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Effects of levcromakalim and nucleoside diphosphates on glibenclamide-sensitive K^+ channels in pig urethral myocytes

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1 Effects of levcromakalim and nucleoside diphosphates (NDPs) on both membrane currents and unitary currents in pig proximal urethra were investigated by use of patch clamp techniques (conventional whole-cell configuration, nystatin perforated patch, cell-attached configuration and inside-out patches).

2 Levcromakalim produced a concentration-dependent outward current at a holding potential of −50 mV. The peak current amplitude showed little variation when measured by either conventional whole-cell or nystatin perforated patch configurations.

3 In conventional whole-cell configuration, the levcromakalim (100 μM)-induced outward current decayed by about 90% in 18 min. In contrast, with the nystatin perforated patch, approximately 86% of the levcromakalim-induced outward current still remained after 18 min.

4 The peak amplitude of the levcromakalim (100 μM)-induced outward membrane current recorded by the conventional whole-cell configuration was greatly reduced by inclusion of 5 mM EDTA in the pipette. The much smaller but significant outward membrane current remaining was abolished by glibenclamide.

5 In conventional whole-cell recordings, inclusion of an NDP in the pipette solution induced a small outward current which slowly reached a maximal amplitude (in 2 to 10 min) and was suppressed by glibenclamide. Addition of 100 μM levcromakalim after the NDP-induced current had peaked activated a further outward current which was larger than that recorded in the absence of NDPs. Approximately 50% of this current still remained at 18 min, even when conventional whole-cell configuration was used.

6 In the cell-attached mode in symmetrical 140 mM K^+ conditions, glibenclamide inhibited the 100 μM levcromakalim-activated 43 pS K^+ channel in a concentration-dependent manner, showing an inhibitory dissociation constant (K_i) of approximately 520 nM.

7 In inside-out patches in which the glibenclamide-sensitive K^+ channel had run down after exposure to levcromakalim, both uridine 5′-diphosphate (UDP) and MgATP were capable of reactivating the channel. Further application of Mg^{2+} to the UDP-reactivated K^+ channels enhanced the channel activity reversibly.

8 In inside-out patches UDP was capable of activating the glibenclamide-sensitive K^+ channel without levcromakalim, providing that there was free Mg^{2+} present (either UDP in 5 mM EGTA or UDP in 5 mM EDTA with Mg^{2+}). Additional application of levcromakalim caused a further reversible activation of channel opening.

9 In the presence of levcromakalim, application of adenosine 5′-triphosphate (ATP) to the inner surface of the membrane patch inhibited UDP-reactivated channel opening in a concentration-dependent manner.

10 Addition of an untreated cytosolic extract of pig proximal urethra reactivated the glibenclamide-sensitive K^+ channel in the presence of 100 μM levcromakalim in inside-out patches.

11 These results demonstrate the presence in the pig proximal urethra of a glibenclamide-sensitive K^+ channel that is blocked by intracellular ATP and can be activated by levcromakalim. Intracellular UDP can reactivate the channel after rundown. Additionally, intracellular Mg^{2+} may play an important role in regulating the channel activity.

Keywords: Glibenclamide; levcromakalim; potassium channel; nucleoside diphosphate; channel run-down; cytosolic extract; intracellular Mg^{2+}

Introduction

Potassium channel openers (KCOs) form a class of drugs with a wide variety of chemical structures and were originally characterized by their ability to increase the opening probability of adenosine 5′-triphosphate (ATP)-sensitive K^+ channels (K_{ATP}). KCOs hyperpolarize the membrane and increase the K^+ conductance in many smooth muscle cells (reviewed by Edwards & Weston, 1993; Kitamura & Kuriyama, 1994). Sulphonylureas, typified by glibenclamide, inhibit not only the membrane hyperpolarization (Seki *et al*., 1992), but also the K^+ ($^{86}Rb^+$) efflux (Trivedi *et al*., 1994) and the muscle relaxation induced by KCOs (Fujii *et al*., 1990; Ito *et al*., 1991; Okada *et al*., 1993). Since these agents are known selectively to inhibit K_{ATP} in pancreatic β-cells (Ashcroft, 1988), it is thought that KCOs also activate K_{ATP} in smooth muscles (Kajioka *et al*., 1991). In addition, it has been shown from an examination of single-channel properties in some smooth muscles that the channel involved is activated by nucleoside diphosphates (NDPs), and as such it has been suggested that this channel would be more appropriately termed an NDP-dependent K^+ channel (K_{NDP}; Beech *et al*., 1993b; Zhang & Bolton, 1995; 1996). However, controversy remains not only as to whether or not an NDP alone can reactivate glibenclamide-sensitive K^+ channels in inside-out patches, but also as to whether NDPs may be the only molecules capable of reactivating the channels, and whether or not intracellular Mg^{2+} is essential for channel reactivation.

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In urinary tract smooth muscle cells, K^+ channels play an important role in regulating the membrane potential and cellular excitability (reviewed by Brading, 1987; Andersson, 1993). We have been interested in functional abnormalities of the urinary bladder, and in particular the condition of detrusor instability in which the smooth muscle becomes hyperexcitable. In this condition, K^+ channel activating drugs potently decrease excitability and may be useful in the treatment of bladder instability (Foster *et al*., 1989). For this reason, KCOs have been introduced to urology in an attempt to treat both unstable detrusor contractions and bladder outflow obstruction (Malmgren *et al*., 1989; Hedlung *et al*., 1991). Since urethral relaxation would be an undesirable side effect, direct investigations of the effect of KCOs on urethral myocytes are therefore necessary. The pig and human urinary bladder and urethra have been shown to have many similarities in both function and structure (Melick *et al*., 1961; Crowe & Burnstock, 1989) and thus the pig has become a useful experimental model. Given this, we have selected the pig proximal urethra as a source of tissue to investigate potassium channels. Recently, we have revealed the presence of a glibenclamide-sensitive 43 pS K^+ channel in this tissue (Teramoto & Brading, 1996). In the present experiments we have investigated further the effects of levcromakalim (the biologically-active (−)-enantiomer of cromakalim) on these channels by use of patch clamp technique, and have studied their re-activation not only by uridine 5′-diphosphate (UDP), but also by MgATP and by a cytosolic tissue extract. Some preliminary results have been communicated to the second joint meeting of the Physiological Societies of Japan and U.K. and Eire (Teramoto & Brading, 1995).

Methods

Fresh urethra, from female pig, still attached to its bladder, was collected from a local abattoir and transported to the laboratory in a cold solution (at 4–6°C) of composition (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, which had previously been bubbled with 97% O_2 and 3% CO_2 (pH 7.25–7.3). The proximal region of the pig urethra (1–2 cm from the bladder neck) was excised and the connective tissue and mucosa removed under a dissection microscope.

Cell dispersion and preparation of cytosol

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, glucose 10, HEPES 10/Tris, titrated to pH 7.35–7.4, containing papain (17 unit mg^{-1} protein, 0.3–0.4 mg ml^{-1}) bubbled with O_2 at 4–6°C for 20 min. 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 I) at 35°C for 10–15 min. Relaxed spindle-shaped cells, with length varying between 400 μm and 500 μm, were isolated by the gentle tapping method and stored at 4°C. The dispersed cells were normally used within 5 h for experiments. Cytosol was prepared from fresh urethral tissue, which was finely diced and homogenized in 5 volumes of ice-cold high potassium pipette solution containing 1 mM Pefabloc (a wide-range peptidase inhibitor, S. Black, Hertfordshire, U.K.), followed by centrifugation for 20 min at 5000 *g* in a precooled centrifuge (4°C). The supernant fraction was filtered through fine nylon mesh (1 × 1 mm), aliquoted, frozen and stored at −70°C. In the present experiments, the supernatant fraction was diluted by 2–4 times with high K^+ bath solution. The heated cytosolic extract was obtained by treating at approximately 90–95°C for 4–5 min.

Drugs and solutions

For recording whole-cell currents (conventional whole-cell and nystatin perforated patch), the following solutions were used: physiological salt solution (PSS) containing (mM): Na^+ 140, K^+ 5, Mg^{2+} 1.2, Ca^{2+} 2, glucose 5, Cl^- 151.4, HEPES 10, titrated to pH 7.35–7.40 with Tris base (21–23°C); pipette solution containing (mM): K^+ 140, Cl^- 140, 5 ethylene glycol-bis (β-aminoethylether) N,N,N′,N′-tetraacetic acid (EGTA), 10 HEPES/Tris (pH 7.35–7.40). In some experiments, 5 mM ethylendiaminetetraacetic acid (EDTA) was added to the pipette solution. ATP or NDPs were occasionally included in the pipette solution as indicated in the Results section. For single channel recording (cell-attached and inside-out configuration), the pipette and bath solution was high potassium solution (mM): K^+ 140, Cl^- 140, EGTA 5, HEPES 10/Tris (pH 7.35–7.40) producing symmetrical 140 mM K^+ conditions. In some experiments, 1.18 mM Mg^{2+} was added to high K^+ solution (as above) in order to achieve 1 mM free Mg^{2+} in the presence of 5 mM EGTA. MgATP 1 mM was also obtained when both 1.17 mM Na_2ATP and 2.18 mM Mg^{2+} were further added to the above high K^+ solution. EDTA (5 mM) solution was achieved by equimolar replacement of EGTA in the high potassium solution to chelate free Mg^{2+} further. In some experiments, 6 mM Mg^{2+} was added to high K^+ solution (as above) in order to achieve 1 mM free Mg^{2+} in the presence of 5 mM EDTA. The concentrations of free ATP, free Ca^{2+} and free Mg^{2+} were calculated by use of the commercial software 'EQCAL' (Biosoft, Cambridge, U.K.). 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 solution system (a concentration jump technique, Teramoto & Brading, 1996), recording the drug application time as a trigger pulse on VHS tape with current and voltage at the same time. The following chemicals were used: BSA, EGTA, ATP, adenosine 5′-diphosphate (ADP), guanosine 5′-diphosphate (GDP), inosine 5′-diphosphate (IDP), uridine 5′-diphosphate (UDP), HEPES, collagenase, glibenclamide, nystatin, DMSO and papain (Sigma, Dorset, U.K.), and EDTA and Tris (BDH Chemicals Ltd., Dorset, U.K.). Levcromakalim was kindly provided by SmithKline Beecham Pharmaceuticals (Harlow, U.K.). NDPs and ATP were added as the relevant Na-salt and 100 mM stock solutions of these were titrated to pH 7.4 and frozen at −70°C. Dilution of the stock solution was made immediately before application. Both levcromakalim and glibenclamide were prepared daily as 100 mM stock solutions in DMSO. The final concentration of DMSO was less than 0.1%, and this concentration was shown not to affect potassium channels in pig urethra.

