100 μM levcromakalim. Secondly, in the presence of 100 μM levcromakalim, the extracellular potassium concentration ($[K^+]_o$) was raised from 5 mM ($E_K = -84.2$ mV) to 60 mM ($E_K = -21.4$ mV) by isosmotic substitution of sodium (Figure 5a (i)). Figure 5a (ii) shows the average of the eight ramp currents before and during application of levcromakalim for the cell shown in Figure 5a (i). In the absence of levcromakalim (5 mM K^+), the mean ramp current changed sign at -33.7 mV and outward rectification was observed. In the presence of levcromakalim, the amplitude of the mean ramp current increased and it changed sign at a more negative potential (-67.7 mV). On raising $[K^+]_o$ from 5 mM to 60 mM, the basal sustained levcromakalim-induced current changed from outward to inward current at the holding potential (-50 mV) and the mean ramp pulse changed sign at -20.1 mV. On returning $[K^+]_o$ to 5 mM, the basal sustained levcromakalim-induced current changed from inward to outward current again and the mean ramp pulse changed sign at -62.0 mV, and on removal of levcromakalim, the current at the holding potential returned to the control level. Similar observations were obtained in four other cells. The effects of iberiotoxin and glibenclamide on the 100 μM levcromakalim-induced outward current were examined using the nystatin-perforated patch condition. Iberiotoxin, a selective blocker of Ca-dependent K channel in smooth muscle, was applied using the concentration-jump method (Figure 5b). Iberiotoxin (300 nM) had little effect on this sustained outward current. However, on application of 10 μM glibenclamide, this outward current was rapidly inhibited and the current noise was reduced. The levcromakalim-induced outward current is thus mainly carried by potassium ions through channels which are insensitive to iberiotoxin, but which are inhibited by glibenclamide.

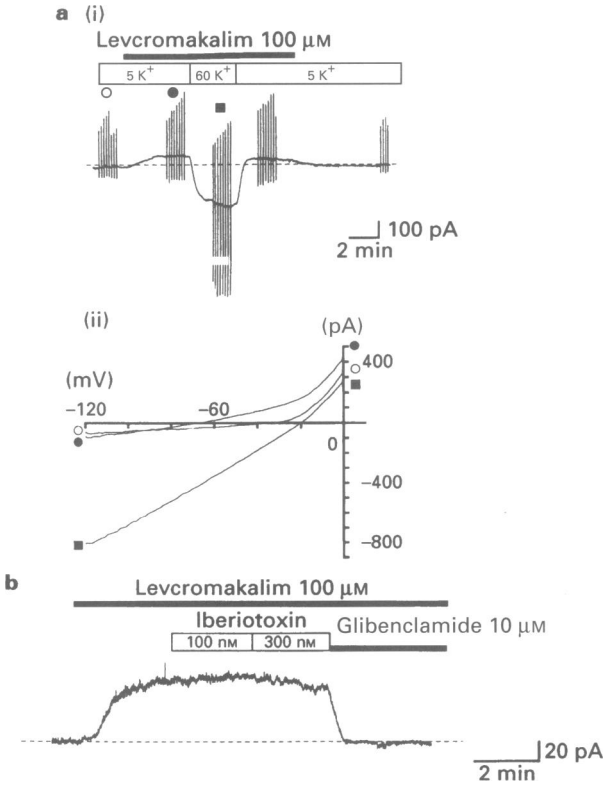

Figure 5 Experiments demonstrating that the outward current induced by levcromakalim is mainly a K-current in pug urethra. (a) (i) A conventional whole-cell record performed with 140 mM K^+ containing 5 mM EGTA in the pipette solution. The holding membrane potential was -50 mV. The bath solution was initially PSS and the external concentration of K^+ was transiently raised to 60 mM K^+ in the presence of 100 μM levcromakalim. The vertical deflections indicate ramp potential pulses. The dashed line indicates the zero current level. (ii) The mean ramp currents induced by the eight ramp potential pulses from -120 mV to 0 mV for 600 ms following a 300 ms conditioning pulse (-120 mV) applied every 10 s before and during application of 100 μM levcromakalim. In the absence of levcromakalim (○) the membrane exhibited outward rectification. Levcromakalim (●) shifted the potential at which the mean ramp current crossed zero current level from -33.7 mV to -67.7 mV; and when $[K^+]_o$ was raised to 60 mM (■), the basal sustained 100 μM levcromakalim-induced current at -50 mV changed from outward to inward and the mean ramp pulse changed sign at -20.1 mV, indicating that the currents were through potassium-selective channels. Similar observations were obtained in four other cells. (b) Effects of iberiotoxin and glibenclamide on 100 μM levcromakalim-induced outward current recorded at -50 mV using the nystatin-perforated patch configuration. The dashed line indicates the current level in the presence of 10 μM glibenclamide.

