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The effects of flecainide on ATP-sensitive K^+ channels in pig urethral myocytes

[1]Takakazu Yunoki, *,[1]Noriyoshi Teramoto, [2]Seiji Naito & [1]Yushi Ito

[1]Department of Pharmacology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, 812-8582, Japan and [2]Department of Urology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, 812-8582, Japan

1 The effects of the antiarrhythmic drug flecainide on levcromakalim-induced hyperpolarization, macroscopic and unitary K^+ currents in pig urethra were investigated using patch-clamp techniques. The effects of flecainide were also examined on currents in inside-out patches of COS7 cells expressing carboxy terminus truncated inwardly rectifying K^+ channel (Kir6.2) subunits (i.e. Kir6.2ΔC36) which form ATP-sensitive K^+ channels (K_{ATP} channels).

2 In current-clamp mode, application of flecainide ($\geqslant 100$ μM) caused a significant depolarization after the membrane potential had been hyperpolarized by levcromakalim.

3 In voltage-clamp experiments, the levcromakalim-induced outward current was suppressed by 300 μM flecainide in quasi-physiological K^+ conditions ($K_i = 51$ μM). In contrast, approximately 20% of the levcromakalim-induced inward current still remained even after application of 300 μM flecainide in symmetrical 140 mM K^+ conditions ($K_i = 126$ μM).

4 In cell-attached configuration, the channel activity of the levcromakalim-induced K_{ATP} channels was reversibly inhibited by flecainide ($\geqslant 30$ μM) at -50 mV. Their activity was also suppressed by either disopyramide or cibenzoline.

5 Flecainide reversibly inhibited the channel activity of Kir6.2ΔC36 expressed in COS7 cells using inside-out configuration.

6 Inhibitory effects of flecainide on the levcromakalim-induced currents became more potent when the value of external pH increased, although this slightly reduced the proportion of drug molecules carrying a positive charge.

7 These results suggest that flecainide inhibits channel activity through blocking the pore site of the K_{ATP} channel in pig urethra.

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Abbreviations: GFP, green fluorescence protein; K_{ATP} channels, ATP-sensitive K^+ channels; E_K theoretical K^+ equilibrium potential; K_a, acid dissociation constant; K_i, inhibitory dissociation constant; Kir, inwardly-rectifying K^+ channel; Kir6.2ΔC36, the last 36 amino acids of the carboxy terminus truncated Kir6.2; n_H. Hill's coefficient; NP_o, channel open state probability; PSS, physiological salt solution; RT–PCR, reverse transcriptase-polymerase chain reaction; SUR, sulphonylurea receptor

Introduction

Antiarrhythmic drugs have been widely used for the treatment of supraventricular and ventricular arrhythmias, by affecting cardiac repolarization and prolonging the duration of action potentials mainly due to the inhibitory effects on Na^+ channels in a voltage- and rate-dependent manner (reviewed by Woosley, 1991). In addition, antiarrhythmic drugs block voltage-dependent Ca^{2+} channels (Scamps *et al*., 1989) and K^+ channels (Slawsky & Castle, 1994). Furthermore, it has been shown that antiarrhythmic drugs also inhibit ATP-sensitive K^+ channels (K_{ATP} channels) in cardiac myocytes and pancreatic ß-cells (Horie *et al*., 1992).

In the urological field, it is well-known that both antiarrhythmic drugs and K^+ channel openers are clinically effective for the treatment of motor urge incontinence (reviewed by Andersson, 1988). However, Danziger & Horn (1983) reported that severe urinary retention is commonly observed as an adverse reaction during antiarrhythmic drug therapy probably due to its antimuscarinic properties. The precise mechanisms of action of these drugs still remain to be elucidated. Furthermore, no data are currently available concerning the effects of antiarrhythmic drugs on either K_{ATP} currents or K_{ATP} channels in smooth muscle. It is thus of interest to investigate the mechanisms of action of these drugs and the direct interaction between them in lower urinary tract cells.

We have previously demonstrated the presence of the glibenclamide-sensitive K^+ channels which are suppressed by intracellular application of ATP ($\geqslant 1$ mM) in pig proximal urethra (i.e. K_{ATP} channels; Teramoto & Brading, 1996). These channels play an important role in regulating not only the resting urethral muscle tone in the pig but also the resting membrane potentials and the basal membrane current even

*Author for correspondence at: Department of Pharmacology, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi Ward, Fukuoka, 812-8582, Japan.
E-mail: noritera@linne.med.kyushu-u.ac.jp

under unstimulated conditions (Teramoto & Brading, 1996; Teramoto *et al*., 1997). In the present experiments, we have investigated the effects of antiarrhythmic drugs (such as flecainide, disopyramide, cibenzoline) on the levcromakalim-induced K_{ATP} currents and K_{ATP} channels in pig proximal urethra. We have further studied the blocking mechanisms of antiarrhythmic drugs on currents in COS7 cells expressing a carboxy terminus truncated Kir6.2 (Kir6.2ΔC36) which can form K^+ channels in the absence of sulphonylurea receptors (SURs).

Methods

Cell dispersion

Fresh urethras from female pigs were collected from a local abattoir. Pig urethral myocytes were isolated by the gentle tapping method (Teramoto & Brading, 1996). Relaxed spindle-shaped cells, with length varying between 400 and 500 μm, were isolated and stored at 4°C. The dispersed cells were normally used within 2 h for experiments.

Molecular biology

Kir6.2ΔC36, in which the last 36 amino acids were truncated from the carboxy terminus (C-terminus), was constructed by PCR, inserting a stop codon at the appropriate position. Kir6.2ΔC36 was subcloned into the pCl vector which contained the CMV-IE promoter/enhancer (Promega, Madison, WI, U.S.A.). pEGFP-N1, enhanced fluorescent mutant green fluorescent protein (GFP) driven by CMV-IE promoter/enhancer (Clontech Laboratories, Inc., Palo Alto, CA, U.S.A.) was co-transfected as a marker and the whole nucleotide sequence of the PCR clone was confirmed by DNA sequencing.

Cell culture and transfection

COS7 cells were plated on coverslips at a density of 1×10^5 per dish (35 mm in diameter) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum. Two days later, a cocktail of pCl-Kir6.2ΔC36 and pEGFP−N1 was co-transfected into the COS7 cells using LipofectAMINE and Opti-MEM (Gibco BRL, Life Technologies, Inc., Rockville, MD, U.S.A.) according to the manufacturer's instructions. Electrophysiological measurements were usually conducted from 2–4 days after transfection.