Recording procedure

The experimental system used was essentially the same as that described previously (Hamill *et al*., 1981; Teramoto & Brading, 1996). In short, patch clamp experiments were performed with an L/M-EPC 7 patch-clamp amplifier (List-Medical-Electronic, Darmstadt, Germany) in conjunction with an AD/DA converter (DT2801A, Data Translation, U.K.). The sampled current data were filtered at 10 kHz and stored together with potential records on videotape by use of 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 with 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. Levcromakalim-induced whole-cell currents were recorded by the perforated patch technique with nystatin as performed in our previous paper (Teramoto & Brading, 1996). The holding potential in all ex-

periments was kept at −50 mV throughout. All experiments were carried out at room temperature (21–23°C).

Data analysis

The whole-cell current data were low-pass filtered at 500 Hz by an 8 pole Bessel filter, sampled at 20 ms and analysed on a computer (Macintosh Quadra 610, Apple Computer UK Limited, Uxbridge, U.K.) by the commercial software 'MacLab 3.4.2' (ADInstruments Pty Ltd., Castle Hill, Australia). For single channel recordings, the stored data were low-pass filtered at 1 kHz (−3 dB) and sampled into the computer with a digitalized interval of 200 μs by use of the 'PAT' programme (kindly provided by Dr J. Dempster, the University of Strathclyde, U.K.); events briefer than 200 μs were not included in the evaluation. The all-point amplitude histogram was obtained from a continuous recording of 1 or 2 min (2 kHz filtration; 80 μs digital sampling interval), and fitted with the Gaussian distribution function using a least-squares fitting. Continuous traces in the figures were obtained from records filtered at 500 Hz for presentation (digital sampling interval, 25 ms). Values for the channel open state probability (P_{open}) were measured at −50 mV for 1 or 2 min.

$$NP_O \quad \sum_{j\ 1}^{N} t_j\, j \quad T$$

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, and N is taken as the maximum number of channels observed in the patch membrane where P_{open} was relatively high. NP_o values (number of channels × open state probability) were calculated for every 2 min segment of the channel recording. Data points were fitted by a least-squares fitting.

Statistics

Statistical analyses were performed with either Student's *t* test for paired values of analysis of variance (ANOVA) test (two-factor with replication). Changes were considered significant at $P<0.01$. Data are expressed as mean with the standard deviation (s.d.).

Results

Time course of decay of levcromakalim-induced current

The time course of 100 μM levcromakalim-induced outward currents in both a conventional whole-cell configuration and a nystatin perforated patch were compared with the whole-cell voltage clamp configuration at −50 mV. When levcromakalim was applied by the concentration jump method, outward current was evoked immediately. There was no significant

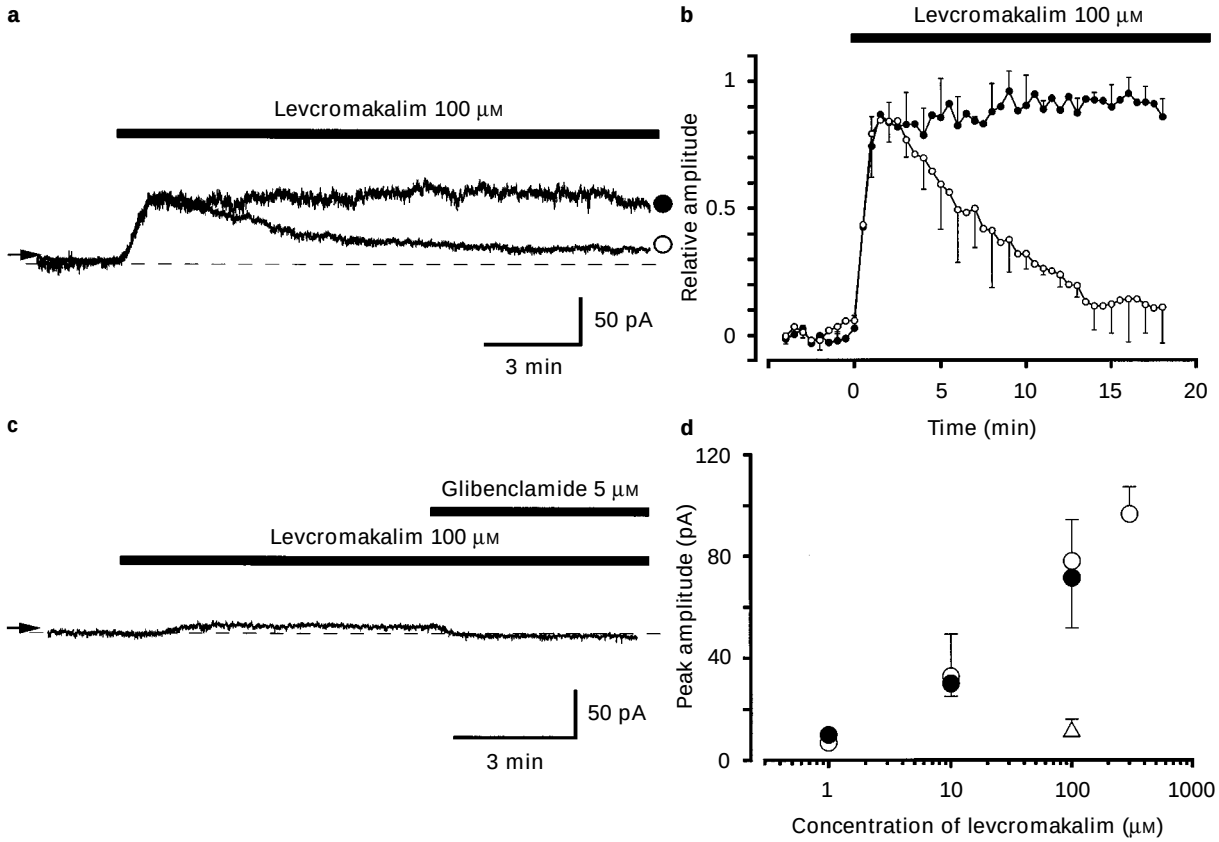

Figure 1 The time course of outward currents induced by 100 μM levcromakalim with two different configurations of whole-cell recording (nystatin perforated patch and conventional whole-cell configuration) at −50 mV. (a) Examples of the outward currents in two cells with the different configurations. (●) Nystatin perforated patch; (○) conventional whole-cell recording. Dashed line: control base current level before levcromakalim. Arrow: zero current level. (b) The time courses of the relative mean amplitude of the outward currents. The peak amplitude for each recording was normalized as 1.0 (●, nystatin perforated patch; ○, conventional whole-cell recording). Time 0 indicates the time when 100 μM levcromakalim was applied by a concentration jump method. Each symbol indicates the mean of 4 observations determined as 30 s intervals. (c) Effects of 100 μM levcromakalim on the membrane current when the pipette solution contained 5 mM EDTA in addition to 5 mM EDTA. Dashed line: control base current level before levcromakalim. Arrow: zero current level. (d) Relationships between peak amplitude of outward currents and concentration of levcromakalim with the two different configurations (●, nystatin perforated patch; ○, conventional whole-cell configuration) in the presence of 5 mM EGTA. Each symbol indicates the mean and vertical lines s.d. of 8–24 observations. (△) Indicates the average of the peak amplitude when 5 mM EDTA was additionally included in the internal solution ($n=5$).

difference in either the rising time course or the peak amplitude of the outward current produced in the two different configurations. In the conventional whole-cell configuration, after reaching a peak, the current gradually decreased to a stable level in about 14 min, despite the continued presence of levcromakalim. At 18 min, the sustained level was 10.9±8.9% of the peak amplitude ($n=4$). In comparison, in the nystatin perforated patch configuration, although the current amplitude showed a small decline, 85.8±7.0% still remained after 18 min ($n=4$). Figure 1b shows the time course of the relative amplitude of the outward currents with the two different configurations. Significantly, when 5 mM EDTA was added to the pipette solution (*i.e.* with 5 mM EDTA and 5 mM EGTA in the pipette), the peak amplitude of the 100 μM levcromakalim-induced current was much smaller (12±4 pA, $n=5$ versus 78±16 pA, $n=24$). This small outward current was suppressed by the additional application of 5 μM glibenclamide (Figure 1c). Figure 1d indicates the relationship between the peak amplitude of the outward current and the concentration of levcromakalim in both of these conditions.