Unitary currents in pig urethra

To investigate further the levcromakalim-induced, glibenclamide-sensitive outward current, single channel recordings were performed in symmetrical 140 mM K^+ conditions using the cell-attached configuration. To minimize activity of the large conductance K channels (235 pS, Teramoto & Brading, 1994), experiments were performed at negative holding potentials (-50 mV) with $[Ca^{2+}]_o$ buffered at a low level (<2 nM), although significant activity still occurred under these conditions. Figure 6a indicates that another single-channel current, which had a much smaller amplitude than the 235 pS K channel, was evoked when 10 μM levcromakalim was applied using the concentration-jump technique. Levcromakalim did not modify the frequency of opening of the 235 pS K channel. When 10 μM glibenclamide was applied additionally, the opening of the smaller-amplitude channel was selectively suppressed without modifying the frequency of opening of the larger conductance K channel. The properties of this channel were also investigated by use of voltage ramps. Figure 6b shows an example of the current recorded in the presence of 10 μM levcromakalim (the bath solution) in response to a 120 ms voltage ramp from -90 mV to $+30$ mV following a 10 ms conditioning pulse (-90 mV) from a patch in symmetrical 140 mM K^+ solutions with one active channel. The current-voltage relationship of this channel was constructed by subtracting the null ramp current traces (averaging the nearest 10 traces in which the channel did not open before and after the event) from the segments of the current record during which the channel was open. To minimize the error in the calculation of the channel conductance, it was obtained only from current traces in which the channel opened for a duration of at least 50% of the ramp pulse (>60 ms). The reversal potential of this channel was very close to the calculated E_K of 0 mV. The average conductance of channels recorded with this method was 43.3 ± 1.7 pS ($n=16$).

CN activates a glibenclamide-sensitive K channel

To increase the driving force for K^+ movement and to minimize the activity of voltage-dependent K channels, whole-cell K currents were investigated at -50 mV in symmetrical