Drugs and solutions

For whole-cell recordings, 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 (sometimes 140 mM K^+ PSS was obtained by replacing 135 mM Na^+ with equimolar K^+); high potassium pipette solution containing (mM): K^+ 140, Cl^- 140, EGTA 5, and HEPES 10/Tris (pH 7.35–7.40). For single-channel recordings, the pipette and bath solution were high potassium solution (mM): K^+ 140, Cl^- 140, EGTA 5, glucose 5, HEPES 10/Tris (pH 7.35–7.40) producing symmetrical 140 mM K^+ conditions. Cells were allowed to settle in the small experimental chamber (150 μl in volume). The bath solution was superfused by gravity throughout the experiments at a rate of 2 ml min^{-1}. The following chemicals were used: collagenase (Wako Pure Chemical Industries Ltd., Osaka, Japan), dimethysulphoxide (DMSO), EGTA, glibenclamide, HEPES, papain and TrisCl (Sigma-Aldrich Japan K.K., Tokyo, Japan), pinacidil (RBI Chemicals Ltd., Natick, MA, U.S.A.) and levcromakalim (kindly provided by SmithKline Beecham Pharmaceuticals, Harlow, U.K.). Pinacidil, levcromakalim, glibenclamide and antiarrhythmic drugs (flecainide, disopyramide, cibenzoline) were prepared as 100 mM stock solutions in DMSO. The final concentration of DMSO was less than 0.3%, and this concentration was shown not to affect K^+ channels in pig urethra. Occasionally, flecainide was directly dissolved in 5 mM K^+ PSS or 140 mM K^+ PSS.

Recording procedure

Patch-clamp experiments were performed at room temperature (21–23°C) as described previously (Teramoto *et al*., 2000a, b). 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. Noise associated with capacitance was kept to a minimum by maintaining the test solution in the electrode (2–3 MΩ) as low as possible. At the beginning of each experiment, the series resistance was compensated. Transfected cells were identified by green fluorescence under a microscope.

Data analysis

The data recording system used was essentially the same as that described previously (Teramoto *et al*., 2000a, b). In short, generation of voltage pulses was performed using a L/M-EPC 8 patch-clamp amplifier (HEKA Elektronik Corp., Lambrecht, Germany) in conjunction with a circuit board which has both an analogue to digital and a digital to analogue conversion function (ITC-16, Instrutech Corp., NY, U.S.A.), using a 'Pulse' software package (HEKA Elektronik Corp., Lambrecht, Germany). The sampled current data were filtered at 10 kHz and stored together with potential records on videotape using a digital data recorder (VR-10B, Instrutech Corp., NY, U.S.A.) coupled to a video recorder (Panasonic, Tokyo, Japan) for subsequent off-line analysis. The whole-cell current data were low-pass filtered at 500 Hz (continuous traces) or 2 kHz (ramp current) by an eight pole Bessel filter, sampled at 25 ms intervals (continuous traces) or 1 ms (ramp current) and analysed on a PowerMac G3 computer (Apple Computers, Tokyo, Japan) using commercial software 'MacLab 3.5.6' (ADInstruments Pty Ltd., Castle Hill, Australia). For single-channel recordings, the stored data were low-pass filtered at 2 kHz (−3 dB) and sampled into the computer with a digitalized interval of 80 μs using the 'PAT' programme (kindly provided by Dr. J. Dempster, University of Strathclyde, U.K.); events briefer than 80 μs were not included in the evaluation. 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_o) were measured at −50 mV for 1 min. According to the equation:

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

where t_j is the time spent at each current level corresponding to $j=0, 1, 2, \ldots N$, T is the duration of the recording, and N was the maximum number of channels observed but the minimal number of channels in the patch. Data points were fitted by least-squares analysis.

Statistics

Statistical analyses were performed with analysis of variance (ANOVA) (two-factor with replication). Changes were considered significant at $P<0.01$ and data are expressed as mean with the standard deviation (s.d.).

Results

Effects of flecainide on the levcromakalim-induced hyperpolarization in pig proximal urethral myocytes

In current-clamp mode, bath application of 100 μM levcromakalim immediately caused a stable hyperpolarization (from −35±5 mV, $n=4$ to −73±5 mV, $n=4$) which was close to the theoretical K^+ equilibrium potential ($E_K=-82$ mV) under quasi-physiological K^+ conditions (the bath solution, PSS; the pipette solution, 140 mM KCl containing 5 mM EGTA). This levcromakalim-induced hyperpolarization was irreversibly inhibited by 1 μM glibenclamide (Teramoto & Brading, 1996; 1998). When flecainide was additionally applied in a cumulative manner, it caused a significant reversal of the hyperpolarization at higher than 100 μM, whereas lower concentrations of flecainide (⩽30 μM) had no significant effect on the levcromakalim-induced hyperpolarization (Figure 1a, b, $n=4$).

Effects of flecainide on the levcromakalim-induced outward and inward currents in pig urethra

To investigate further the inhibitory effects of flecainide on the levcromakalim-induced stable hyperpolarization, whole-cell voltage-clamp experiments were performed at a holding potential of −50 mV under quasi-physiological K^+ conditions. As shown in Figure 2a, application of levcromakalim (100 μM) immediately caused an outward current which, after reaching a peak value, sustained in amplitude at constant membrane potential. When 300 μM flecainide was additionally applied, the levcromakalim-induced outward current was rapidly inhibited to 8±1% ($n=5$) of the peak amplitude when the mean amplitude of the levcromakalim-induced outward current was taken as one 30 s before application of flecainide. On removal of flecainide, the amplitude of the levcromakalim-induced current showed a rapid recovery towards the steady state value it would have reached. Subsequent application of 5 μM glibenclamide suppressed the outward current.