Outward current induced by NDPs

When 1 mM ATP was included in the pipette solution, little appreciable outward current was induced (3.6±1.6 pA, $n=25$) 10 min after the conventional whole-cell configuration had become established. However, when 1 mM GDP was present, an outward current slowly developed (Figure 2a). In some cells, transient outward currents, similar to spontaneous transient outward currents (STOCs; Benham & Bolton, 1986) occurred, and overlapped the GDP-induced outward current whilst the pipette solution was diffusing into the cell. The GDP-induced outward current reached its maximum amplitude in 2 to 10 min, (26.2±6.9 pA in 1 mM GDP, $n=5$; 10.2±4.2 pA in 0.3 mM GDP, $n=4$) and was sustained for more than 20 min. Application of glibenclamide suppressed this outward current. Concentrations (1 mM) of other NDPs (such as ADP, IDP and UDP) and control pipette solution (with neither ATP nor NDP) were also tested. To minimize cell-to-cell variation, we tested each NDP on cells from the same animal under the same conditions and the maximum amplitude of outward current was measured (Figure 2b). These outward currents were all abolished by glibenclamide (10–100 μM). As a small but detectable outward current was gradually evoked after the establishment of whole-cell configuration even in the absence of NDP (control) or in the presence of ATP (1 mM), the peak amplitude of NDP-induced outward current was measured from the current level in 100 μM glibenclamide, a method used by Beech *et al.* (1993a). All four NDPs (1 mM) induced outward currents that were significantly larger than that of the control (Figure 2c).

Additional effects of NDP on levcromakalim-induced outward currents

When 100 μM levcromakalim was applied after the NDP-induced outward current had reached its maximum value and become stable, an additional outward current was induced.

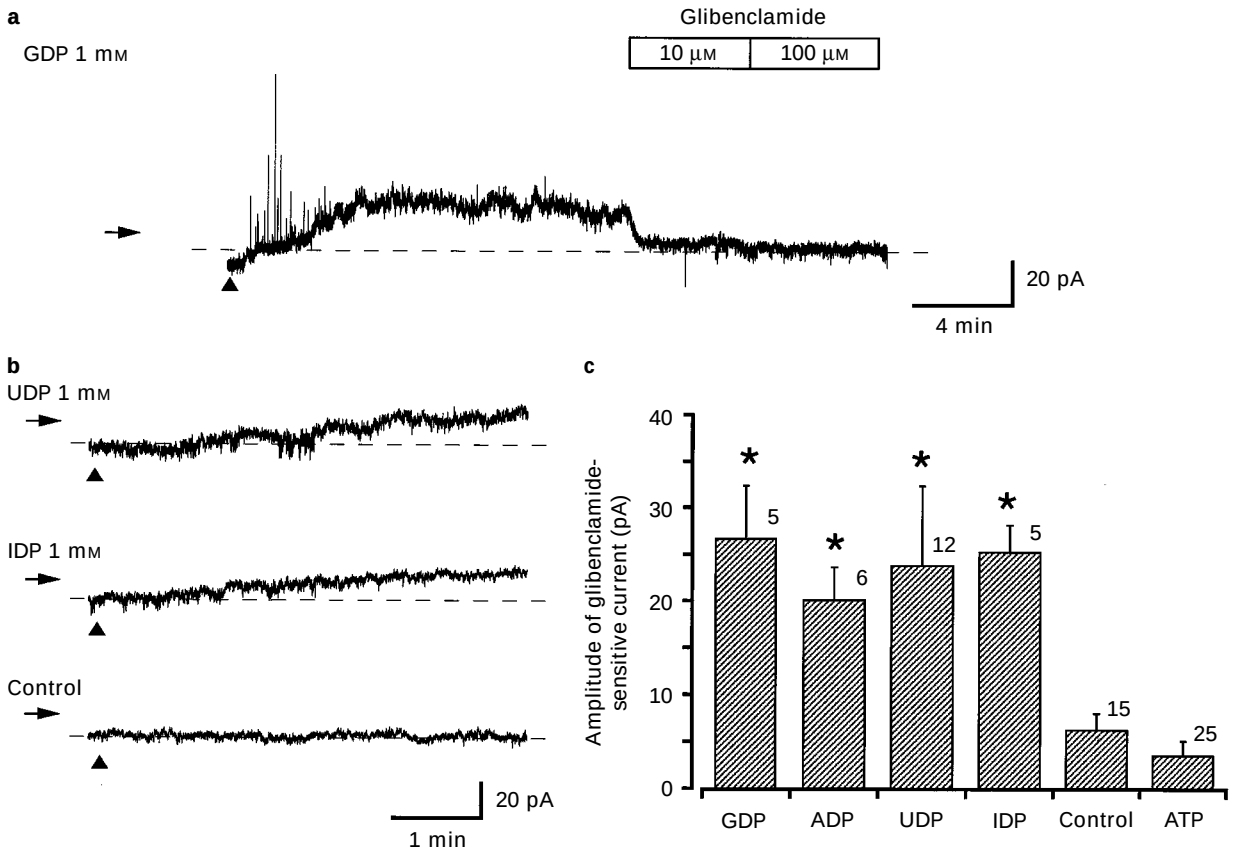

Figure 2 Effects of NDPs on the membrane current in a conventional whole-cell configuration at −50 mV when either NDP or ATP (1 mM) was included in the pipette solution. Note that control indicates the current when neither NDPs nor ATP were present in the pipette solution (control solution). (a) Effects of 1 mM GDP on the membrane current. Glibenclamide suppressed the 1 mM GDP-induced outward current. (▲) Indicates the time when a conventional whole-cell configuration was established. The dashed line indicates the current level in the presence of 100 μM glibenclamide. (b) Effects of 1 mM UDP, 1 mM IDP and control solution on the membrane current. (▲) Indicates the time when whole-cell configuration was established. The dashed line indicates the current level in the presence of 100 μM glibenclamide. (c) The maximum current amplitude in the presence of 1 mM NDP, measured from the current level in 100 μM glibenclamide. Each column indicates the mean with s.d. The number of observations (n) is shown above each column. *Significantly different from the control solution (ANOVA, $P<0.01$).

This is illustrated for 1 mM ADP in Figure 3a. The total outward current reached a peak which was much larger than that observed in the absence of ADP and then showed a gradual decay. Significant outward current still remained after 18 min (74.0 ± 8.7 pA, $47.3 \pm 4.2\%$ of the peak amplitude, $n=4$) despite the conventional whole-cell configuration. When 10 μM glibenclamide was applied, the outward current was suppressed to the control level. The same results were observed when other NDPs (GDP, IDP or UDP) were included in the pipette solution (1 mM). Figure 3b summarizes both the peak amplitude of the outward current and the amplitude of the outward current 18 min after application of 100 μM levcromakalim for each NDP included in the pipette solution. The order of potency of the NDPs was UDP > ADP > GDP > IDP.

Effects of glibenclamide on levcromakalim induced-unitary channel currents in cell-attached configuration

To study further the glibenclamide-sensitive outward current which was evoked by either levcromakalim or intracellular application of NDPs, single-channel recordings were performed in symmetrical 140 mM K^+ conditions. When the holding potential was changed from -90 mV to 0 mV in the presence of 100 μM levcromakalim, the conductance, obtained from the amplitude of K^+ channel currents, was 43.1 ± 2.8 pS ($n=21$) measured from the all-points amplitude histograms at every membrane potential (data not shown). Figure 4 shows the effects of glibenclamide on the 43 pS K^+ channel when 100 μM levcromakalim was present in the bath at -50 mV. Brief openings of the large conductance K^+ channel (240 ± 18 pS, Teramoto & Brading, 1994) are also seen. To exclude the possibility of delayed contact between glibenclamide and its binding site on the patch membranes, we applied glibenclamide for 7 min at each concentration before assessing blocking efficacy (Figure 4a). The NP_o value of the 43 pS K^+ channel was calculated to be 0.044. Glibenclamide (1 μM) suppressed the channel opening without changing the amplitude (control, 2.17 pA, 1 μM glibenclamide, 2.17 pA) and the NP_o value was estimated to be 0.013 (Figure 4b (i)). On removal of 1 μM glibenclamide, channel opening recovered to the control level (NP_o value, 0.045). On applying a higher concentration of glibenclamide (10 μM), the NP_o value was nearly 0 (Figure 4b (ii)). After washing out the 10 μM glibenclamide, the 43 pS K^+ channel reappeared, although the NP_o value did not completely recover to the control level (NP_o value, 0.038). Figure 4c shows the relationships between the NP_o for the 100 μM levcromakalim-activated 43 pS K^+ channel and the concentration of glibenclamide at -50 mV ($n=4$). Glibenclamide selectively blocked the 43 pS K^+ channel in a concentration-dependent manner.

UDP-induced reactivation of the glibenclamide-sensitive K^+ channel in inside-out patches

When a cell-attached patch showed openings of the glibenclamide-sensitive K^+ channel with 100 μM levcromakalim