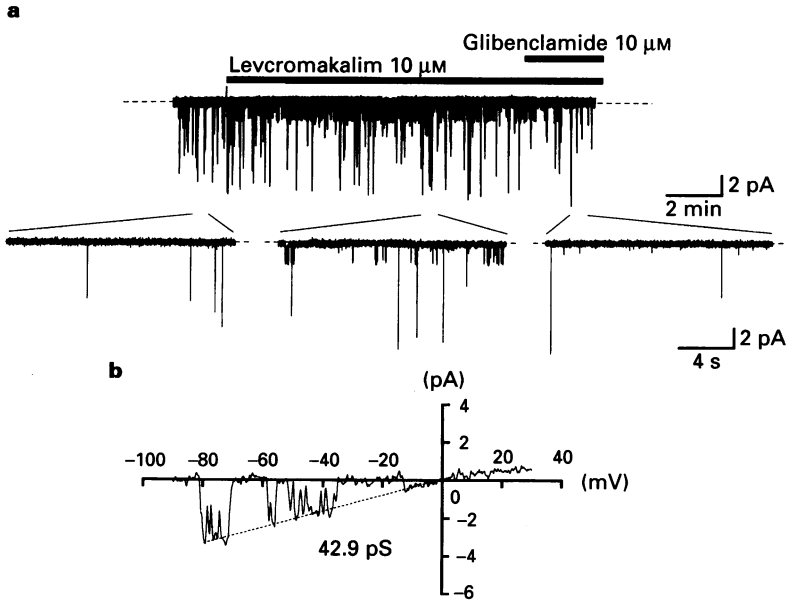

Figure 6 Effects of 10 μM levcromakalim on the unitary K channel current recorded using cell-attached patches (symmetrical 140 mM K^+ conditions) at a holding potential of −50 mV. Patch membranes contained Ca-activated K channels (large downward deflections). (a) When 10 μM levcromakalim was applied using the concentration-jump technique, a small conductance K channel started to activate; 10 μM glibenclamide selectively blocked this channel. The lower traces show expansions of the upper trace. The dashed line indicates the current base line. (b) Single channel current recorded in response to a linear voltage ramp in the presence of 10 μM levcromakalim, from a patch containing only one active channel, which opened and closed during the ramp. The pipette potential was ramped from −90 mV to +30 mV over 120 ms following a 10 ms conditioning pulse (−90 mV). The average ramp currents from 20 null traces (the nearest 10 traces before and after the event in which the channel did not open) were subtracted. The conductance of this channel was 43.3 ± 1.7 pS (n = 16). The dashed line was fitted by the least-squares method at negative membrane potentials.

140 mM K^+ conditions (internal solution also containing 5 mM EGTA). K currents were therefore inward in this experimental condition. When 1 mM cyanide (CN), an aerobic-metabolism inhibitor, was applied by the concentration-jump method, a small inward current was observed (Figure 7a). Increasing the concentration to 3 mM CN resulted in a larger inward current with increased current noise (n = 5). Glibenclamide in the presence of 3 mM CN, suppressed the CN-induced current in a concentration-dependent manner and the current noise was considerably reduced.

To investigate further the glibenclamide-sensitive CN-induced current in pig urethra, single channel recordings were performed (Figure 7b). In symmetrical 140 mM K^+ conditions using the cell-attached patch, 5 mM CN activated a small-amplitude channel (2.14 pA at −50 mV) but with a delay of about a 3 min despite using the concentration-jump technique. The same delay was observed in other cells (n = 5). Glibenclamide 10 μM reversibly inhibited the opening of this channel. When CN was then washed out, the glibenclamide-sensitive channel remained opened for a while and then disappeared. These results suggest that inhibition of aerobic metabolism by CN, caused a glibenclamide-sensitive membrane current due to the opening of 43 pS K channels.

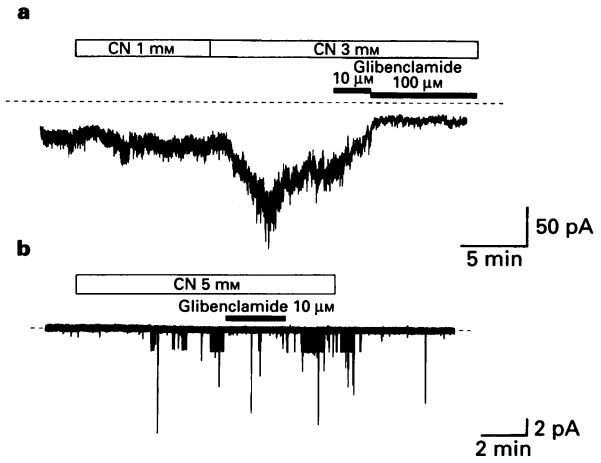

Figure 7 Figure demonstrating that the metabolic poison, cyanide (CN), activated a current due to the opening of glibenclamide-sensitive K channels in pig urethra. The membrane potential was held at −50 mV. (a) Continuous current recording in a conventional whole-cell configuration. The bath solution was 140 mM K^+ PSS and the pipette solution was 140 mM K^+ containing 5 mM EGTA. After a delay, CN induced a concentration-dependent inward current which was inhibited by the application of glibenclamide. The dashed line indicates the zero current line. (b) CN (5 mM) activated glibenclamide-sensitive K channel recorded using the cell-attached configuration in symmetrical 140 mM K^+ condition at −50 mV. Note that there was about a 3 min delay after the application of 5 mM CN despite using the concentration-jump method. The dashed line indicates the current base line.