In order to change the direction of the basal sustained levcromakalim-induced current at −50 mV from outward to inward, $[K^+]_o$ was raised from 5 mM to 140 mM (the bath solution, 140 mM K^+ PSS; the pipette solution, 140 mM KCl containing 5 mM EGTA, i.e., symmetrical 140 mM K^+ conditions). Under these conditions, levcromakalim (100 μM) caused an inward current which, as expected, was reduced in amplitude by 300 μM flecainide to 25±4% ($n=5$). Figure 2c shows the concentration-dependent inhibitory effects of flecainide on the 100 μM levcromakalim-induced outward and inward currents at −50 mV, demonstrating a significantly different inhibitory potency (outward current, $K_i=51$ μM; inward current, $K_i=126$ μM). When the holding membrane potential was raised to −30 mV, the inhibitory curve was shifted to the left in comparison with −50 mV ($K_i=79$ μM, Figure 3). On the other hand, lowering the holding potential to −80 mV, shifted the curve to the right ($K_i=173$ μM). Both inward and outward levcromakalim-induced K^+ currents were suppressed by application of 5 μM glibenclamide whether the membrane potential was held at −80 or −30 mV (data not shown).

Further studies were carried out of the effects of 100 μM flecainide on the levcromakalim-induced inward currents using triangular ramp potential pulses (see inset in Figure 4a). Four of these were applied from −120 to +80 mV, obtaining the current-voltage relationships in the absence and presence of 100 μM flecainide. Figure 4a shows the expected reduction of the levcromakalim-induced inward current on application of 100 μM flecainide. Figure 4b shows the averaged membrane currents during the falling phase of the ramp pulses under the various experimental conditions. The levcromakalim-induced membrane current was obtained by subtracting the averaged control current from the membrane current in the presence of 100 μM levcromakalim and demonstrated an inwardly rectifying property at positive

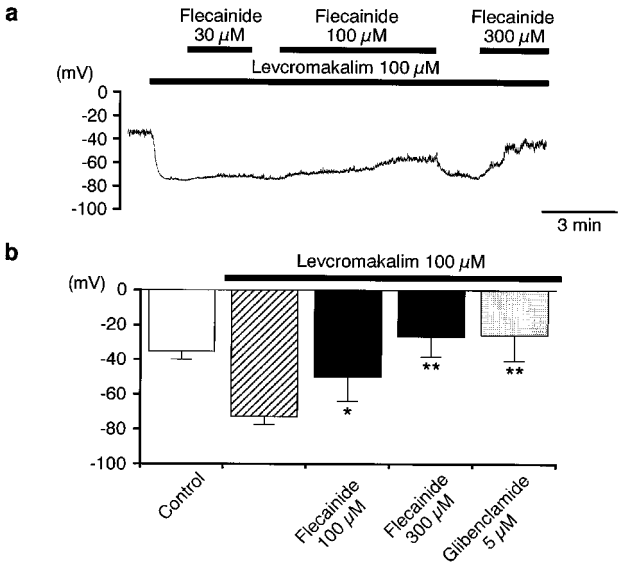

Figure 1 Concentration-dependent inhibitory effects of flecainide on the 100 μM levcromakalim-induced hyperpolarization in dispersed urethral smooth muscle cells recorded in current-clamp mode. (a) Recording from a single cell. (b) Concentration-response relationships for the inhibitory effects of flecainide and glibenclamide. Control indicates the membrane potential just before the application of 100 μM levcromakalim. Each column shows the mean of 3–4 observations with s.d. The potentials in the presence of flecainide and glibenclamide were significantly different from that in levcromakalim alone (*$P<0.05$, **$P<0.01$).

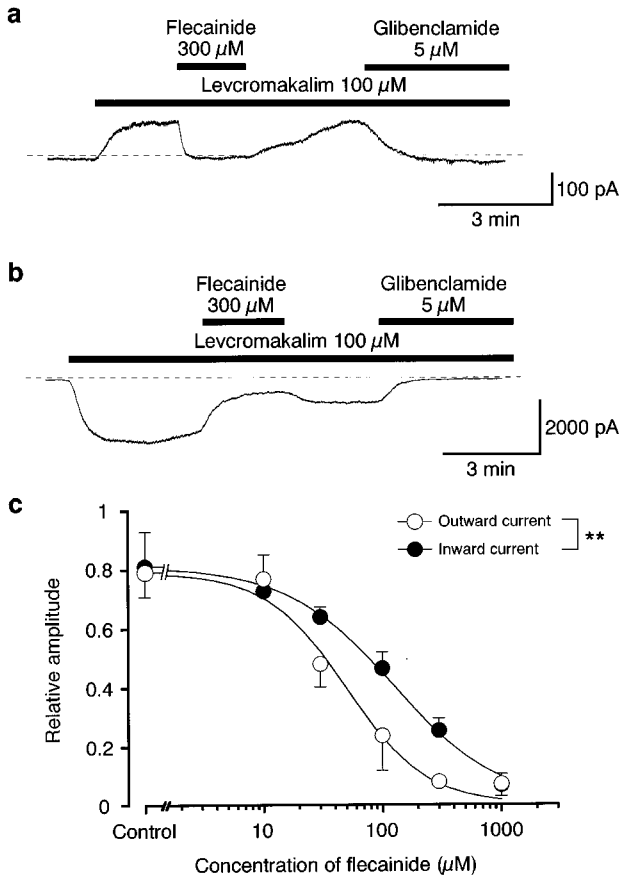

Figure 2 Effects of flecainide (300 μM) on the 100 μM levcromakalim-induced K^+ current in pig urethral myocytes at a holding potential of -50 mV. The pipette solution was 140 mM KCl containing 5 mM EGTA. The bath solution was either 5 mM K^+ PSS (a) or 140 mM K^+ PSS (b). The dashed lines indicate zero current. (c) Concentration-response curves of flecainide. The mean amplitude of the 100 μM levcromakalim-induced current (measured over the final 30 s before application of flecainide) was taken as one. In the absence of flecainide, after the levcromakalim-induced K^+ current had reached a maximum value, the amplitude of the current gradually decreased. The rate of the current decay was determined from measurements at 30 s intervals for 8 min after the peak amplitude. Since flecainide was applied within 2 min from the peak in all experiments, the value of the levcromakalim-induced K^+ current 1.5 min after the peak was taken as one, measuring from the current level in the presence of 5 μM glibenclamide. The relative control value was roughly estimated by measuring the current decay 2.5 min after the peak amplitude (outward current, 0.79 ± 0.08, $n=5$; inward current, 0.81 ± 0.12, $n=5$). The curve was drawn by fitting the equation using the least-squares method:

$$\text{Relative amplitude} = 1/\{1+(K_i/D)n_H\}$$

where K_i, D and n_H are the inhibitory dissociation constant, concentration of flecainide (μM) and Hill's coefficient, respectively. The following values were used for the curve fitting: outward, $K_i=51$ μM, $n_H=1.3$; inward, $K_i=126$ μM, $n_H=1.0$. ** The two curves were significantly different (ANOVA, $P<0.01$).

