British Journal of Pharmacology (2001) 133, 154–164

www.nature.com/bjp

Dual action of ZD6169, a novel K^+ channel opener, on ATP-sensitive K^+ channels in pig urethral myocytes

*,[1]Noriyoshi Teramoto, [1]Takakazu Yunoki, [2]Makoto Takano, [3]Yoshikazu Yonemitsu, [3]Ichiro Masaki, [3]Katsuo Sueishi, [4]Alison F. Brading & [1]Yushi Ito

[1]Department of Pharmacology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, 812-8582, Japan; [2]Department of Physiology and Biophysics, Graduate School of Medicine, Kyoto University, Kyoto, 606-8501, Japan; [3]Department of Pathology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, 812-8582, Japan and [4]University Department of Pharmacology, Mansfield Road, Oxford OX1 3QT

1 The effects of ZD6169, a novel K^+ channel opener, on both membrane and unitary currents in pig urethra were investigated using patch-clamp techniques. Its effect was also examined on currents in inside-out patches of COS7 cells expressing carboxy terminus truncated inwardly rectifying K^+ channel (Kir6.2) subunits (Kir6.2ΔC36) which form ATP-sensitive K^+ channels (K_{ATP} channels).

2 In current-clamp mode, ZD6169 ($\leqslant 10\ \mu$M) induced a concentration-dependent membrane hyperpolarization. Higher concentrations ($\geqslant 30\ \mu$M) caused a transient membrane hyperpolarization, followed by a gradual membrane depolarization. On removal of ZD6169, an after hyperpolarization was observed.

3 In conventional voltage-clamp configuration, at -50 mV in symmetrical 140 mM K^+ conditions, ZD6169 (100 μM) caused a transient inward current which gradually decayed. Removal of ZD6169 evoked a much larger amplitude K^+ current with a similar time course.

4 ZD6169 produced an inward glibenclamide-sensitive K^+ current, demonstrating a bell-shaped concentration-response relationship.

5 In cell-attached configuration in symmetrical 140 mM K^+ conditions, ZD6169 ($\leqslant 30\ \mu$M) activated an K_{ATP} channel which was reversibly suppressed by application of glibenclamide. In contrast, ZD6169 (100 μM) inhibited the activity of the levcromakalim-induced K_{ATP} channels.

6 ZD6169 (100 μM) had no significant effect on the channel activity of Kir6.2ΔC36 in inside-out configuration, although cibenzoline greatly suppressed the channel activity.

7 These results demonstrate that ZD6169 possesses a dual effect on the activity of the K_{ATP} channel; activating at low concentration and inhibiting at higher concentration.

British Journal of Pharmacology (2001) **133,** 154–164

Keywords: Carboxy terminus truncated Kir6.2; dual action; K^+ channel opener; unstable urinary bladder; urinary bladder-selectivity; ZD6169

Abbreviations: E_K, theoretical equilibrium potential of K^+; GFP, green fluorescence protein; *I-V* relationships, current-voltage relationships; K_{ATP} channels, ATP-sensitive K^+ channels; Kir, inwardly-rectifying K^+ channel; Kir6.2ΔC36, the last 36 amino acids of the carboxy terminus truncated Kir6.2; NDP, nucleoside diphosphate; NP_o, channel open state probability; PSS, physiological salt solution; RT–PCR, reverse transcriptase-polymerase chain reaction; SUR, sulphonylurea receptor

Introduction

ZD6169, the *S*-enantiomer of the racemic compound *N*-(4-benzoylphenyl)-3,3,3-trifluoro-2-hydroxy-2-methylpropionamide, induced a potent reduction of mechanical activity in isolated guinea-pig detrusor (Li *et al*., 1995). It has been reported that ZD6169 shows *in vivo* selectivity for relaxing detrusor smooth muscle over cardiovascular parameters (such as heart rate, blood pressure etc.) in comparison with (±)-cromakalim (Howe *et al*., 1995) and that ZD6169 induces ATP-sensitive K^+ currents (i.e. K_{ATP} currents) in guinea-pig urinary bladder smooth muscle cells, leading to membrane hyperpolarization and inhibition of the contractile force of the urinary bladder (Li *et al*., 1995; Trivedi *et al*., 1995). Although the detrusor-selectivity of ZD6169 makes it likely to be established as a unique K_{ATP} channel opener, the target K^+ channels for ZD6169 in detrusor smooth muscle cells at normal resting potential levels have not yet been identified by use of single-channel recordings. Moreover, since reduction in urethral smooth muscle tone and thus urethral pressure would be undesirable in a drug used to treat urinary bladder instability, we have suggested that potential detrusor-selective K_{ATP} channel openers should be screened against urethral as well as vascular smooth muscle (Teramoto *et al*., 1997a). Therefore, we believe that direct investigations of the effects of detrusor-selective K_{ATP} channel openers on urethral myocytes are essential to further consider ZD6169 as a possible K_{ATP} channel opener for the treatment of unstable bladders.