Discussion

In the present experiments, we have demonstrated that levcromakalim concentration-dependently induces hyperpolarization through activation of a 43 pS glibenclamide-sensitive K channel and that CN also activates this channel in smooth muscle cells of pig proximal urethra.

The membrane potential and the effects of levcromakalim

Using the current-clamp recording configuration, the resting membrane potential in pig urethra (−36.1 mV) was similar to that measured with micro-electrodes in circular smooth muscles of the urethra of other species (guinea-pig; −42 mV, Callahan & Creed, 1981, rabbit; −40 mV, Ito & Kimoto, 1985), and to the value of −35 mV recorded in pig urethra

with micro-electrodes (personal communication from M. Bridgewater). This suggests that the electrophysiological properties of the single cells had not been changed by the dispersal procedure.

In all smooth muscles, KCOs effectively hyperpolarize the resting membrane as well as membranes depolarized by agonists or increased K^+ (<20 mM) and they produce muscle relaxation. The effects of KCOs on the resting membrane potential in intact smooth muscle have been mainly investigated using micro-electrodes. In guinea-pig bladder, cromakalim reduces spike activity with a threshold concentration of around 500 nM, and hyperpolarizes the membrane at higher concentrations, approaching E_K at about 10 μM; spontaneous contractions are abolished at 500 nM (Foster *et al.*, 1989a; Fujii *et al.*, 1990). In the pig and human bladder, spontaneous contractions are also abolished at this concentration of cromakalim (Foster *et al.*, 1989b). The effects of levcromakalim on pig urethral smooth muscle seen in the present studies follow the similar pattern as KCOs in other tissues (cromakalim: guinea-pig mesenteric artery and vein, Nakao *et al.*, 1988; pinacidil: guinea-pig bladder, Seki *et al.*, 1992).

Levcromakalim ($\geq$100 nM) relaxed the tissue and induced a concentration-dependent hyperpolarization which is close to E_K at a concentration of 10 μM; both effects were completely antagonized by glibenclamide. The effects of KCOs on the urinary tract have also been studied *in vivo* (Foster *et al.*, 1989b; Malmgren *et al.*, 1989; Hedlund *et al.*, 1991; Kontani *et al.*, 1993a,b). They are certainly effective in abolishing unstable bladder contractions, but also have marked and undesirable effects on the cardiovascular system. Our results suggest that they will also have effects on the urethral smooth muscle, which could result in the lowering of urethral pressure, and might reduce the effectiveness of KCOs in the treatment of incontinence due to bladder instability. A bladder-selective KCO will be necessary for effective treatment for the instability, but urethral relaxation could be valuable for treatment of outflow obstruction.

The target K channel for levcromakalim in pig proximal urethra

Levcromakalim activates a membrane current which can be seen as a time-independent current additional to that evoked by voltage steps from −120 mV to −40 mV (except −80 mV), a result similar to that described in rabbit portal vein (Russell *et al.*, 1992), and in rabbit pulmonary artery (Clapp *et al.*, 1993) when the holding potential was near to the resting membrane potential. At potentials more positive than −30 mV, the records are complicated by variable activation of Ca-dependent K channels due to their voltage and Ca^{2+} sensitivity, and the contribution of Ca^{2+} entry through voltage-gated Ca channels. We have therefore not investigated the effects of levcromakalim on the outward current at positive potentials in any detail. However, iberiotoxin, a specific large conductance Ca-dependent K channel blocker (Giangiacomo *et al.*, 1991; Garcia & Kaczorowski, 1992), had little effect on the levcromakalim-induced outward current at a concentration which effectively blocked the 235 pS Ca-dependent K channel in pig urethra (300 nM, unpublished observation) at −50 mV. Moreover, levcromakalim also did not affect the opening of the 235 pS K channel, but increased the open probability of a 43 pS glibenclamide-sensitive K channel in single channel recordings. The large conductance Ca-dependent K do not seem to be targets for levcromakalim in pig proximal urethra. Since we have not yet tested an intracellular ATP sensitivity of the 43 pS K channel in pig urethra, we use the term 'glibenclamide-sensitive' instead of 'ATP-sensitive'. However, glibenclamide blocks this channel in the same submicromolar concentration as it does the K_{ATP} which have been reported in many vascular smooth muscle cells (reviewed by Edwards & Weston, 1993; Kitamura & Kuriyama, 1994). The conductance of the urethral K channel opened by levcromakalim is approximately 43 pS in symmetrical 140 mM K^+ conditions, and the outward current can be evoked even when 5 mM EGTA is present in the pipette solution, suggesting little intracellular Ca sensitivity. Given this, the glibenclamide-sensitive 43 pS K channel has many similarities to K_{ATP} in vascular smooth muscles. Further characteristics of this channel (such as ATP-sensitivity etc.) may elucidate whether or not the glibenclamide-sensitive 43 pS K channel in pig urethra may belong to the category of K_{ATP} in vascular smooth muscles.