potentials (Figure 4c). The 100 μM flecainide-sensitive membrane current in the presence of levcromakalim was obtained by subtracting the mean membrane current in the presence of 100 μM flecainide from the membrane current in its absence. Although the amplitude of the flecainide-sensitive current was smaller than that of the levcromakalim-induced current at negative potentials, its amplitude was much larger

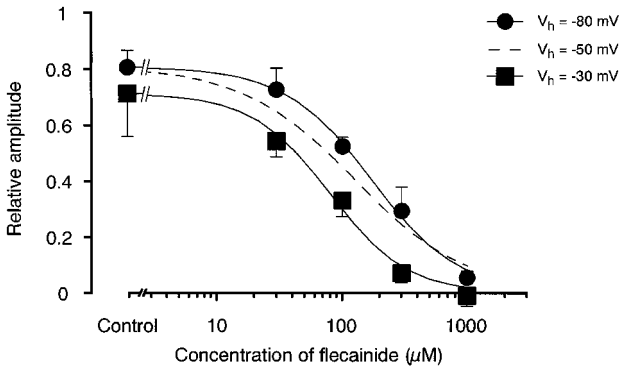

Figure 3 Relationship between the relative peak amplitude of the levcromakalim-induced inward K^+ current and the concentration of flecainide at various holding membrane potentials (-30, -50 and -80 mV). The mean amplitude of the levcromakalim-induced current over 30 s before application of flecainide was normalized to one and 90 s after application of flecainide, the mean membrane current was measured for 30 s from the current level in the presence of 5 μM glibenclamide. The curves are drawn as in Figure 2, and the current decay of the levcromakalim-induced current in the absence of flecainide was estimated as 0.71 ± 0.15 at -30 mV, ($n=5$); and 0.81 ± 0.06 at -80 mV, ($n=4$). The following values were used for the curve fitting: $K_i=79$ μM, $n_H=1.4$, -30 mV and $K_i=173$ μM, $n_H=1.2$, -80 mV. Each symbol indicates the mean of 3–6 observations with s.d.s shown by vertical lines. Some of the s.d. bars are less than the size of the symbol. The curve with the broken line was obtained from Figure 2 (c).

than that of the levcromakalim-induced current at positive potentials. A similar inhibitory effect of flecainide was observed on the inward current induced by 100 μM pinacidil at -50 mV (Figure 5a, 0.32 ± 0.06, $n=4$). The amplitude of the 100 μM levcromakalim-induced inward current was also suppressed by application of the other antiarrhythmic drugs cibenzoline 100 μM (Figure 5b, 0.15 ± 0.03, $n=4$) and disopyramide (100 μM, 0.31 ± 0.08, $n=4$; 300 μM, 0.19 ± 0.09, $n=4$, Figure 5c). On removal of the antiarrhythmic drugs, the levcromakalim-induced current gradually increased to a steady value that was less than the original level.

Effects of flecainide on the levcromakalim-activated K^+ channels at -50 mV in cell-attached configuration

The effects of flecainide (100 μM) on the K^+ channels activated by 100 μM levcromakalim were investigated in cell-attached patches in which small numbers of the 2.1 pA channels were opened at a holding membrane potential of -50 mV. In Figure 6a, the K^+ channel had an NP_o value of 0.22. Application of 100 μM flecainide reversibly inhibited the channel activity to an NP_o value of 0.03 (Figure 6b). If the control 100 μM levcromakalim-induced NP_o value was taken as one, the relative NP_o value was 0.25 ± 0.2 ($n=4$) in the presence of 100 μM flecainide. On removal of flecainide, both the channel activity and the unitary amplitude completely recovered to the control level. Subsequently, bath application of glibenclamide (5 μM) reversibly abolished the channel activity (data not shown). In Figure 6c, disopyramide (100 μM) also inhibited the activity of the levcromakalim-activated K^+ channels (the relative inhibitory ratio, 0.21 ± 0.08, $n=5$) when the control 100 μM levcromakalim-induced NP_o value was normalized as one. On removal of