We have investigated the effects of several types of K_{ATP} channel openers on the ATP-sensitive K^+ currents and

*Author for correspondence;
E-mail: noritera@linne.med.kyushu-u.ac.jp

channels in pig proximal urethral myocytes and demonstrated the pharmacological and electrophysiological properties of the ATP-sensitive K^+ channel (i.e. K_{ATP} channel, Teramoto *et al*., 1997b). The present study was designed to identify the target K^+ channels for ZD6169 by use of single-channel recordings and to investigate a dual action exerted by ZD6169 on the pig urethra. We discuss further the inhibitory mechanisms of ZD6169 (100 μM) 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 urethra from female pigs was collected from a local abattoir. Pig urethral myocytes were freshly 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, Palo Alto, CA, U.S.A.) was used as a co-transfection member and the whole nucleotide sequence of the PCR clone as expected was confirmed by a DNA sequencer.

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, Grand Island, NY, U.S.A.) according to the manufacturer's instruction. Electrophysiological measurements were usually conducted 2–4 days after transfection.

Recording procedure

Patch-clamp experiments were performed at room temperature (21–23°C) as described previously (Teramoto *et al*., 2000). 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. Transfected cells were identified by green fluorescence under a microscope.

Drugs and solutions

For whole-cell recording, 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, glucose 5, EGTA 5, and HEPES 10/Tris (pH 7.35–7.40). For cell-attached 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. For recording the maxi K^+ channels in inside-out configuration, the bath solution was initially (mM): K^+ 140, Cl^- 140, EGTA 5, glucose 5, HEPES 10/Tris (pH 7.35–7.40), with varying free intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$). The concentrations of free $[Ca^{2+}]$ were calculated using the commercial software 'EQCAL' (Biosoft, Cambridge, U.K.). ATP (3 mM) was occasionally included in the pipette solution. 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}. In some experiments, for rapid drug application, the flow pipe system (concentration-jump technique) was used as described by Yellen (1982), recording the drug application time as a trigger pulse on VHS tape with current and voltage at the same time. All drugs were obtained from Sigma Chemical (Sigma Chemical K.K., Tokyo, Japan). Levcromakalim (SmithKline Beecham Pharmaceuticals, Harlow, U.K.), ZD6169 (AstraZeneca Pharmaceuticals, Cheshire, U.K.), cibenzoline (Fujisawa Pharmaceuticals, Osaka, Japan) and glibenclamide were prepared daily 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.

Data analysis and statistics

The whole-cell recording data were low-pass filtered at 500 Hz by an 8 pole Bessel filter (E-3201B, NF Electronic Instruments, Yokohama, Japan), sampled at 25 ms intervals and analysed on a computer (Macintosh PowerPC G3, Apple Computer Japan Limited, Tokyo, Japan) by the commercial software 'MacLab 3.5.2' (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 'PAT' program (kindly provided by Dr J. Dempster, the 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_{open}) were measured for 2 min or 30 s (Figure 9a),

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

where tj 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. Data points were fitted using a least-squares fitting. Statistical analyses were performed with Student's *t*-test for paired values. Changes were considered significant at $P<0.05$. Data are expressed as mean with the standard deviation (s.d.).

Results

ZD6169-induced hyperpolarization in pig urethral myocytes

In Figure 1a, the effects of increasing concentrations of ZD6169 (10–100 μM) on the membrane potential are shown in one cell in current-clamp mode (bath solution, 5 mM K^+ physiological salt solution (PSS); pipette solution, 140 mM KCl containing 5 mM EGTA). Application of 10 μM ZD6169 rapidly caused a stable hyperpolarization (from -37 ± 4 to -74 ± 4 mV, $n=6$). After wash-out of the drug, the membrane potential gradually recovered to its previous level. However, higher concentrations of ZD6169 ($\geqslant30$ μM) induced a biphasic change in the membrane potential, namely, a transient hyperpolarization (30 μM, -79 ± 3 mV, $n=6$; 100 μM, -80 ± 2 mV, $n=6$), followed by a significant depolarization during the continuous presence of ZD6169. On removal of the drug, a rapid after hyperpolarization occurred (30 μM, -76 ± 3 mV, $n=6$; 100 μM, -78 ± 2 mV, $n=6$) which then gradually recovered to the control level. Figure 1b summarizes the relationships between the values of the ZD6169-induced membrane potentials (the peak amplitude of the hyperpolarization and mean value of the membrane potential just before wash-out) and the concentration of ZD6169. Using other cells, after 10 min continuous application of ZD6169, mean value of the ZD6169-induced membrane potential was also measured at each concentration of ZD6169 (Figure 1b, $n=6$). Figure 1c shows that the 10 μM ZD6169-induced hyperpolarization was markedly inhibited by additional application of 1 μM glibenclamide. Similar observations were obtained in four other cells. In Figure 1d, bath application of ZD6169 (100 μM) caused a transient hyperpolarization. When ZD6169 was removed, the after hyperpolarization was also suppressed by pretreatment of 1 μM glibenclamide ($n=7$). These results indicate that the hyperpolarization evoked by application and removal of ZD6169 are due to an activation of glibenclamide-sensitive