Metabolic poison activates the glibenclamide-sensitive K channel

Activation of K current by metabolic poisons such as 2-deoxy-D-glucose (2-DG), cyanide (CN), 2,4-dinitrophenol (DNP) and iodoacetic acid has been reported with whole-cell recordings in smooth muscles (Silberberg & van Breemen (1992), rabbit mesenteric artery; Beech *et al.* (1993), rabbit portal vein; Bonev & Nelson (1993), guinea-pig urinary bladder). In pig urethra in symmetrical 140 mM K^+ conditions, we observed a steady CN-induced inward current at −50 mV and could observe a small CN-induced outward current under quasi-physiological conditions (unpublished observations).

Using single channel recordings, Zhang & Bolton (1995) have reported that CN and 2-DG activate glibenclamide-sensitive K channels in rat mesenteric artery. In our experiments, CN activated a small amplitude K channel in pig urethra, which was glibenclamide-sensitive and passed a current of 2.14 pA at −50 mV (channel conductance 43 pS). Levcromakalim activated a channel of similar amplitude in the same patches (data not shown), and the reversal potential of the channels was very close to 0 mV in 140 mM K^+ symmetrical conditions. Although we used the concentration-jump method for the application of CN, a time-delay (about 3 min) for activating the channel was always observed ($n=5$), in contrast to the immediate effect of levcromakalim. Metabolic poisons (such as CN, 2-DG and DNP) have many effects on both intracellular metabolites and cytosolic organs (reviewed by Paul, 1989; Silberberg & van Breemen, 1992). In rat pancreatic β-cells, Misler *et al.* (1986) suggest that metabolic inhibitors change $[ATP]_i$ and the ratio of ATP/ADP concentration in the intracellular space. In our experiments, we cannot be sure of the exact targets of cyanide and whether it has direct effects on the glibenclamide-sensitive K channel or not. However, presumably cyanide's main effect will be to inhibit aerobic metabolism resulting in time-dependent changes, which would explain the onset delay of CN-induced currents in pig urethra. Recently, Dart & Standen (1995) have indicated that hypoxia activates K_{ATP} channels in pig coronary artery, suggesting that these channels may play a role in the relaxation to lowered P_{O_2} levels. Our results suggest that the glibenclamide-sensitive K channel in pig urethra might play an important role in regulating the resting tone and resting membrane potential in hypoxic conditions.

In conclusion, the glibenclamide-sensitive K channel in pig urethra seems to play a role in regulating the resting membrane potential, and may also be the target which mediates the effects of metabolic inhibition. The glibenclamide-sensitive K channels in the urinary tract may play some role both in the pathophysiology of abnormal micturition and its clinical treatment. We suggest that any K-channel openers developed for the treatment of bladder instability should be examined not only on the cardiovascular system but also on the tone of the urethra.

We are grateful to the SmithKline Beecham Pharmaceuticals for the generous gift of levcromakalim (BRL 38227). We thank the staff of Banbury Meat Ltd. for supplying pig urethra. This work was supported by the Wellcome Trust.

References

ANDERSSON, K.-E. (1993). Pharmacology of lower urinary tract smooth muscles and penile erectile tissues. *Pharmacol. Rev.*, **45**, 253–308.

ASHFORD, M.L.J., STURGESS, N.C., TROUT, N.J., GARDNER, N.J. & HALES, C.N. (1988). Adenosine-5′-triphospate-sensitive ion channels in neonatal rat cultured central neurones. *Pflügers Arch.*, **412**, 297–304.