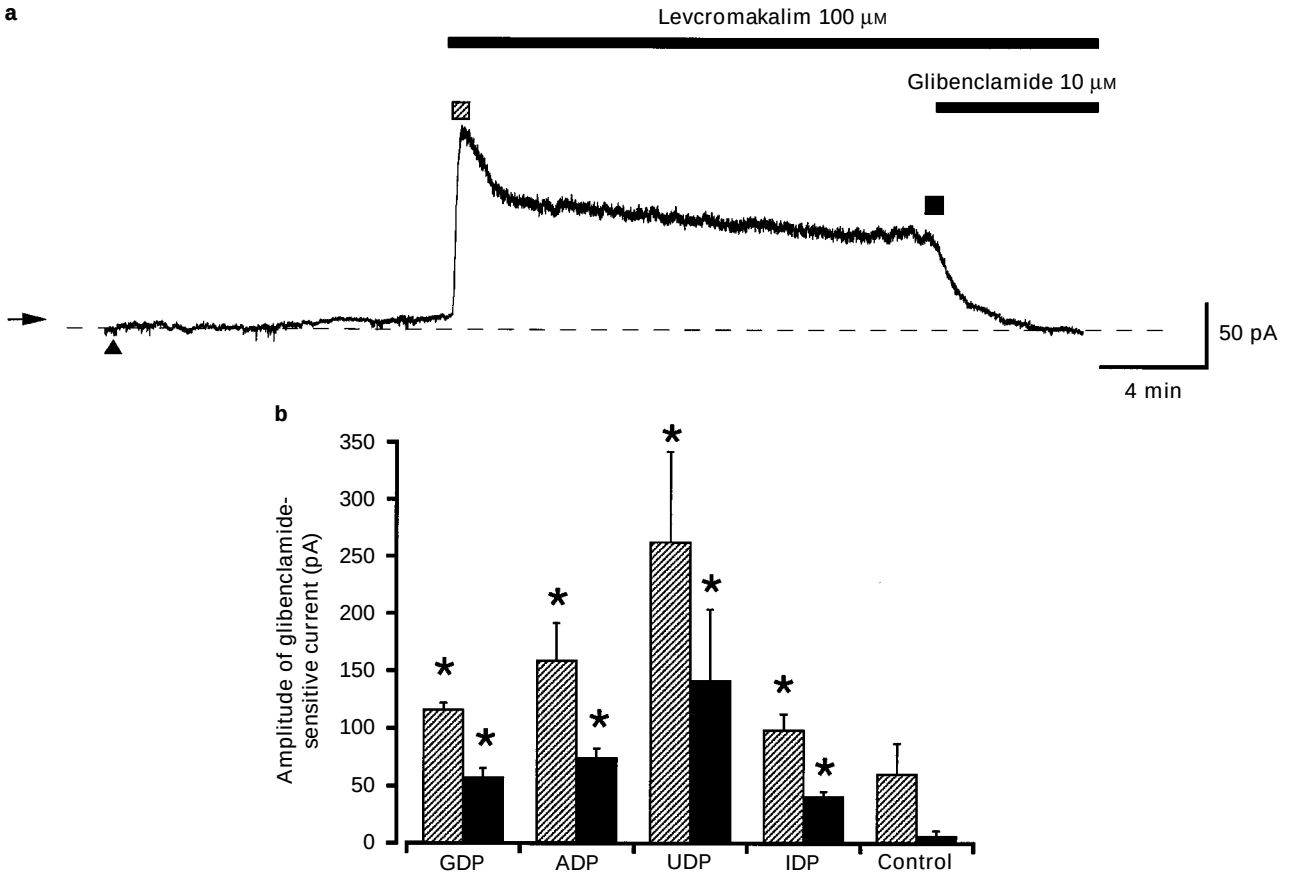

Figure 3 Effect of 100 μM levcromakalim on NDP-induced outward current by use of a conventional whole-cell configuration at -50 mV. (a) When 1 mM ADP was present in the pipette, a small outward current developed slowly after the establishment of the conventional whole-cell configuration (indicated by the ▲). Levcromakalim 100 μM caused an additional outward current. The dashed line indicates the current level after suppression with 10 μM glibenclamide. (b) The peak amplitude (hatched columns) and the amplitude 18 mm from the application of levcromakalim (solid columns) of the levcromakalim-induced current with various NDPs, in the pipette solution. Currents were measured from the 10 μM glibenclamide-sensitive level in each pipette condition. Control shows results with no addition of NDPs to the pipette solution. Each column indicates the mean of 3–10 observations with s.d. The current amplitudes with GDP ($n=3$), ADP ($n=4$), UDP ($n=4$) and IDP ($n=6$) were significantly different from control ($n=10$), with $P<0.01$ (ANOVA).

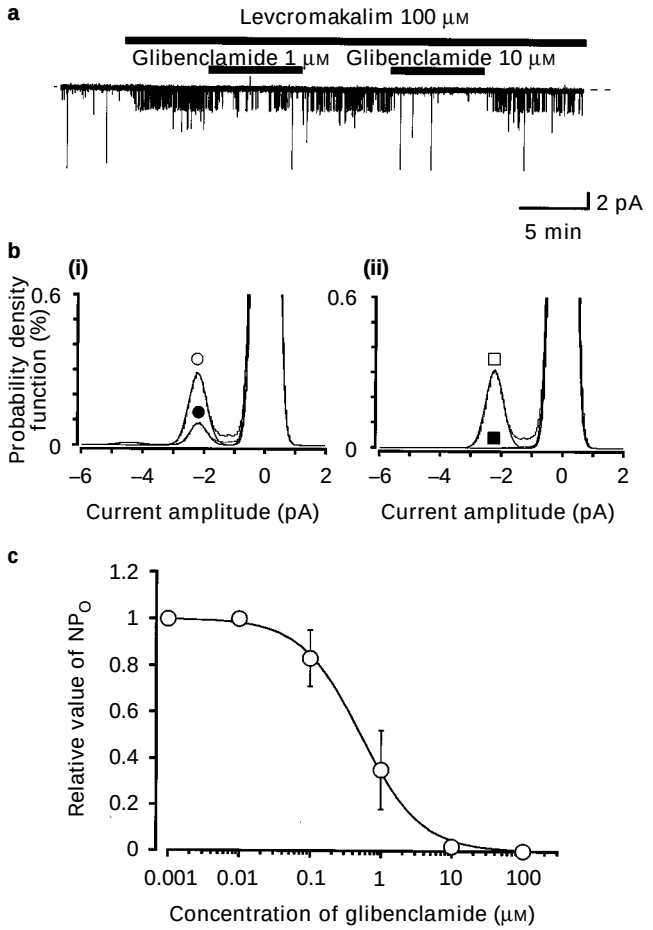

Figure 4 Effects of glibenclamide on the 43 pS K^+ channel activated by 100 μM levcromakalim. Both 100 μM levcromakalim and glibenclamide were applied in the bath (symmetrical 140 mM K^+ conditions). Each concentration of glibenclamide was applied for 7 min at -50 mV. (a) Current traces at the indicated concentration of glibenclamide are shown. The dashed line indicates the current base line where the channel is not open. (b) The all-point amplitude histogram for each concentration of glibenclamide ((i) 1 μM or (ii) 10 μM) was obtained during the last 2 min of a 7 min application. The all-point amplitude histograms were superimposed in the absence (control; ○ or □, just before the application of each concentration of glibenclamide) or presence of glibenclamide (●, 1 μM; ■, 10 μM). Continuous lines in the histograms are theoretical curves fitted with the Gaussian distribution, by the least-squares methods. The abscissa scale shows the amplitude of the current (pA) and the ordinate scale shows the percentage value of the probability density function (%) for recording period (2 min). (c) Relationships between relative inhibition of the NP_o value for the 43 pS K^+ channel in the presence of 100 μM levcromakalim and the concentration of glibenclamide. The NP_o value just before applying glibenclamide was normalized as 1.0. The curve was drawn by fitting the equation by the least-squares method, relative value of $NP_o = 1/\{1+(K_i/D)\}^{n_H}$ where K_i, D and n_H are inhibitory dissociation constant, concentration of glibenclamide (nM) and Hill coefficient, respectively. The following values were used for the curve fitting: $K_i = 518$ nM, $n_H = 1.03$ ($n = 4$). Each symbol indicates the mean with s.d. shown by vertical lines.

present in the bath, patch excision resulted in a rapid 'run-down' of channel opening in the inside-out patch. Channel run-down took nearly 60 s (although there was cell-to-cell variation), suggesting levcromakalim alone could not prevent the run-down of this channel activity ($n = 48$). Figure 5a (i) shows that, after the run-down was complete, application of 1 mM UDP to the inner surface of the membrane patch re-activated the channel in the presence of 100 μM levcromakalim (the bath). Other NDPs (such as ADP, GDP, IDP *etc.* at 1 mM) reactivated the same amplitude K^+ channel in a similar manner (data not shown). An additional application of 1 mM Mg^{2+} further increased the channel opening activity. On removal of Mg^{2+}, the channel activity recovered to the previous level (Figure 5a, $n = 6$). In the presence of 100 μM levcromakalim, UDP started to reactivate the glibenclamide-sensitive K^+ channel at a concentration of 0.1 mM and increased the channel activity in a concentration-dependent manner (Figure 5b). Glibenclamide 10 μM inhibited the channel opening reversibly (Figure 5c).

Effects of UDP, ATP and Mg^{2+} on glibenclamide-sensitive K^+ channel in inside-out patches

Figure 6a shows an experiment in which, after an inside-out patch had been established, no channel opening was observed at -50 mV for about 10 min. Addition of 1 mM UDP in a solution containing 5 mM EGTA caused a slight activation of the glibenclamide-sensitive K^+ channel. An additional application of 100 μM levcromakalim to the bath caused channel opening to increase. After both levcromakalim and UDP had been washed out, channel activity was still observed for a short while before gradually disappearing ($n = 5$). A similar experimental protocol was performed in the presence of 5 mM EDTA (in the bath solution) in Figure 6b. Under these conditions addition of 1 mM UDP did not stimulate openings of the glibenclamide-sensitive K^+ channel. However, subsequent application of 1 mM Mg^{2+} resulted in occasional channel opening, and application of 100 μM levcromakalim caused a much higher channel opening. After the channel had run-down, the application of Na_2ATP to the internal surface of the patch membrane in inside-out configuration did not stimulate the channel activity, as shown in Figure 6c. Subsequent application of Mg^{2+} to the bath solution caused an increment of the glibenclamide-sensitive K^+ channel activity. On washing-off Mg^{2+}, the channel activity gradually disappeared. Similar observations were made in four other patches. In inside-out patches, changing the intracellular chelator of the bath solution from 5 mM EGTA to 5 mM EDTA during UDP-induced channel reactivation, resulted in the disappearance of channel activity. The activity did not reappear even when the chelator was changed back to EGTA (Figure 7a). However, when 3 mM Mg^{2+} was applied, even in the presence of 5 mM EDTA, the UDP-reactivated channel activity reappeared (Figure 7b).