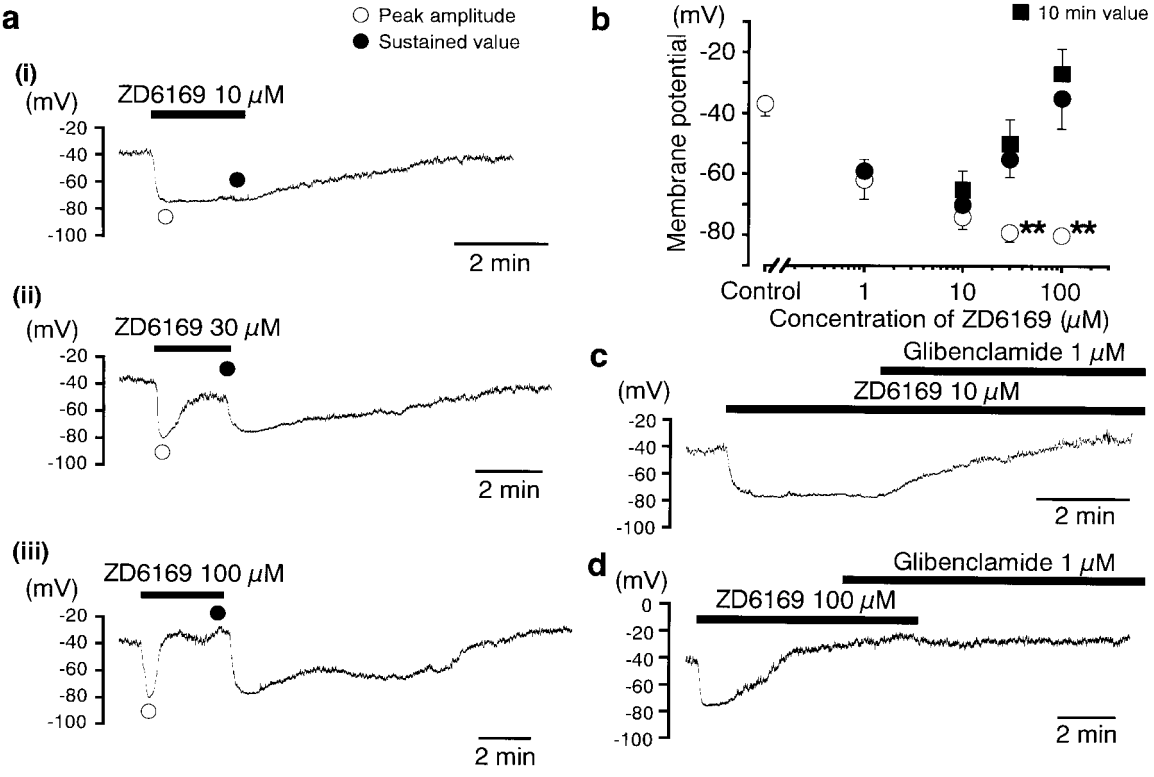

Figure 1 Dual action of ZD6169 on the membrane potential of dispersed urethral smooth muscle cells (whole-cell recordings, current-clamp mode). The bath contained 5 mM K^+ PSS and the pipette solution was 140 mM KCl containing 5 mM EGTA. (a) Effects on the membrane potential of ZD6169 (10, 30 and 100 μM) applied using the concentration-jump method. Data were obtained from the same cell. (b) Concentration-response relationships for the effects of ZD6169 on membrane potential. Control indicates the condition just before the application of ZD6169. The amplitude of the membrane potential was measured as the peak amplitude of the ZD6169-induced hyperpolarization (open circles) and the mean value (20 s duration) of the membrane potential just before wash-out of each concentration of ZD6169 (filled circles) as shown in the traces for (a). Filled squares show the mean value (20 s duration) of the ZD6169-induced membrane potential measured after 10 mins application. At higher concentrations of ZD6169 ($\geqslant30$ μM), there was a significant difference between the peak amplitude of the ZD6169-induced hyperpolarization and mean value of the ZD6169-induced membrane potentials (**t-test, $P<0.01$). Each symbol shows the mean of six observations with s.d. (c) The hyperpolarization induced by 10 μM ZD6169 was blocked by 1 μM glibenclamide. (d) The 100 μM ZD6169-induced rapid after hyperpolarization was suppressed by pretreatment of 1 μM glibenclamide when ZD6169 was removed.

mechanisms and that ZD6169 possesses a dual action (an activation and inhibition) on the membrane potential, depending upon its concentration.

ZD6169-induced glibenclamide-sensitive current in pig urethra

To investigate further the mechanisms involved in the ZD6169-induced hyperpolarization, whole-cell voltage-clamp experiments were performed at a holding potential of −50 mV (bath solution, 140 mM K^+ PSS; pipette solution, 140 mM KCl solution containing 5 mM EGTA; i.e., symmetrical 140 mM K^+ conditions). As shown in Figure 2a, application of ZD6169 (20 μM) caused an inward current which was suppressed by application of 5 μM glibenclamide. Before and during application of 20 μM ZD6169, current-voltage (*I-V*) relationships were obtained by applying four ramp pulses (from −120 to +80 mV for 1 s duration, see inset in Figure 2a) every 15 s at −50 mV (Figure 2b). The net membrane current activated by 20 μM ZD6169 was obtained by subtracting the averaged control current from the mean ZD6169-induced current, demonstrating an inwardly rectifying property (Figure 2c). The reversal potential of the ZD6169-induced membrane current in this cell in symmetrical 140 mM K^+ conditions was approximately 0 mV (0±1 mV, $n=6$). This value was very close to E_K in the present experimental conditions ($E_K=0$ mV). When 3 mM ATP was added to the pipette solution, the peak amplitude of the ZD6169 (20 μM)-induced current was much smaller (17±8 pA, $n=4$ with ATP *versus* 205±29 pA, $n=4$ without ATP). This small inward current was also suppressed by additional application of 5 μM glibenclamide (data not shown). These results suggest that the ZD6169-induced membrane currents are carried mainly by K^+ through channels that are inhibited by glibenclamide and intracellular ATP.