BEECH, D.J., ZHANG, H., NAKAO, K. & BOLTON, T.B. (1993). K channel activation by nucleotide diphosphates and its inhibition by glibenclamide in vascular smooth muscle cells. *Br. J. Pharmacol.*, **110**, 573–582.

BONEV, A.D. & NELSON, M.T. (1993). ATP-sensitive potassium channels in smooth muscle cells from guinea pig urinary bladder. *Am. J. Physiol.*, **264**, C1190–C1200.

BRADING, A.F. & TURNER, W.H. (1994). The unstable bladder: towards a common mechanism. *Br. J. Urol.*, **73**, 3–8.

CALLAHAN, S.M. & CREED, K.E. (1981). Electrical and mechanical activity of the isolated lower urinary tract of the guinea-pig. *Br. J. Pharmacol.*, **74**, 353–358.

CLAPP, L.H., DAVEY, R. & GURNEY, A.M. (1993). ATP-sensitive K^+ channels mediate vasodilation produced by lemakalim in rabbit pulmonary artery. *Am. J. Physiol.*, **264**, H1907–H1915.

CLAPP, L.H. & GURNEY, A.M. (1991). Outward currents in rabbit pulmonary artery cells dissociated with a new technique. *Exp. Physiol.*, **76**, 677–693.

COOK, D.L. & HALES, C.N. (1984). Intracellular ATP directly blocks K^+ channels in pancreatic B-cells. *Nature*, **311**, 271–273.

DART, C. & STANDEN, N.B. (1995). Activation of ATP-dependent K^+ channels by hypoxia in smooth muscle cells isolated from the pig coronary artery. *J. Physiol.*, **483**, 29–39.

EDWARDS, G. & WESTON, A.H. (1993). The pharmacology of ATP-sensitive potassium channels. *Annu. Rev. Pharmacol. Toxicol.*, **33**, 597–637.

FOSTER, C.D., FUJII, K., KINGDON, J. & BRADING, A.F. (1989a). The effect of cromakalim on the smooth muscle of guinea-pig urinary bladder. *Br. J. Pharmacol.*, **97**, 281–291.

FOSTER, C.D., SPEAKMAN, M.J., FUJII, K. & BRADING, A.F. (1989b). The effect of cromakalim on the detrusor muscle of human and pig urinary bladder. *Br. J. Urol.*, **63**, 284–294.

FUJII, K., FOSTER, C.D., BRADING, A.F. & PAREKH, A.B. (1990). Potassium channel blockers and the effects of cromakalim on the smooth muscle of the guinea-pig bladder. *Br. J. Pharmacol.*, **99**, 779–785.

GARCIA, M.L. & KACZOROWSKI, G.J. (1992). High conductance calcium-activated potassium channels: molecular pharmacology, purification and regulation. In *Potassium Channel Modulators*, ed. Weston, A.H. & Hamilton, T.C., pp. 76–109. Oxford: Blackwell Scientific Publication.

GIANGIACOMO, K.M., GARCIA, M.L. & MCMANUS, O.B. (1991). Mechanism of iberiotoxin block of the high conductance Ca^{2+}-activated K^+ channel from aortic smooth muscle. *Biophys. J.*, **59**, 79a.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.*, **391**, 85–100.

HEDLUND, H., MATTIASSON, A. & ANDERSSON, K.-E. (1991). Effects of pinacidil on detrusor instability in men with bladder outlet obstruction. *J. Urol.*, **146**, 1345–1347.

HORN, R. & MARTY, A. (1988). Muscarinic activation of ionic currents measured by a new whole-cell recording method. *J. Gen. Physiol.*, **92**, 145–159.

INOUE, R. & BRADING, A.F. (1990). The properties of the ATP-induced depolarization and current in single cells isolated from the guinea-pig urinary bladder. *Br. J. Pharmacol.*, **100**, 619–625.

ITO, Y. & KIMOTO, Y. (1985). The neural and non-neural mechanisms involved in urethral activity in rabbits. *J. Physiol.*, **367**, 57–72.