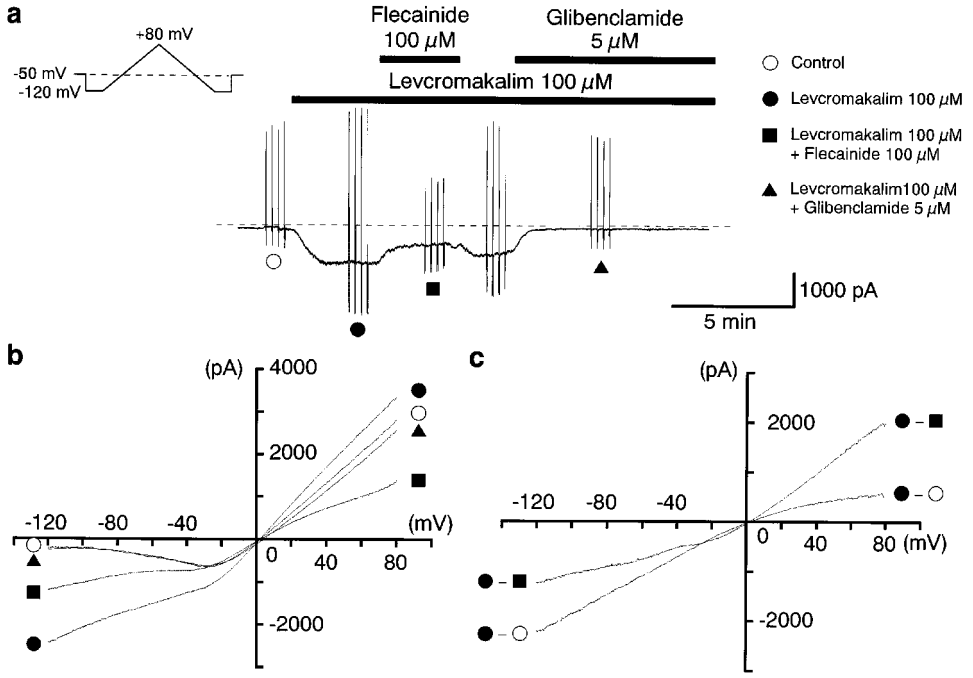

Figure 4 Inhibitory effects of flecainide on the levcromakalim-induced glibenclamide-sensitive inward K^+ current at -50 mV (bath solution, 140 mM K^+ PSS; pipette solution, 140 mM K^+ containing 5 mM EGTA). (a) Current trace. The vertical lines are responses to triangular ramp potential pulses of 200 mV s^{-1} from -120 to $+80$ mV, applied after an initial 100 ms conditioning pulse to -120 mV (see inset). Levcromakalim (100 μM) caused an inward membrane current (peak amplitude approximately 1 nA). The current was suppressed by application of 100 μM flecainide, recovered to a steady state amplitude after flecainide was removed, and was then suppressed by 5 μM glibenclamide. The dashed line indicates zero current line. (b) The current-voltage curves measured from the negative-going limb (the falling phase) of the ramp pulse. Each symbol is the same as in the current trace. The lines are mean membrane currents from the four ramps in each condition. (c) Net membrane currents. The levcromakalim-induced membrane current was obtained by subtraction of the two mean ramp currents recorded before and during application of 100 μM levcromakalim. The flecainide-sensitive membrane current was obtained from the membrane currents in the absence and presence of 100 μM flecainide when levcromakalim was present in the bath solution.

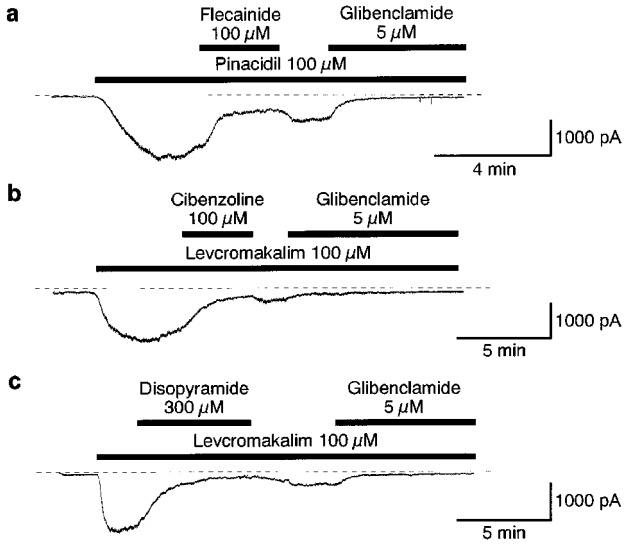

Figure 5 Effects of antiarrhythmic drugs on K_{ATP} opener-induced glibenclamide-sensitive inward K^+ currents at a holding potential of -50 mV. Bath solution 140 mM K^+ PSS, pipette solution 140 mM K^+ containing 5 mM EGTA. The dashed lines indicate the zero current level. (a) Application of flecainide 100 μM inhibited the 100 μM pinacidil-induced glibenclamide-sensitive inward K^+ current. (b), (c) The 100 μM levcromakalim-induced membrane current was suppressed by subsequent application of 100 μM cibenzoline (b) or 300 μM disopyramide (c).

disopyramide, the activity did not recover to the control level even after 20 min washing-out (0.63 ± 0.2, $n=5$).

Inhibitory effects of flecainide and disopyramide on channel activity of Kir6.2ΔC36

In excised membrane patches from GFP-positive COS7 cells which expressed Kir6.2ΔC36, application of flecainide caused a reversible inhibition of channel activity in a concentration-dependent manner at -50 mV (Figure 7a, b, $n=4$). However, glibenclamide (10 μM) had no significant effect (data not shown). Similarly, disopyramide (100 μM) suppressed the channel activity (Figure 7c).

Effects of pH on the flecainide-induced inhibition of the membrane currents in pig urethra

Flecainide is a strongly hydrophobic drug and possesses a pK_a value of 9.3. In the present experiments, although flecainide was initially dissolved in DMSO, it was then directly diluted in an aqueous extracellular solution at three different values of pH (6.7, 7.4 and 8.1) in order to investigate the effects of the charged form of flecainide on the levcromakalim-induced currents. Figure 8a shows the experimental protocol. Application of 100 μM levcromakalim caused an outward current in 5 mM K^+ PSS at pH 6.7 at -50 mV. In Figure 8c, application of 300 μM flecainide