Time course of 100 μM ZD6169-induced membrane currents

Figure 3a shows a typical time course of 100 μM ZD6169-induced membrane current in symmetrical 140 mM K^+ conditions at −50 mV. When 100 μM ZD6169 was applied by a concentration jump technique, ZD6169 induced a transient inward current (205±50 pA, $n=4$) which gradually recovered to the original level. On removal, the basal current was transiently increased, followed by a gradual recovery to the control level after approximately 3 min. Similar observations were obtained in eight other cells (the peak amplitude, 460±170 pA, $n=8$). In the same experi-

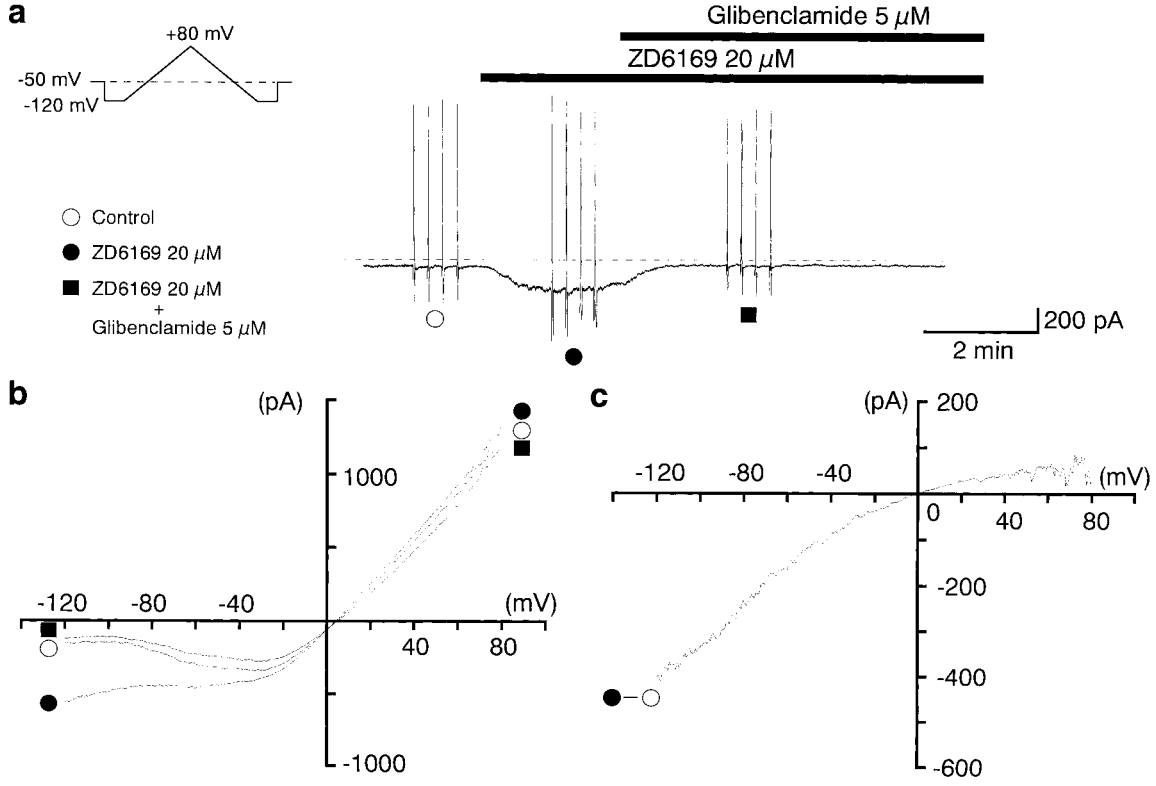

Figure 2 Effects of glibenclamide on ZD6169-induced membrane currents in pig urethral myocytes (whole-cell recordings, voltage-clamp mode). (a) Inhibitory effect of glibenclamide on the ZD6169-induced membrane current at a holding potential of −50 mV (symmetrical 140 mM K^+ conditions). The vertical 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 in (a), every 15 s). The dashed line indicates the zero current level. (b) *I-V* curves measured from the negative-going limb (the falling phase) of the ramp pulse. The mean ramp membrane currents on an expanded time scale under three conditions. Each symbol is the same as in (a). (c) Net membrane current evoked by ZD6169 (20 μM). Net membrane current was obtained by subtraction of the two ramp membrane currents (shown in (b)) recorded before and during application of 20 μM ZD6169. The reversal potential of ZD6169-induced current was approximately 0 mV.