KITAMURA, K. & KURIYAMA, H. (1994). Molecular mechanisms of action of antihypertensive agents Ca-. Ca-antagonists and K-channel openers on vascular smooth muscle. In *Pharmacology of Smooth Muscle*, ed. Szekeres, L. & Papp, J.G. Chapter 16, pp. 595–630. Berlin: Springer-Verlag.

KONTANI, H., GINKAWA, M. & SAKAI, T. (1993a). A simple method for measurement of ureteric peristaltic function in vivo and the effects of drugs acting on ion channels applied from the ureter lumen in anesthetized rats. *Jpn. J. Pharmacol.*, **62**, 331–338.

KONTANI, H., JINKAWA, M. & SAKAI, T. (1993b). Effects of levcromakalim on ureteral peristaltic function and cystometrogram in rats. *Jpn. J. Pharmacol.*, **63**, 503–511.

MALMGREN, A., ANDERSSON, K.-E., SJÖGREN, C. & ANDERSSON, P.O. (1989). Effects of pinacidil and cromakalim (BRL 34915) on bladder function in rats with detrusor instability. *J. Urol.*, **142**, 1134–1138.

MELICK, W.F., NARYKA, J.J. & SCHMIDT, J.H. (1961). Experimental studies of ureteral peristaltic patterns in the pig: 1. similarity of pig and human ureter and bladder physiology. *J. Urol.*, **85**, 145–148.

MISLER, S., FALKE, L.C., GILLIS, K. & MCDANIEL, M.L. (1986). A metabolite-regulated potassium channel in rat pancreatic B cells. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 7119–7123.

NAKAO, K., OKABE, K., KITAMURA, K., KURIYAMA, H. & WESTON, A.H. (1988). Characteristics of cromakalim-induced relaxations in the smooth muscle cells of guinea-pig mesenteric artery and vein. *Br. J. Pharmacol.*, **95**, 795–804.

NOMA, A. (1983). ATP-regulated K^+ channels in cardiac muscle. *Nature*, **305**, 147–148.

PAUL, R.J. (1989). Smooth muscle energetics. *Annu. Rev. Physiol.*, **51**, 331–349.

RUSSELL, S.N., SMIRNOV, S.V. & AARONSON, P.I. (1992). Effects of BRL 38227 on potassium currents in smooth muscle cells isolated from rabbit portal vein and human mesenteric artery. *Br. J. Pharmacol.*, **105**, 549–556.

SEKI, N., KARIM, O.M.A. & MOSTWIN, J.L. (1992). Effect of pinacidil on the membrane electrical activity of guinea pig detrusor muscle. *J. Pharmacol. Exp. Ther.*, **263**, 816–822.

SILBERBERG, S.D. & VAN BREEMEN, C. (1992). A potassium current activated by lemakalim and metabolic inhibition in rabbit mesenteric artery. *Pflügers Arch.*, **420**, 118–120.

SPRUCE, A.E., STANDEN, N.B. & STANFIELD, P.R. (1985). Voltage-dependent ATP-sensitive potassium channels of skeletal muscle membrane. *Nature*, **316**, 736–738.

TERAMOTO, N. & BRADING, A.F. (1994). Effects of niflumic acid on calcium-activated potassium channels in smooth muscle cells isolated from pig urethra. *J. Physiol.*, **481**, 56P.

TERAMOTO, N. & BRADING, A.F. (1995). Levcromakalim activates glibenclamide and tolbutamide sensitive-potassium channels in smooth muscle cells isolated from pig urethra. *Proceeding of The Second Joint Meeting of the Physiological Societies of Japan and U.K. and Eire*, (Nagoya Symposium), **P4-32**, 180.

YELLEN, G. (1982). Single Ca^{2+}-activated nonselective cation channels in neuroblastoma. *Nature*, **296**, 357–359.

ZHANG, H. & BOLTON, T.B. (1995). Activation by intracellular GDP, metabolic inhibition and pinacidil of a glibenclamide-sensitive K-channel in smooth muscle cells of rat mesenteric artery. *Br. J. Pharmacol.*, **114**, 662–672.

(Received September 7, 1995
Revised February 19, 1996
Accepted February 21, 1996)