Inhibitory action of intracellular ATP on the UDP-reactivated glibenclamide-sensitive K^+ channel

When both 1 mM UDP and 100 μM levcromakalim were present in the bath, an application of 300 μM ATP to the inner (bath) surface of the membrane patch inhibited channel opening (Figure 8). An increase in the concentration of ATP to 1 mM further reduced the NP_o value. On removal of ATP, the channel opening recovered to be control level.

Untreated cytosolic extract reactivates the glibenclamide-sensitive K^+ channel

When an untreated cytosolic extract from pig proximal urethra was applied to the inner surface of the membrane patch by the inside-out configuration, no channel was activated (data not shown, $n = 10$). However, with the same patch, in the presence of 100 μM levcromakalim, application of the untreated cytosolic extract reactivated the 2.14 pA channel at -50 mV. Heated cytosolic extract applied after removal of the untreated extract caused no channel reactivation. When the untreated extract was again applied in place of the heated extract, channel reactivation was elicited (Figure 9a). Similar observations were obtained in seven other patches. The untreated cytosolic extract-induced channel had a conductance of 43 pS (43.1 ± 0.2 pS, $n = 4$) and 10 μM glibenclamide inhibited the channel reversibly. To investigate further the effects of intracellular Mg^{2+} on the untreated cytosolic extract-induced K^+ channels, EGTA (5 mM) in the bath was replaced with EDTA (5 mM) in order to minimize the concentration of free Mg^{2+}. Under these conditions the extract did not stimulate the

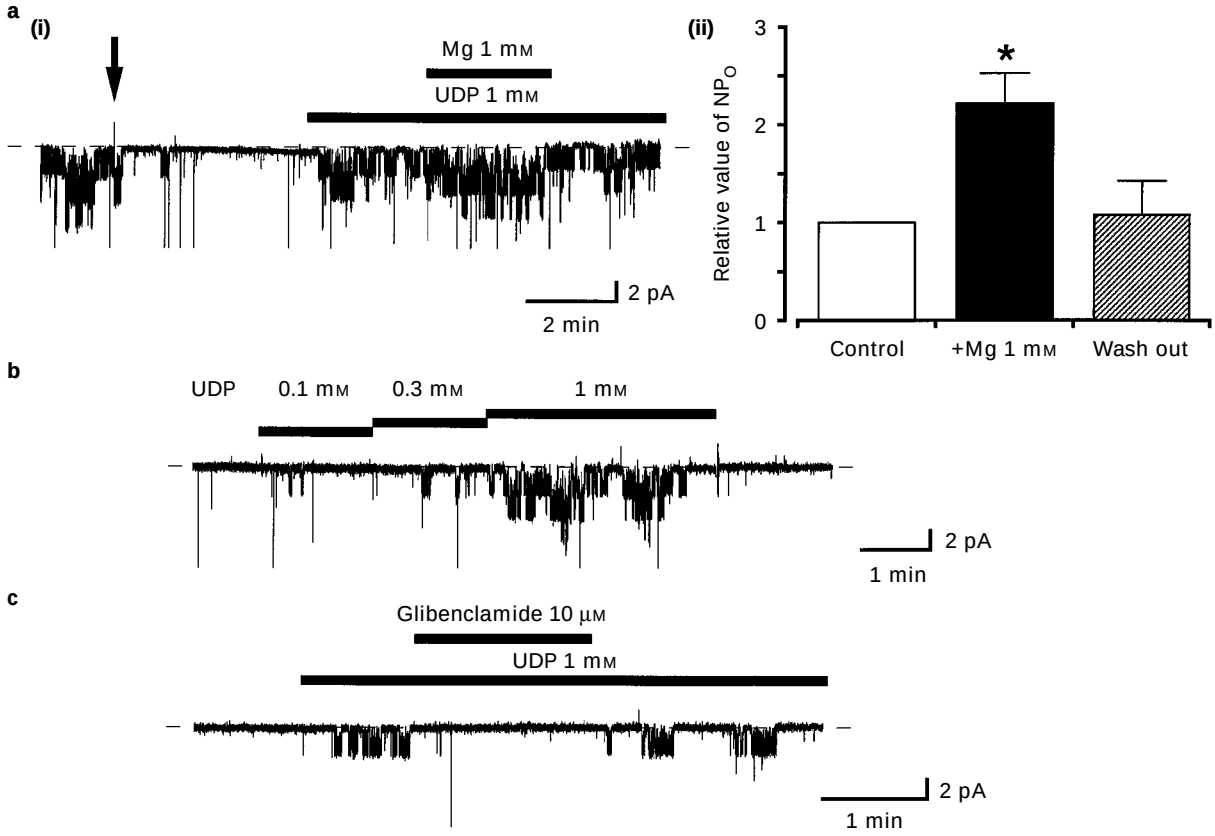

Figure 5 Effects of 1 mM UDP and 1 mM Mg^{2+} on the excised membrane patch in inside-out configuration when 100 μM levcromakalim was present in the bath solution (symmetrical 140 mM K^+ conditions). The patch membrane was held at -50 mV. The dashed line indicates the current base line when the channel is not open. (a) (i) When an inside-out patch was established from the cell-attached configuration (excision of the patch membrane at the arrow), the levcromakalim-induced K^+ channel showed a rapid run-down even in the presence of 100 μM levcromakalim. The application of 1 mM UDP to the inner surface of the membrane patch reactivated the same amplitude of K^+ channel. An additional application of 1 mM Mg^{2+} further accelerated the UDP-induced channel opening activity. (ii) The channel activity (NP_o) of the UDP-reactivated K^+ channel in the absence of 1 mM Mg^{2+} was normalized as 1.0 (control; open column). The columns indicate the relative NP_o values (+s.d.) under control conditions, in the presence of 1 mM Mg^{2+} (solid column) and after washing-out Mg^{2+} (hatched column) ($n=6$). *Significantly different from the control ($P<0.01$, t test). (b) UDP ($\geqslant$0.1 mM) reactivated channel opening in a concentration-dependent manner. (c) Glibenclamide 10 μM reversibly inhibited the 1 mM UDP-induced channel opening activity.

2.14 pA K^+ channel. After the cytosol extract in 5 mM EDTA-containing solution had been washed out, reapplication of the cytosol extract in solution containing 5 mM EGTA reactivated this channel (Figure 9b).

Discussion

In the present experiments, we have demonstrated that both levcromakalim and NDPs induce a glibenclamide-sensitive K^+ current in smooth muscle cells of the pig proximal urethra, that this current flows through K^+ channels with a 43 pS conductance and that not only UDP but also MgATP and an untreated cytosolic extract can reactivate this channel activity even in inside-out configuration.

The different time course of the levcromakalim-induced current decay between conventional whole-cell recording and nystatin perforated patch recording

In our experiments, there was no significant difference in the peak amplitude of the outward currents which were evoked by the application of levcromakalim by either conventional whole-cell configuration or nystatin perforated patches when 5 mM EGTA was included in the pipette solution. However, the time courses of the outward current decay at -50 mV were distinctly different. About 90% of the levcromakalim-induced K^+ current was lost within 18 min in the conventional whole-cell experiments, whilst the current was better maintained with the nystatin perforated patch whole-cell recording. Zhang & Bolton (1995) have demonstrated that the intracellular concentration of NDPs and ATP are important in the regulation of the glibenclamide-sensitive current. In the present experiments, when NDPs (1 mM) were included in the recording pipette solution, the amplitude of the levcromakalim-induced K^+ current was larger than that in the absence of NDPs and was better maintained, even with the conventional whole-cell configuration. These results suggest the possibility that some intracellular organic molecules or metabolic regulators, lost by diffusion during whole-cell recordings, may be necessary to maintain the outward current evoked by levcromakalim in pig urethra.

Similarities and differences between the properties of the glibenclamide-sensitive channels in vascular and urinary tract smooth muscles

Although at a submicromolar concentration, glibenclamide has been considered a selective blocker for not only NDP-reactivated K^+ channels but also K_{ATP} (Edwards & Weston, 1993; Kitamura & Kuriyama, 1994), the K_i value of the glibenclamide inhibitory effect in smooth muscles seems to vary from tissue to tissue (rabbit portal vein: 25 nM, GDP-induced K^+ current, Beech *et al*., 1993a; rat portal vein: 3 μM, LK