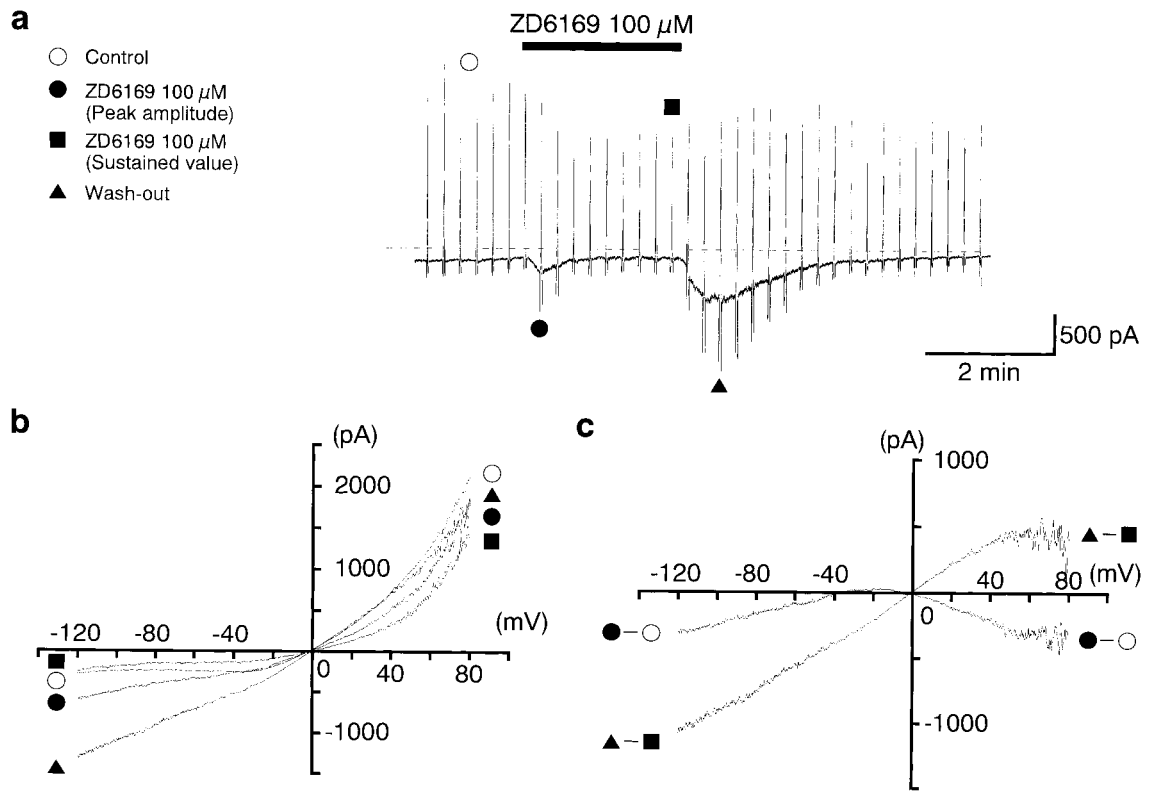

Figure 3 The effects of 100 μM ZD6169 on the membrane current in pig urethra (whole-cell recording, symmetrical 140 mM K^+ conditions, holding potential, -50 mV). (a) The vertical deflections indicate triangular ramp potential pulses (see inset in Figure 2a). The dashed line indicates zero current line. (b) *I-V* curves measured from the negative-going limb (the falling phase) of the ramp pulse in several conditions. Each symbol is the same as in (a). (c) Net current component in each condition, obtained by subtraction of the two ramp membrane currents (shown in (b)) recorded before and during application of 100 μM ZD6169. On removal of 100 μM ZD6169, the amplitude of the basal membrane current was transiently enhanced (the peak amplitude, 637 pA). The reversal potential of wash-out component was -1 mV, very close to the calculated E_K value, and the current demonstrated an inwardly rectifying property.

ment, ramp pulses (see inset in Figure 2a) were applied at 15 s intervals in order to obtain *I-V* relationships in the absence and presence of 100 μM ZD6169. Figure 3b shows the ramp currents under various experimental conditions. The 100 μM ZD6169-induced membrane current was obtained by subtracting the averaged control current from the peak membrane current in the presence of ZD6169 (Figure 3c). The net membrane current which was activated after washing-out of ZD6169 was also obtained by subtracting the membrane current after removal of ZD6169 from the membrane current in the presence of ZD6169, demonstrating an inwardly rectifying property at positive potentials.

When the concentration of ZD6169 was increased in a cumulative manner, the peak amplitude of the ZD6169-induced basal membrane current at -50 mV first increased in a concentration-dependent manner up to 10 μM (Figure 4a). However, further application of ZD6169 (100 μM) caused an inhibitory effect on the membrane current. On removal of ZD6169, a transient inward current was elicited, gradually declining to the control level. Figure 4b indicates the relationships between the peak amplitude of the inward current and the concentration of ZD6169, resulting in a bell-shaped curve when ZD6169 was applied in a cumulative manner. In contrast, single application of ZD6169 ($\geqslant$30 μM) elicited a transient inward current, followed by a gradual decay of the membrane current.