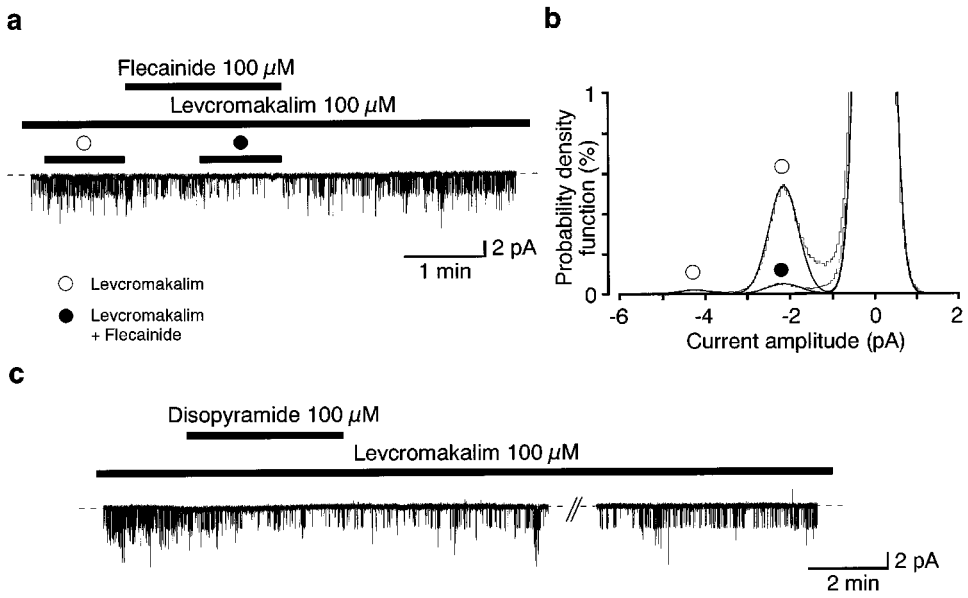

Figure 6 Effects of flecainide and disopyramide on the 100 μM levcromakalim-induced K^+ channel. Cell-attached patch at -50 mV. Levcromakalim 100 μM was added to the bath solution (symmetrical 140 mM K^+ conditions). (a) Application of 100 μM flecainide (1 min duration) reversibly reduced the activity of the 2.1 pA K^+ channel. The dashed line indicates the current when the channel is not open. (b) All-point amplitude histograms in the presence of 100 μM flecainide. Histograms in the absence (control; just before the application of flecainide) and presence of 100 μM flecainide are superimposed. Continuous lines in the histograms are theoretical curves fitted with the Gaussian distribution, by the least-squares method. The abscissa scales show the amplitude of the current (pA) and the ordinate scales show the percentage value of the probability density function (per cent) for the recording period (1 min). The relative NP_o value ($\pm$s.d.) in the presence of 100 μM flecainide was 0.25 ± 0.2 ($n=4$). The value in the presence of flecainide was significantly different from the control ($P<0.01$, ANOVA). Each symbol is the same as in the current trace. (c) Application of 100 μM disopyramide (4 min duration) reduced the activity of the 2.1 pA K^+ channel. On removal of disopyramide, the channel activity did not recover to the control level even after approximately 12 min wash-out. The dashed line indicates the current when the channel is not open.

caused a reversible inhibition which was significantly less than that observed at pH 7.4. Similar results were obtained on the 100 μM levcromakalim-induced inward currents (pH 6.7, 0.52 ± 0.06, $n=6$; pH 7.4, 0.33 ± 0.03, $n=6$). Although the levcromakalim-induced outward current was suppressed by 100 μM flecainide, the inhibitory ratio for flecainide was much more potent at pH 8.1 than that at pH 7.4 (pH 7.4, 0.28 ± 0.05, $n=6$; pH 8.1, 0.08 ± 0.06, $n=6$, Figure 8d). Note that the amplitude of the levcromakalim induced K^+ currents at -50 mV were not significantly different between pH 6.7 and pH 8.1 in pig urethra and that application of 5 μM glibenclamide suppressed the levcromakalim-induced currents to the same level.

Effects of external pH on the flecainide-induced inhibition of Kir6.2ΔC36 currents

The effects of external pH was examined in outside-out patches from GFP-positive COS7 cells which expressed Kir6.2ΔC36 in symmetrical 140 mM K^+ conditions. Figure 9a shows that the channel activity was unaffected by changing the external pH from 6.7 to 8.1 at a holding potential of -50 mV. Similar observations were obtained in nine other patches. In Figure 9b, application of 100 μM flecainide (dissolved in an aqueous extracellular 140 mM K^+ solution) caused an inhibitory effect on the activity of Kir6.2ΔC36, demonstrating a external pH-dependent effect (pH 6.7, 0.83 ± 0.1, $n=5$; pH 7.4, 0.33 ± 0.1, $n=5$; pH 8.1, 0.13 ± 0.04, $n=5$; Figure 9c).

Discussion

In the present experiments, we have been able to demonstrate the inhibitory actions of flecainide, one of the potent Vaughan–Williams class Ic antiarrhythmic drugs, on the activity of K_{ATP} channels in dispersed smooth muscle cells. The drug was able to block both inward and outward currents through these channels, showing little rectification.

Blocking mechanisms of antiarrhythmic drugs on K_{ATP} channels in pig urethra

Recent studies have shown that various types of antiarrhythmic drugs block K_{ATP} channels in both ventricular myocytes (cibenzoline, Horie *et al*., 1992; disopyramide, Horie *et al*., 1992; De Lorenzi *et al*., 1995; flecainide, Wang *et al*., 1995) and pancreatic ß-cells (cibenzoline, Horie *et al*., 1992; Kakei *et al*., 1993, disopyramide, Horie *et al*., 1992), suggesting that the inhibitory effects of the antiarrhythmic drugs on the channel activity of K_{ATP} channels may account for the prolongation of action potential in conditions of hypoxia and metabolic deprivation. In the present experiments, the levcromakalim-induced glibenclamide-sensitive K^+ currents were inhibited by several types of the antiarrhythmic agents (flecainide, cibenzoline and disopyramide) in pig urethra. Similarly, in cell-attached configuration, the levcromakalim-induced K_{ATP} channels were also inhibited by these antiarrhythmic drugs. Given this, these results strongly