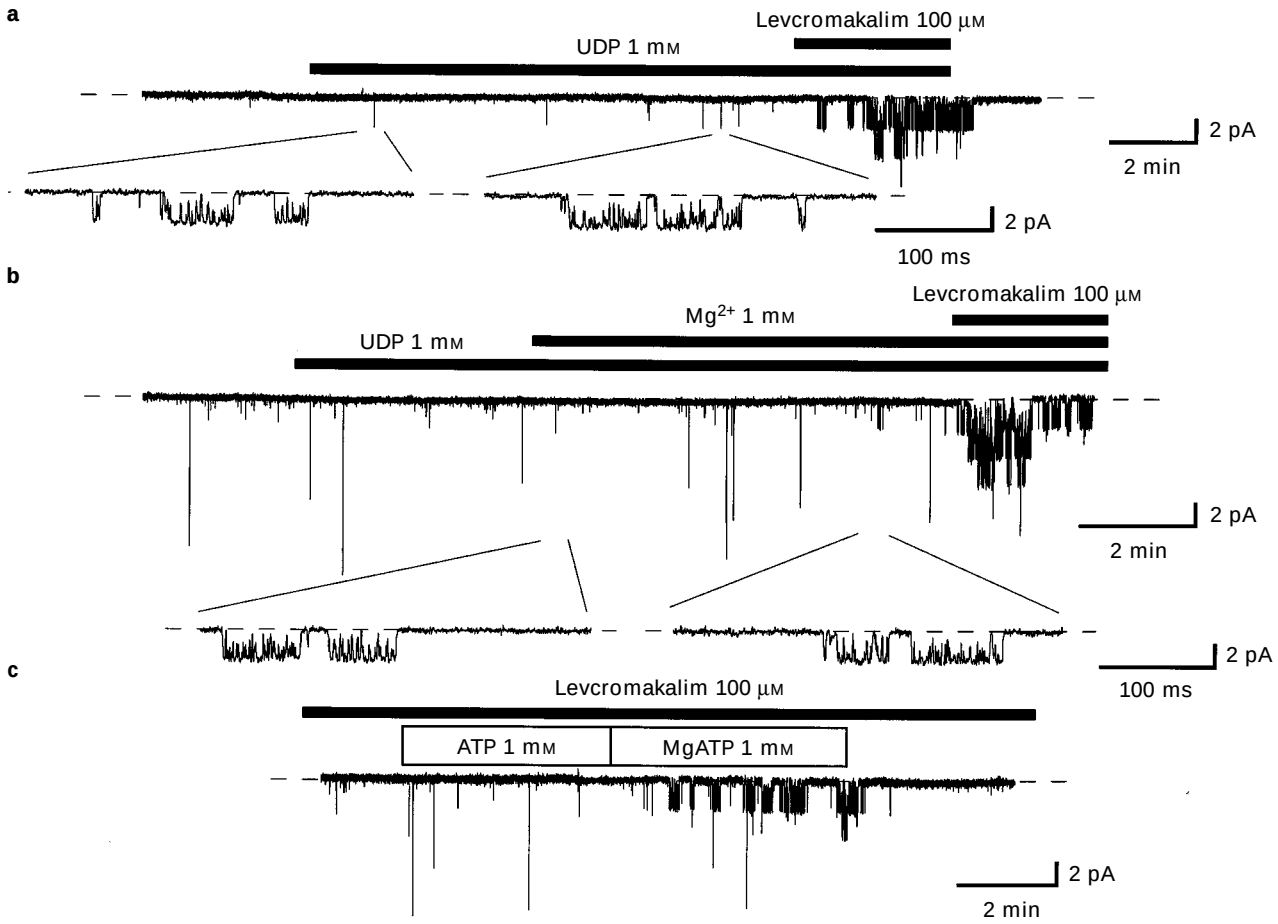

Figure 6 Activation of the glibenclamide-sensitive K^+ channel in inside-out patches at -50 mV. The dashed line indicates the current base line when the channel is not open. (a) UDP 1 mM alone activated the channel with brief openings even in the absence of levcromakalim (140 mM KCl containing 5 mM EGTA, in symmetrical K^+ conditions, $n=5$). An additional application of 100 μM levcromakalim further stimulated the channel activity. Lower traces show expansions of the upper trace. (b) UDP 1 mM did not activate the channel in the presence of 5 mM EDTA (symmetrical K^+ conditions). An additional application of free Mg^{2+} (1 mM) stimulated the channel with a small but significant channel opening. An additional application of 100 μM levcromakalim further stimulated the channel activity. Lower traces show expansions of the upper trace. (c) Recording after run-down of the glibenclamide-sensitive K^+ channel in the presence of 100 μM levcromakalim. Free ATP (1 mM) alone did not reactivate the channel although after the additional application of Mg^{2+} the channel reversibly reactivated ($n=5$).

channel, Zhang & Bolton, 1996). In pig proximal urethra, 10 μM glibenclamide completely abolished not only the levcromakalim-induced K^+ outward current but also the NDP-induced outward currents, the UDP-reactivated 43 pS K^+ channel and the untreated cytosolic extract-stimulated 43 pS K^+ channel. We have obtained a value of about 520 nM for the K_i of glibenclamide on the 100 μM levcromakalim-stimulated 43 pS K^+ channel in cell-attached configuration.

Apart from its sensitivity to glibenclamide, the K^+ channel in pig urethra has many properties in common with the glibenclamide-sensitive channels seen in vascular smooth muscles (K_{ATP} channel: rabbit portal vein, Kajioka *et al*., 1991; Kamouchi & Kitamura, 1994, K_{NDP}: rabbit portal vein, Beech *et al*., 1993b; rat mesenteric artery, Zhang & Bolton, 1995). (1) Levcromakalim selectively activated the channel. (2) Channel run-down was observed when the membrane was excised, even in the presence of KCOs. (3) Intracellular Mg^{2+} (1 mM) enhanced the UDP-induced reactivation in the presence of KCOs. (4) ATP on the cytoplasmic side of the plasma membrane inhibited the channel. However, in a number of other properties, the glibenclamide-sensitive K^+ channel in pig urethra seemed to have quite different characteristics. (1) The conductance of K_{ATP} channel in rabbit portal vein was found by Kamouchi & Kitamura (1994) to be approximately 26 pS in symmetrical 140 mM K^+ conditions when activated by pinacidil, (although under somewhat different conditions a channel of 50 pS (GDP-reactivated K_{ATP}; Kajioka *et al*., 1991), a value similar to that found in the pig urethra was found). (2) Although MgATP clearly reactivates the glibenclamide-sensitive K^+ channel in both pig urethra (present study) and rabbit portal vein (Kamouchi & Kitamura, 1994), MgATP had no effect on the GDP-reactivated K_{ATP} in rabbit portal vein (Kajioka *et al*., 1991). The ability of MgATP to restore channel activity in run-down patches is widely acknowledged to be a general characteristic of K_{ATP} in other tissues (cardiac cells: Tung & Kurachi, 1991; Terzic *et al*., 1994; insulin-secreting cell line: Findlay, 1987). Recently, Zhang & Bolton (1996) have identified two types of K_{ATP} with different conductances (LK channel; 50 pS, MK channel; 22 pS) in rat portal vein. The LK channel is blocked by glibenclamide (K_i 3 μM) and inhibited by $[ATP]_i$ but is also insensitive to KCOs, resembling K_{ATP} in other tissues (cardiac myocytes, pancreatic β-cells etc.), although MgATP dramatically suppressed LK channel activity. The MK channel is blocked by lower concentrations of glibenclamide, and is activated by NDPs, KCOs and MgATP, and is thus similar to the channel observed in both rabbit portal vein and rat mesenteric artery (K_{NDP}, Beech *et al*., 1993a; Zhang & Bolton, 1995). In pig urethra, the glibenclamide-sensitive channel clearly differs from the LK channel in that it is more sensitive to glibenclamide (K_i 520 nM) and is activated by KCOs and Mg ATP. Although the MK channel has a similar glibenclamide sensitivity to the urethral channel, and can be activated by NDPs in a similar concentration range, it has a much higher channel activity than the urethral channel in the

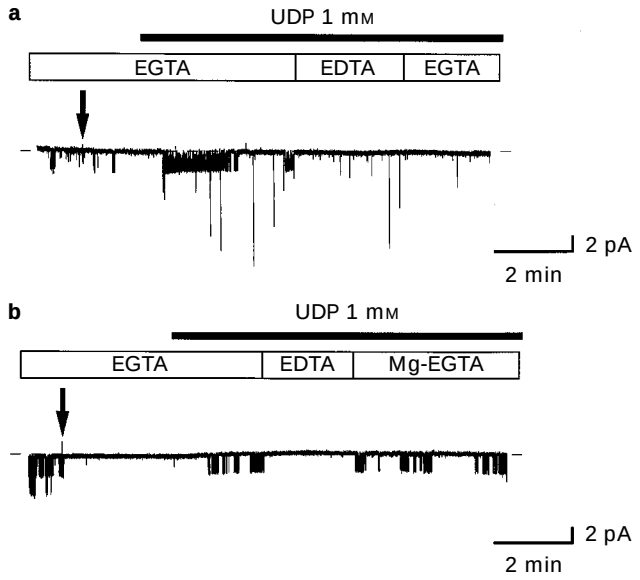

Figure 7 Intracellular Mg^{2+} regulated the channel activity of the 100 μM levcromakalim-activated K^+ channel. With 100 μM levcromakalim present in the bath solution, an inside-out patch was excised from the cell-attached configuration at the arrow. The holding potential was -50 mV, and the dashed lines indicate the current base line where the channel is not open. (a) UDP 1 mM reactivated the K^+ channel after run-down in EGTA containing intracellular solution, but the channel opening was suppressed when 5 mM EDTA was added to the solution to chelate any free Mg^{2+}, and did not recover when the EDTA-containing solution was removed. (b) Addition of 1 mM free Mg^{2+} to the EDTA-containing bathing solution resulted in the reappearance of the 1 mM UDP-reactivated K^+ channel even in the presence of 5 mM EDTA.

absence of KCOs, suggesting that the glibenclamide-sensitive K^+ channel in pig urethra may not belong to the MK type channel.

Bonev & Nelson (1993) have described a levcromakalim-induced glibenclamide-sensitive K_{ATP} in guinea-pig bladder myocytes. However, the unitary current conductance of this channel was 7.3 pS (bath 6 mM K^+/pipette 140 mM K^+, outside-out patch) which is much smaller than that in pig urethra under similar conditions (20 pS, Teramoto, unpublished observation). Moreover, Bonev & Nelson (1993) state that an intracellular application of GDP does not reactivate K_{ATP} in inside-out patches.

Thus the glibenclamide-sensitive K^+ channel in pig urethra appears to differ from K_{ATP} in guinea-pig bladder, despite both being activated by levcromakalim and sensitive to glibenclamide. The glibenclamide-sensitive K^+ channel in pig urethra is the first NDP-reactivated K^+ channel to be demonstrated in urinary tract smooth muscle cells.

Intracellular Mg^+ modulates channel activity

In whole-cell recordings, when Mg^{2+} was omitted from the pipette solution, both levcromakalim (>10 μM) and NDPs induced significant outward current at -50 mV. However, when 5 mM EDTA was included in the Mg^{2+}-free pipette solution in order to chelate intracellular Mg^{2+}, the peak amplitude of the levcromakalim induced current was much smaller. These results suggest that intracellular Mg^{2+} may play an important role in the activation mechanisms of the levcromakalim-induced outward current in pig proximal urethra. This is consistent with the findings of Edwards *et al.* (1993) that levcromokalim ($\leqslant 10$ μM) does not induce significant outward current in rat portal vein if Mg^{2+} is absent from the pipette solution.