Inhibitory effects of 100 μM ZD6169 on the levcromakalim-induced glibenclamide-sensitive membrane currents

The effect of ZD6169 on the levcromakalim-induced membrane currents was investigated by applying additional 100 μM ZD6169 after the membrane current had been maximally activated by 100 μM levcromakalim at -50mV. As shown in Figure 5a, ZD6169 (100 μM) immediately inhibited the levcromakalim-induced inward current to a relative value of 0.02 ± 0.01, ($n=5$, the amplitude of the levcromakalim-induced inward K^+ current just before application of ZD6169 was taken as one, measuring from the current level in the presence of 5 μM glibenclamide). On removal of ZD6169, the amplitude of the levcromakalim-induced current at -50 mV showed a gradual recovery towards a steady state value that was much less than that of the original level, but probably similar to the level that would have occurred without application of ZD6169, due to run down of the current under these experimental conditions (Teramoto *et al.*, 1997b). Subsequent application of 5 μM glibenclamide suppressed the inward current to a value close

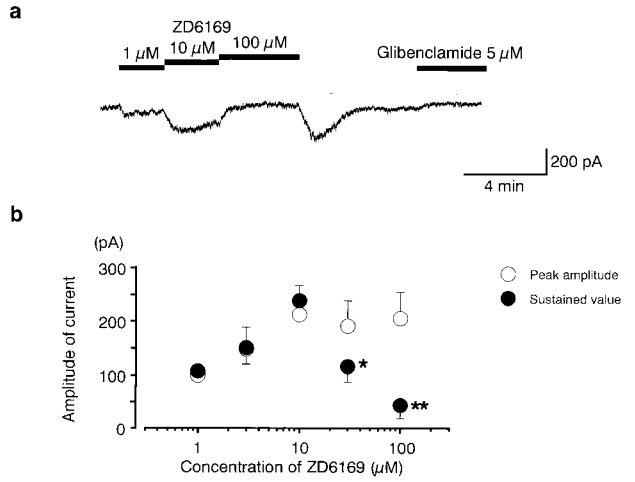

Figure 4 Concentration-response relationships of ZD6169-induced inward current at −50 mV in pig urethra (symmetrical 140 mM K^+ conditions). (a) A cumulative application of ZD6169 caused a dual effect on the membrane current. The dashed line indicates the zero current level. (b) Relationship between peak amplitude of inward currents and concentration of ZD6169. Note bell-shaped curve when ZD6169 was applied in a cumulative manner (filled circle). Open circle indicates the peak amplitude when each concentration of ZD6169 was applied alone. Filled circle indicates the peak value when each concentration of ZD6169 was applied separately. The amplitudes of the ZD6169-induced currents were measured from the current level in 5 μM glibenclamide in each condition. Each symbol indicates mean and s.d. of 6–8 observations. *Significantly different from the peak value (*t*-test, $P<0.05$). **Significantly different from the peak value (*t*-test, $P<0.01$).

to that seen in the presence of ZD6169. These observations show that ZD6169 possesses an antagonistic action on the levcromakalim-induced glibenclamide-sensitive membrane currents.

In the same experiment, eight triangular ramp potential pulses (see inset in Figure 2a) were applied in order to obtain *I-V* relationships under each condition. Figure 5b shows the averaged membrane currents during the falling phase of the ramp pulses under the various experimental conditions of the experiment. The 100 μM ZD6169-sensitive membrane current in the presence of levcromakalim was obtained by subtracting the mean membrane current in the presence of 100 μM ZD6169 from the membrane current in its absence, demonstrating an inwardly rectifying property at positive potentials (Figure 5c, the reversal potential was -1 ± 2 mV, $n=6$).

ZD6169 activates and inhibits a glibenclamide-sensitive 43 pS K^+ channel in pig urethra

Single-channel recordings were performed in symmetrical 140 mM K^+ conditions by use of cell-attached configuration. To minimize activity of maxi K^+ channels, experiments were performed at −50 mV. In Figure 6a, application of 1 μM ZD6169 caused openings of a 2.1 pA K^+ channel (calculated from an all-amplitude histogram). When the concentration of ZD6169 was increased in a cumulative manner, the open probability of the K^+ channel was enhanced in a concentration-dependent manner ($\leqslant$30 μM). In contrast, application of higher concentrations of ZD6169 ($\geqslant$50 μM) reduced the channel openings. Although brief channel openings occasionally appeared in the presence of 100 μM ZD6169, the open probability (NP_o) was negligible in comparison to that of 100 μM levcromakalim. On removal of the drug, channel activity transiently reappeared and then gradually disappeared. Subsequent application of levcromakalim (100 μM) caused opening of the same amplitude K^+ channel, increasing NP_o in the same membrane patch (0.52 ± 0.2, $n=4$). Figure 6b indicates the relationships between the channel activity and the concentration of ZD6169. Again, a bell-shaped curve is observed. In the presence of 30 μM ZD6169, *I-V* relationships in symmetrical 140 mM K^+ conditions, were obtained by changing the holding membrane potential from −120 mV to +40 mV, indicating that the conductance of the channel is approximately 43 pS (43.2 ± 0.8 pS, $n=15$) with an inwardly rectifying property at positive membrane potentials (data not shown).

In Figure 7a, application of glibenclamide (0.5 and 5 μM) reversibly inhibited the activity of the 10 μM ZD6169-induced K^+ channel in a concentration-dependent manner. In the presence of 100 μM levcromakalim, application of 10 μM ZD6169 caused a reversible inhibition of the channel activity to an NP_o of 0.7 ± 0.15 ($n=5$) (NP_o value normalized as 1 just before application of ZD6169, Figure 7c). On removal of 10 μM ZD6169, the channel activity recovered to the control level. Application of 100 μM ZD6169 completely but reversibly abolished the levcromakalim-induced channel activity (NP_o value; 0.05 ± 0.05, $n=5$, Figure 7d) although occasional channel opening events were still observed. Thus ZD6169 possesses a dual (an agonistic and antagonistic) action on the glibenclamide-sensitive 43 pS K^+ channels.