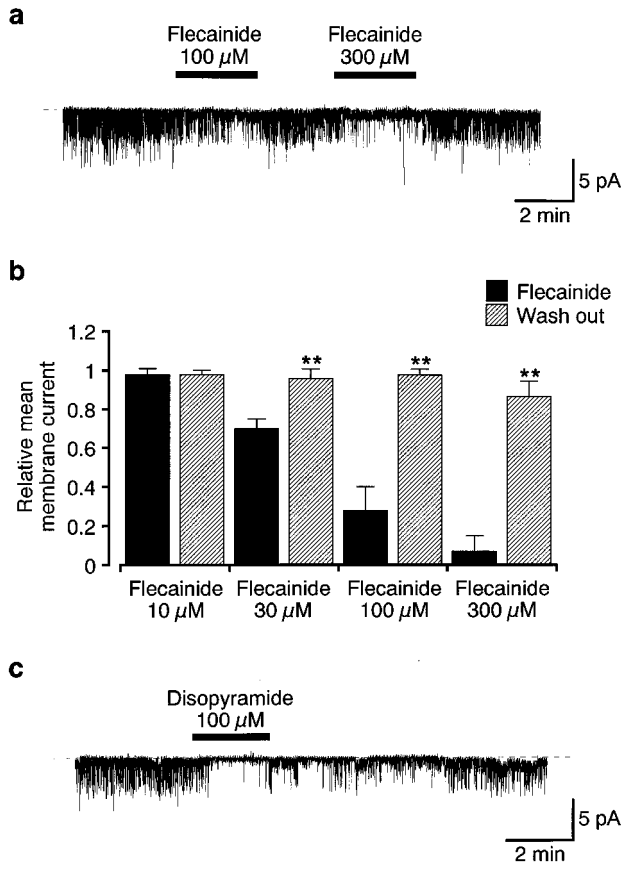

Figure 7 Effects of flecainide and disopyramide on channel activity of Kir6.2ΔC36. (a) Current trace of the inhibitory effects of flecainide (100 and 300 μM, 3 min duration) on channel activity of Kir6.2ΔC36 in inside-out configuration at a holding potential of −50 mV. The dashed line indicates the current when the channel is not open. (b) Effects of flecainide (presence and wash-out) on channel activity of Kir6.2ΔC36 at −50 mV. Each column shows the relative level of the channel activity of Kir6.2ΔC36 (mean value with + s.d.) when the mean amplitude of the channel activity of Kir6.2ΔC36 was normalized as one just before application of flecainide ($n=6$). The mean membrane current was measured from the current level for 30 s duration when the channel was not open. The wash-out value was significantly different from the relative amplitude in the presence of three concentrations of flecainide (*t*-test, $^{**}P<0.01$). (c) Effects of 100 μM disopyramide (2 min duration) on channel activity of Kir6.2ΔC36 at −50 mV. The dashed line indicates the current when the channel is not open.

suggest that antiarrhythmic drugs may inhibit K_{ATP} channel opener-induced membrane currents due to their ability to block K_{ATP} channels.

Inagaki *et al.* (1995) reported that the cloned K_{ATP} channels of pancreatic ß-cells comprise a complex of at least two different proteins: a sulphonylurea receptor 1 (SUR1) and an inwardly rectifying K^+ channel subunit (Kir6.2). Recently, significant currents were recorded from *Xenopus* oocytes injected with mRNA encoding a truncated from of Kir6.2 in which the last 26 or 36 amino acids of the C-terminus had been deleted (i.e. Kir6.2ΔC26 or Kir6.2ΔC36) in the absence of SURs (Tucker *et al.*, 1997). This made it possible to investigate the direct effects of certain drugs on the pore forming Kir6.2 subunits themselves (Proks & Ashcroft, 1997). It has been reported that [^{3}H]-cibenzoline binds to Kir6.2 and inhibits the activity of Kir6.2ΔC26, suggesting that cibenzoline may inhibit K_{ATP} channels through a mechanism in which cibenzoline directly affects the pore-forming Kir6.2 rather than SUR1 subunit (Mukai *et al.*, 1998). Recently, we have reported the expression of Kir6.2 subunit in pig urethral myocytes by use of RT–PCR analysis (Teramoto *et al.*, 2000b). Kir6.2 is also dominantly expressed in a wide variety of smooth muscle cells (murine colon, Koh *et al.*, 1998; A-10 cells, Miller *et al.*, 1999; guinea-pig urinary bladder, Gopalakrishnan *et al.*, 1999). We have demonstrated that flecainide suppressed the channel activity of Kir6.2ΔC36 expressing in COS7 cells using excised membrane patches. Flecainide inhibited the activity of Kir6.2ΔC36 at concentrations similar to those observed in K_{ATP} channels of pig urethra. These results suggest that flecainide may directly block the pore forming K^+ channel subunits of K_{ATP} channels although we cannot be certain of the precise binding sites of flecainide on K^+ channels.

Does flecainide block K_{ATP} channels in an inwardly rectifying manner?

In guinea-pig ventricular cells, flecainide (3–300 μM) blocks the channel opening of outward but not inward unitary K^+ currents through K_{ATP} channels, demonstrating an inwardly rectifying and a voltage-dependent block (Wang *et al.*, 1995). Since intracellular monovalent and divalent cations (such as Na^+, Mg^{2+}, Ca^{2+} etc.) block K_{ATP} channels in a voltage-dependent manner and predominantly decrease the unitary amplitude of the outwardly directed K_{ATP} channels, Wang *et al.* (1995) concluded that the inhibitory effects of flecainide on K_{ATP} channels are due to the same blocking mechanisms as those of monovalent and divalent cations, and depend on its potent positive charge (positive charge hypothesis). Thus, flecainide has been thought to be the only drug that demonstrates an inwardly rectifying inhibitory action on K_{ATP} channels.

In the present experiments, we have shown that flecainide possesses a weak voltage-dependent inhibitory effect on the levcromakalim-induced K^+ currents between −80 and −30 mV and that the inhibitory effects on the outward K^+ currents are relatively more potent than on the inward K^+ currents at −50 mV. However, flecainide suppressed the levcromakalim-induced inward and outward K^+ currents at −50 mV in a concentration-dependent manner, showing no inwardly rectifying inhibition. In this respect, the inhibitory effects of flecainide on K_{ATP} channels were totally different between pig urethral myocytes and guinea-pig cardiac cells. Furthermore, since flecainide has a pK_a value of 9.3, nearly 98.7% of flecainide is ionized at pH 7.4 under the present experimental conditions. When the proportion of the charged form of flecainide was slightly increased, by reducing extracellular pH from 7.4 to 6.7 (to 99.7% at pH 6.7), the inhibitory effects of flecainide on the levcromakalim-induced K^+ currents at pH 6.7 become less potent. Similarly, the inhibitory potency of flecainide was further enhanced when the pH of the extracellular solution was increased (95% of flecainide is charged at pH 8.1).

Furthermore, in the present experiments, similar external pH-dependent inhibitory effects of flecainide were observed in the excised patches expressing Kir6.2ΔC36 which may form