In inside-out patches, UDP reactivated the levcromakalim K^+ channel after run-down even when Mg^{2+} was not added to the intracellular solution. In ventricular cells, Tung & Kurachi

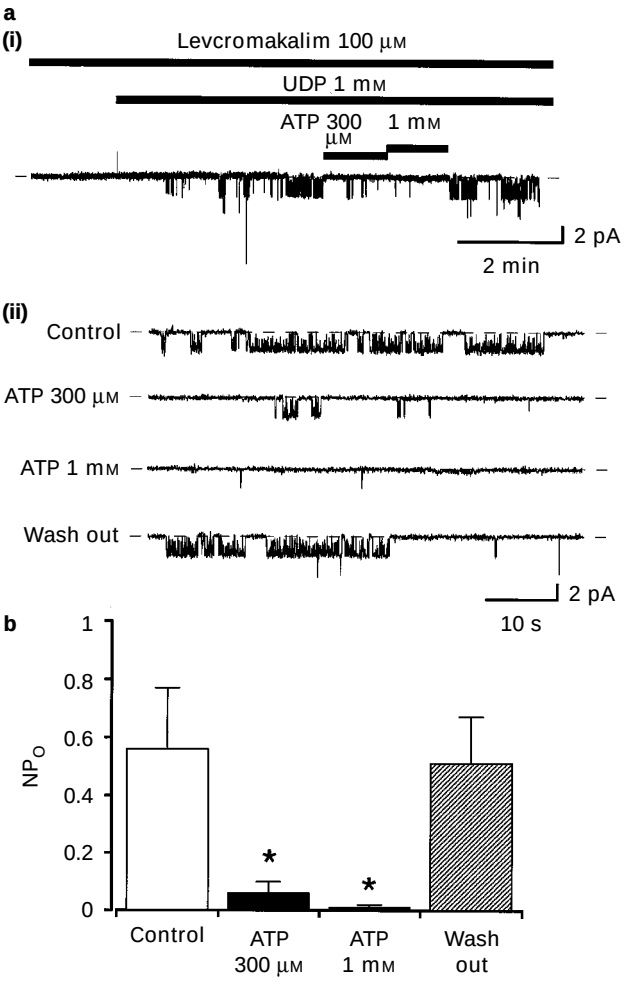

Figure 8 Reactivation of the glibenclamide-sensitive K^+ channel and the effect of ATP. The dashed line indicates the current base line where the channel is not open. (a) (i) The application of ATP to the inner surface of the membrane patch suppressed the UDP-reactivated 43 pS K^+ channel in a concentration-dependent manner. After ATP had been washed out, the channel opening recovered to the control level. (ii) Traces show expansions of the upper trace at the indicated conditions. (b) Histogram summarizing the inhibition due to the application of ATP. Each column indicates the NP_o value (+s.d.) for control (open column), the indicated concentration of ATP (solid columns), and after washing out ATP (hatched column) ($n=5$). *Significantly different from the control ($P<0.01$, t test).

(1991) have shown that the NDP-induced openings of K_{ATP} are dependent on intracellular Mg^{2+}, suggesting that the channel may have a Mg^{2+}-dependent NDP-binding site. In the present experiments, when the internal chelator was exchanged from 5 mM EGTA to 5 mM EDTA to chelate Mg^{2+} to an extremely low concentration, UDP-induced reactivation of the channel activity in inside-out patches disappeared. However, the channel activity reappeared with the additional application of 1 mM Mg^{2+} in the continued presence of 5 mM EDTA. It has been postulated that intracellular Mg^{2+} may remain tightly bound not only to membrane proteins, but also to intracellular proteins and organelles (reviewed by Murphy *et al.*, 1991). Our results suggest that a translatable Mg^{2+} source may be located either on the UDP-binding site or on the intracellular surface of the patch membrane close by. We conclude that intracellular Mg^{2+} may be essential for the reactivation mechanisms of the UDP-stimulated channels in pig urethra.

Terzic *et al.* (1994) have shown that the regulatory model of the K_{ATP} channel in guinea-pig ventricular myocytes has an ATP-inhibitory binding site, an NDP-binding site and two phosphorylation sites. In the present experiments, although we cannot be sure how many phosphorylation sites are present in

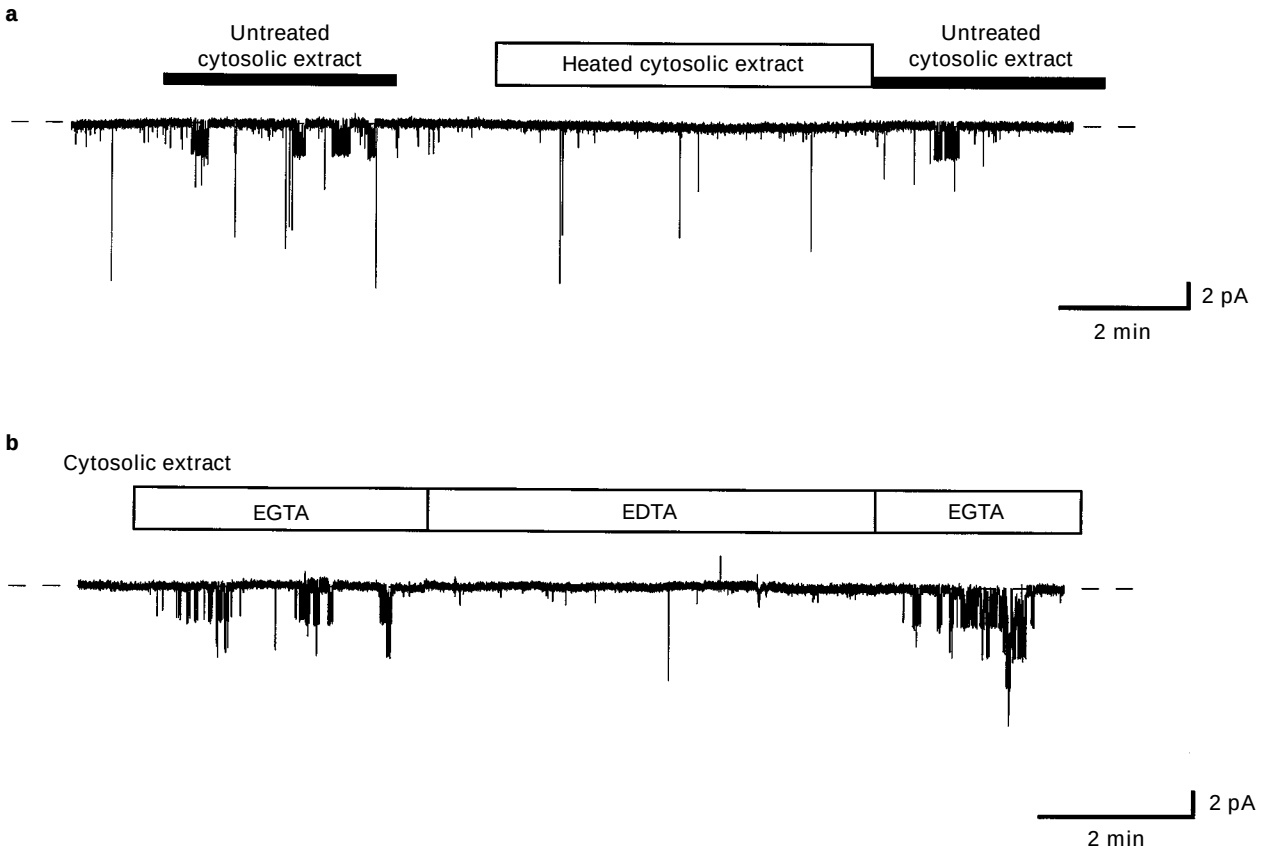

Figure 9 Effects of cytosolic extract on the excised membrane patch by use of inside-out configuration after run-down, in the presence of 100 μM levcromakalim in the bath solution. The patch membranes were held at -50 mV in symmetrical 140 mM K^+ conditions. The dashed line indicates the current base line where the channel is not open. (a) The application of the untreated cytosolic extract to the internal surface of the patch membrane reactivated the 2.14 pA K^+ channel. In contrast, after the untreated extract had been washed, about 1 min later, the application of the cytosol extract preparation which had been heated to 90–95°C for 4–5 min did not reactivate the glibenclamide-sensitive K^+ channel, even in the same patch. Reapplying the untreated extract reactivated the channel. (b) The untreated cytosolic extract dissolved in 140 mM KCl containing 5 mM EGTA reactivated the glibenclamide-sensitive K^+ channel. On the other hand, untreated cytosolic extract dissolved in 140 mM KCl containing 5 mM EDTA did not stimulate the glibenclamide-sensitive K^+ channel.

the glibenclamide-sensitive K^+ channel of pig proximal urethra, at least one phosphorylation site may be present to account for the reactivation of the channel by MgATP. Moreover, this phosphorylation site may be different from the NDP-modulatory site, since ATP alone does not reactivate the channel even in the presence of levcromakalim. Dunne & Peterson (1986) suggest the possibility that NDPs may antagonize the inhibitory effect of ATP on K_{ATP} in the insulin-secreting cell line RINm5F and concluded that the channel opening may be more closely related to the ATP/ADP ratio, than the ATP concentration. Terzic *et al.* (1994) also suggested that NDPs antagonize channel inhibition by ATP in guinea-pig ventricular myocytes. This might account for the ability of NDPs to enhance the levcromakalim-induced current and prevent run-down of channel activity in the pig proximal urethra.