Effects of ZD6169, cibenzoline and glibenclamide on Kir6.2ΔC36 channel activity

COS7 cells expressing a carboxy terminus truncated Kir6.2 (Kir6.2ΔC36) which can form K^+ channels even in the absence of SURs were utilized to investigate further the mechanisms involved in the inhibitory effects of 100 μM ZD6169 on K^+ currents. Functional channel activity of Kir6.2ΔC36 was constantly observed in cell-attached patches on GFP-positive COS7 cells at −50 mV (Figure 8a). When the membrane patch was excised in inside-out configuration, channel activity was dramatically enhanced. After the channel activity had stabilized, application of 100 μM ZD6169 had no significant effect (approximately 4 min duration). In contrast, cibenzoline (100 μM), a potent pore blocker of Kir6.2, reversibly suppressed the channel activity, but glibenclamide (10 μM), like ZD6169 also had no significant effect. Figure 8b summarizes the effects of ZD6169, cibenzoline and glibenclamide on the Kir6.2ΔC36 channel activity, showing the relative mean current amplitude (30 s duration) at −50 mV before and after application of each drug ($n=4$).

Effects of ZD6169 on the activity of maxi K^+ channels in pig urethra

Figure 9a shows the effects of ZD6169 (10 and 100 μM) on the activity of 6.7 pA K^+ channels (maxi K^+ channels) at a holding membrane potential of +30 mV in inside-out configuration (pipette solution, 140 mM K^+; bath solution, 140 mM K^+, $[Ca^{2+}]_i$, 10 nM). Under these experimental conditions, the channel conductance of the maxi K^+ channels

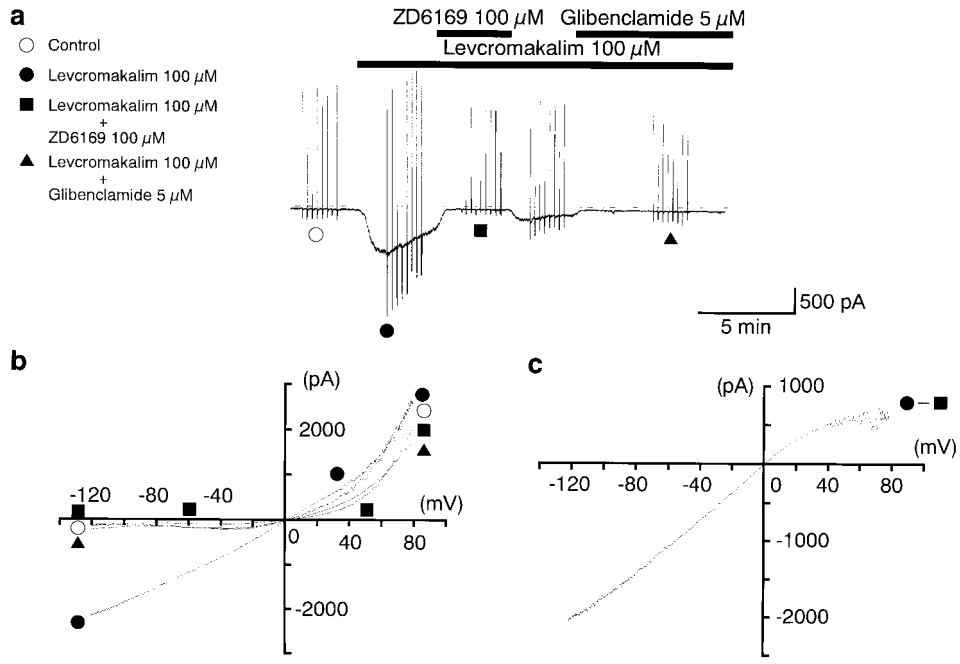

Figure 5 Inhibitory effects of ZD6169 on the levcromakalim-induced glibenclamide-sensitive inward membrane current (whole-cell recording, symmetrical 140 mM K^+ conditions, holding potential -50 mV). (a) Levcromakalim (100 μM) caused an inward membrane current (peak amplitude about 950 pA) which gradually decayed. The current was suppressed by additional application of 100 μM ZD6169, recovered to a steady state amplitude after ZD6169 was removed, and was then suppressed to the same level by 5 μM glibenclamide. The vertical deflections indicate triangular ramp potential pulses (see inset in Figure 2a). The dashed line indicates zero current line. (b) The *I-V* relationships of the levcromakalim-induced current was obtained from that of the first pulse due to the gradual rundown of the current. In the rest of the conditions, the lines are the mean membrane currents from the eight ramps in each condition. (c) Net membrane currents. The 100 μM ZD6169-sensitive membrane current was obtained by subtraction of the membrane currents in the absence and presence of 100 μM ZD6169 when levcromakalim was present in the bath solution.