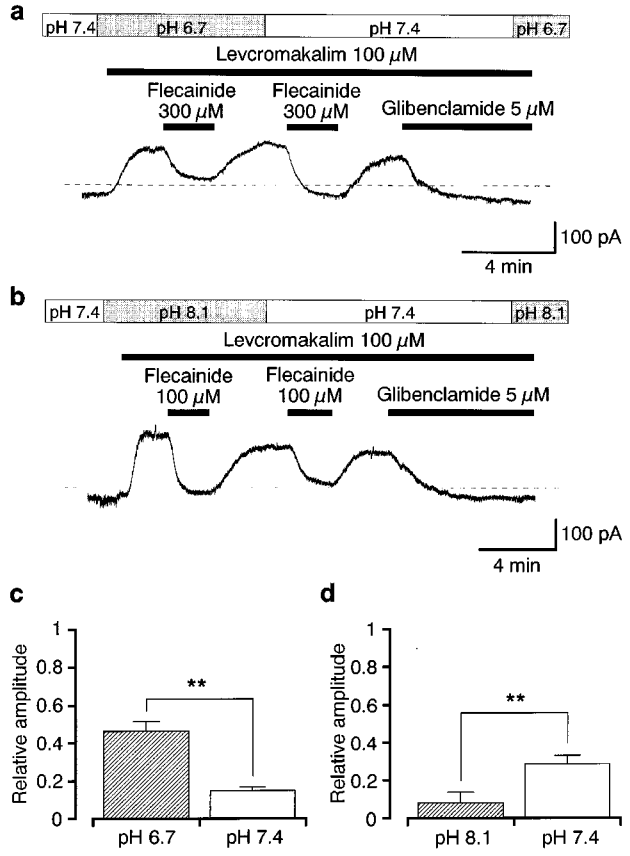

Figure 8 Effects of extracellular pH on the actions of flecainide on 100 μM levcromakalim-induced currents at -50 mV. Bath solution 5 mM K^+ PSS, pipette solution 140 mM K^+ containing 5 mM EGTA. (a) Inhibitory effects of 300 μM flecainide on 100 μM levcromakalim-induced outward currents at the indicated extracellular pH value (6.7 and 7.4). The dashed lines indicate the zero current level. (b) Effects of 100 μM flecainide on the 100 μM levcromakalim-induced outward currents at two different extracellular pH values (7.4 and 8.1). The dashed lines indicate the zero current level. (c, d) Each column shows the relative amplitude of the levcromakalim-induced K^+ current at -50 mV (mean value with + s.d.). The amplitude of the levcromakalim-induced current was taken as one just before application of each concentration of flecainide. Currents were measured from the 5 μM glibenclamide-sensitive level in each experimental condition. There was a significant difference between inhibition obtained at either pH 6.7 or pH 8.1 with that at pH 7.4 (ANOVA, $^{**}P<0.01$).

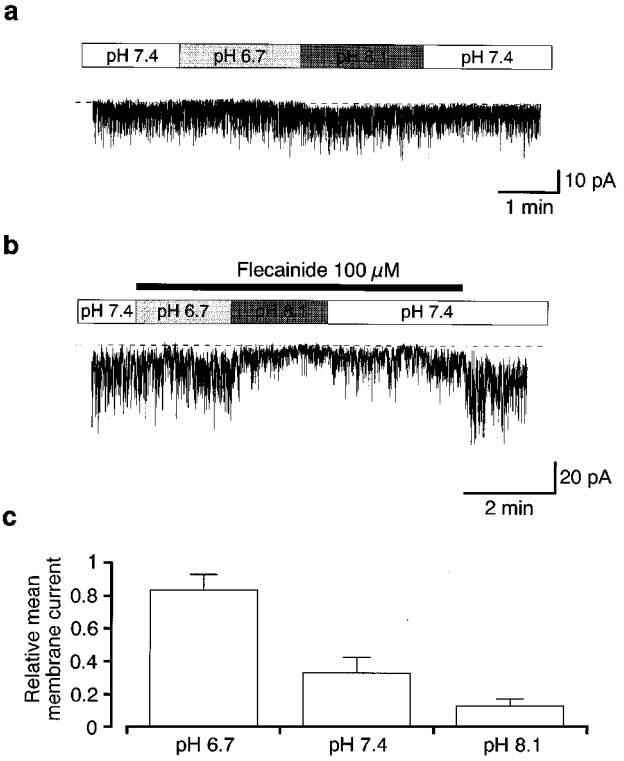

Figure 9 Effects of flecainide on activity of Kir6.2ΔC36 (symmetrical 140 mM K^+ conditions) at various external pH values. (a) Current trace of the effects of external pH (from pH 6.7 to pH 8.1, 2 min duration) on channel activity of Kir6.2ΔC36 in outside-out configuration at a holding potential of -50 mV. The dashed line indicates the current when the channel is not open. (b) Effects of 100 μM flecainide on the activity of Kir6.2ΔC36 at three different external pH values (6.7, 7.4 and 8.1). The dashed line indicates the current when the channel is not open. (c) Effects of flecainide (100 μM) on channel activity of Kir6.2ΔC36. Each column shows the relative level of the channel activity of Kir6.2ΔC36 (mean + s.d.) at each indicated pH value. The mean amplitude of the channel activity of Kir6.2ΔC36 was normalized as one just before application of flecainide at pH 7.4 ($n=6$). The mean membrane current was measured from the current level when the channel was not open.

the pore of K_{ATP} channels. Thus, it is somewhat difficult to explain the inhibitory effects of flecainide on the activity of K_{ATP} channels based on the 'positive charge hypothesis' in pig urethra. The peak amplitude of the levcromakalim-induced K^+ currents was unaffected by either raising the external pH from 6.7 to 8.1 or lowering from pH 8.1 to pH 6.7 in the present experiments, suggesting that changes in external pH may have little effect on the levcromakalim-induced K^+ currents in pig urethra or the activity of Kir6.2ΔC36. Similar results were observed in K_{ATP} channels of mouse pancreatic ß-cells (Proks *et al*., 1994). We have previously reported the inhibitory effects of Ca^{2+} channel blockers (nifedipine, diltiazem and verapamil) on K_{ATP} channels in pig urethra (Teramoto & Brading, 1998). These Ca^{2+} channel blockers inhibit the activity of K_{ATP} channels at 10–1000 times higher concentrations than needed to block voltage-dependent Ca^{2+} channels and Na^+ channels (Horie *et al*., 1992; Mukai *et al*., 1998; Teramoto & Brading, 1998). Given this, we suggest that flecainide may bind to the K_{ATP} channel pore which may possess a pH-sensitivity in pig urethra although further studies may cast light upon the exact nature of the binding sites and the stoichiometry of the interactions between the pore form of the K^+ channels and these channel blockers.

Since we have shown that K_{ATP} channels are present and active in the smooth muscle of the pig urethra (Teramoto *et al*., 1997), and thus may play a physiological role in the relaxation of the urethra during micturition, the potential of the antiarrhythmic drugs to block this channel may account for their ability to precipitate urinary retention.

In conclusion, we have been able to demonstrate that application of different types of antiarrhythmic agents inhibit the levcromakalim-induced glibenclamide-sensitive K^+ currents in smooth muscle myocytes from pig urethra through a blockade of K_{ATP} channel pore.

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