The density of the levcromakalim-activated K^+ channel

Estimation of the number of channels in a cell (N) was made from measurements of the macroscopic currents in the presence of 100 μM levcromakalim by use of the following expression (Sigworth, 1980),

$$^{2} \quad iI \quad I^{2} \; N$$

Where i is the unitary current, σ^2 is the variance of the macroscopic current, and I is the mean macroscopic current. With a quasi-physiological potassium gradient (5 mM K^+ pipette-140 mM K^+ bath), the conductance of the unitary current was 20.1 ± 1.8 pS ($n=7$) in pig urethra. At -50 mV a rough estimation of N per cell was 162 ± 32 ($n=24$). The surface area of single smooth muscle cells in pig urethra can be estimated from the cell membrane capacitance (62.5 ± 9.5 pF, $n=58$) to be 6250 μm^2, assuming a specific capacitance of 1.0 $\mu F\ cm^{-2}$. If it is assumed that the glibenclamide-sensitive K^+ channels are evenly distributed over the membrane, this corresponds to a channel density of one channel per 38.6 μm^2. The channel density can also be estimated from the single channel data. Approximately 15% of membrane patches contained between one and three channels, but maximally five channels could be observed. However, in about 85% of the patches, glibenclamide-sensitive K^+ channel openings were not observed, even in the presence of levcromakalim. The average number of channels in one patch was 0.22 ± 0.03 ($n=285$). The density of the glibenclamide-sensitive K^+ channel may be roughly estimated to be one channel per >4.5 μm^2 (Sakmann & Neher, 1995). The density of this channel in pig urethra is much lower than that of rabbit pulmonary artery (one channel per <1.0 μm^2, Clapp *et al.*, 1994) or rabbit portal vein (one channel per $\geqslant 1.77$ μm^2, Kamouchi & Kitamura, 1994). The low density of the levcromakalim-activated K^+ channel in pig urethra may account for the fact that the levcromakalim-induced whole-cell current amplitude was smaller than obtained in other smooth muscle cells (Beech *et al.*, 1993a, b; Edwards *et al.*, 1993) and might explain the difficulty in investigating the activity of the glibenclamide-sensitive K^+ channel in single channel recordings. When NDPs were included in the pipette solution, the peak outward current amplitude evoked by the application of levcromakalim was increased. One interpretation could be that the glibenclamide-sensitive K^+ channel may have at least two states (non-operative state and operative state) when the channel is closed, and levcromakalim may only

be able to open channels which are in the operative state. NDPs may shift the equilibrium from the non-operative state towards the operative state, although NDPs alone caused some opening of the channel even in the absence of levcromakalim. This two state hypothesis is consistent with the results of the single channel recordings.

Physiological roles and clinical implications of the glibenclamide-sensitive K^+ channels in pig urethra

It is still uncertain whether glibenclamide-sensitive K^+ channels play a role in unstimulated physiological conditions, and whether or not NDPs can activate the channels in the absence of KCOs. In the present experiments, we are certain that in the absence of KCOs, UDP is capable of activating the channel. Our results suggest that in physiological conditions the glibenclamide-sensitive K^+ channel may play a minor role in determining resting membrane potential and urethral tone, and thus the concentration of NDPs, may be around the threshold for channel-opening activity. In the present experiments, a cytosolic extract of pig proximal urethra was also capable of reactivating the glibenclamide-sensitive K^+ channel in inside out patches in the presence of 100 μM levcromakalim, but not in its absence. We have not been able to establish what kind of molecule in the cytosolic extract is regulating the channel activity, although it is a heat-sensitive compound (such as a peptide or NDP etc.) and also intracellular Mg^{2+}-dependent.

The ideal goal for drug treatment of patients with detrusor instability is to reduce unstable contractions without affecting normal micturition. In *in vivo* experiments, cromakalim and pinacidil have been demonstrated to abolish the unstable contractions associated with bladder outflow obstruction in pig and rat without affecting the ability to void urine (Foster *et al*., 1989; Malmgren *et al*., 1989; Hedlund *et al*., 1991) and KCOs thus have potential as a therapeutic treatment of instability. Our results show that levcromakalim is capable of activating K^+ channels in pig urethra. Since reduction in urethral smooth muscle tone and thus urethral pressure would also be undesirable in a drug used to treat bladder instability, potential bladder-selective KCOs should be screened against urethral, as well as vascular smooth muscle.

In conclusion, after run-down, the levcromakalim-induced glibenclamide-sensitive K^+ channels in urethral myocytes can be reactivated by intracellular application not only of UDP but also MgATP and an untreated cytosolic extract. The activation mechanism may be dependent on intracellular Mg^{2+}.

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References

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

ASHCROFT, F.M. (1988). Adenosine 5′-triphosphate-sensitive potassium channels. *Annu. Rev. Neurosci*., **11,** 97–118.

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

BEECH, D.J., ZHANG, H., NAKAO, K. & BOLTON, T.B. (1993b). Single channel and whole-cell K-currents evoked by levcromakalim in smooth muscle cells from the rabbit portal vein. *Br. J. Pharmacol*., **110,** 583–590.

BENHAM, C.D. & BOLTON, T.B. (1986). Spontaneous transient outward currents in single visceral and vascular smooth muscle cells of the rabbit. *J. Physiol*., **381,** 385–406.

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. (1987). Physiology of bladder smooth muscle. In *The Physiology of the Lower Urinary Tract*, ed. Torrens, M. & Morrison, J.F.B. pp. 161–191. Berlin: Springer-Verlag.

CLAPP, L.H., GURNEY, A.M., STANDEN, N.B. & LANGTON, P.D. (1994). Properties of the ATP-sensitive K^+ current activated by levcromakalim in isolated pulmonary arterial myocytes. *J. Memb. Biol*., **140,** 205–213.

CROWE, R. & BURNSTOCK, G. (1989). A histochemical and immunohistochemical study of the autonomic innervation of the lower urinary tract of the female pig. Is the pig a good model for the human bladder and urethra? *J. Urol*., **141,** 414–422.

DUNNE, M.J. & PETERSEN, O.H. (1986). Intracellular ADP reactivates K^+ channels that are inhibited by ATP in an insulin-secreting cell line. *FEBS Lett*., **208,** 59–62.

EDWARDS, G., IBBOTSON, T. & WESTON, A.H. (1993). Levcromakalim may induce a voltage-independent K-current in rat portal veins by modifying the gating properties of the delayed rectifier. *Br. J. Pharmacol*., **110,** 1037–1048.

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

FINDLAY, I. (1987). The effects of magnesium upon adenosine triphosphate-sensitive potassium channels in a rat insulin-secreting cell line. *J. Physiol*., **391,** 611–629.

FOSTER, C.D., SPEAKMAN, M.J., FUJII, K. & BRADING, A.F. (1989). The effects 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.

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.

ITO, S., KAJIKURI, J., ITOH, T. & KURIYAMA, H. (1991). Effects of lemakalim on changes in Ca^{2+} concentration and mechanical activity induced by noradrenaline in the rabbit mesenteric artery. *Br. J. Pharmacol*., **104,** 227–233.

KAJIOKA, S., KITAMURA, K. & KURIYAMA, H. (1991). Guanosine diphosphate activates an adenosine 5′-triphosphate-sensitive K^+ channel in the rabbit portal vein. *J. Physiol*., **444,** 397–418.

KAMOUCHI, M. & KITAMURA, K. (1994). Regulation of ATP-sensitive K^+ channels by ATP and nucleotide diphosphate in rabbit portal vein. *Am. J. Physiol*., **266,** H1687–H1698.

KITAMURA, K. & KURIYAMA, H. (1994). Molecular mechanisms of action of antihypertensive agents, 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.

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.

MURPHY, E., FREUDENRICH, C.C. & LIEBERMAN, M. (1991). Cellular magnesium and Na/Mg exchange in heart cells. *Annu. Rev. Physiol*., **53,** 273–287.

OKADA, Y., YANAGISAWA, T. & TAIRA, N. (1993). BRL 38227 (levcromakalim)-induced hyperpolarization reduces the sensitivity to Ca^{2+} of contractile elements in canine coronary artery. *Naunyn-Schmiedeberg's Arch. Pharmacol*., **347,** 438–444.

SAKMANN, B. & NEHER, E. (1995). Geometric parameters of pipettes and membrane patches. In *Single-Channel Recording Second Edition*, ed. Sakmann, B. & Neher, E., Chapter 21, pp. 637–650, New York, London: Plenum Press.

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

SIGWORTH, F.I. (1980). The variance of sodium current fluctuations at the node of Ranvier. *J. Physiol.*, **307,** 97–129.

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, P,** 56P.

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

TERAMOTO, N. & BRADING, A.F. (1996). Activation by levcromakalim and metabolic inhibition of glibenclamide-sensitive K channels in smooth muscle cells of pig proximal urethra. *Br. J. Pharmacol.*, **118,** 635–642.

TERZIC, A., FINDLAY, I., HOSOYA, Y. & KURACHI, Y. (1994). Dualistic behaviour of ATP-sensitive K^+ channels toward intracellular nucleoside diphosphates. *Neuron*, **12,** 1049–1058.

TRIVEDI, S., STETZ, S., LEVIN, R., LI, J. & KAU, S. (1994). Effect of cromakalim and pinacidil on ^{86}Rb efflux from guinea pig urinary bladder smooth muscle. *Pharmacology*, **49,** 159–166.

TUNG, R.T. & KURACHI, Y. (1991). On the mechanism of nucleotide diphosphate activation of the ATP-sensitive K^+ channel in ventricular cell of guinea-pig. *J. Physiol.*, **437,** 239–256.

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.

ZHANG, H.-L. & BOLTON, T.B. (1996). Two types of ATP-sensitive potassium channels in rat portal vein smooth muscle cells. *Br. J. Pharmacol.*, **118,** 105–114.

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