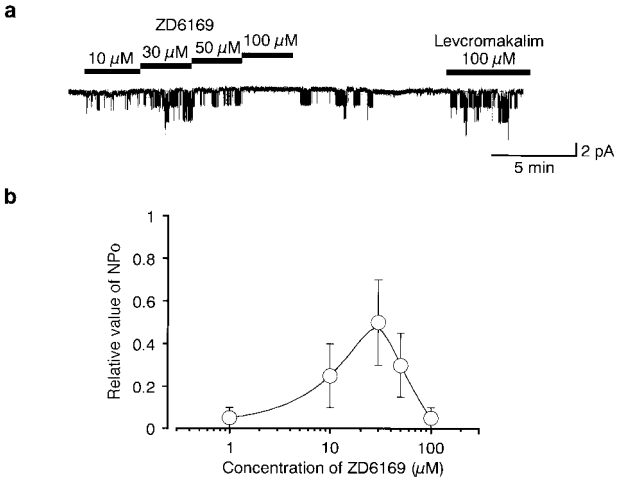

Figure 6 Recording in a cell-attached configuration (symmetrical 140 mM K^+ conditions) at a holding membrane potential of -50 mV. (a) Examples of the currents. ZD6169 at different concentrations was cumulatively applied to the same membrane patch using the concentration-jump technique. The dashed line indicates the current when the channel is not open. (b) Concentration-response curve for relative mean NP_o value$\pm$s.d. ($n=4$), demonstrating a bell-shaped curve. The relative mean NP_o value was obtained when the NP_o value of the 100 μM levcromakalim-induced K^+ channels was normalized as one. The line was drawn by eye.

in pig urethra was approximately 225 pS (225$\pm$15 pS, $n=40$, Teramoto & Brading, 1994). Application of 10 μM ZD6169 showed little effect on the unitary amplitude and the activity of maxi K^+ channels in pig urethra (Figure 9a,b ; 2.5 min duration). However, when the NP_o value just before application of ZD6169 (control) was normalized as 1, it could be seen that 100 μM ZD6169 inhibited the channel activity (relative NP_o; 0.69$\pm$0.07, $n=6$). Figure 9c summarizes the effects of ZD6169 on maxi K^+ channel activity, showing the relative NP_o value at $+30$ mV before and during application of ZD6169 ($n=6$). Similarly, ZD6169 (100 μM) caused an inhibitory effect on the channel activity when $[Ca^{2+}]_i$ was increased to 400 nM (data not shown).

Discussion

Target K^+ channels for ZD6169 in lower urinary tract cells

In lower urinary tract smooth muscle cells, two distinct types of K_{ATP} channels have been identified as target K^+ channels for levcromakalim by use of single-channel recordings (guinea-pig urinary bladder, K_{ATP} channel, Bonev & Nelson, 1993; pig urethra, K_{ATP} channel, Teramoto & Brading, 1996). Interestingly, it has been demonstrated clearly that these two K_{ATP} channels possess different channel conductance, nucleoside diphosphate (NDP)-sensitivity and ATP-sensitivity despite of their similar glibenclamide-sensitivity. Thus, we believed that the significant differences in property between these two K_{ATP} channels hold out some hope for the

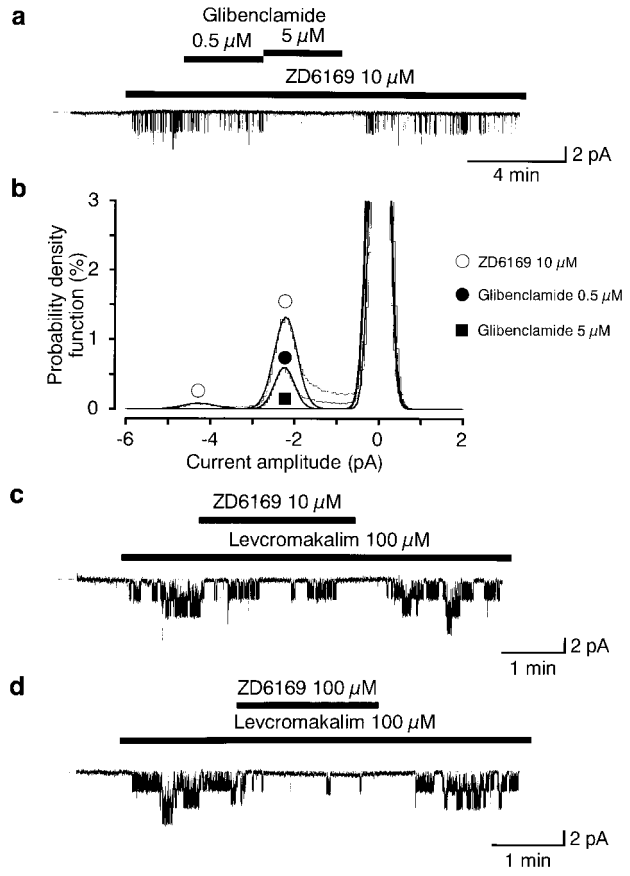

Figure 7 Glibenclamide-sensitivity of ZD6169-induced K^+ channel at a holding potential of −50 mV in cell-attached configuration. (a) Inhibitory effects of glibenclamide (0.5 and 5 μM) on the channel activity of the 10 μM ZD6169-induced K^+ channel. Additional application of glibenclamide (3 min duration) reversibly reduced the activity of the 2.1 pA K^+ channel in a concentration-dependent manner. (b) The all-point amplitude histogram for glibenclamide was obtained during the last 2.5 min of a 3 min application (30 s duration). The all-point amplitude histograms are superimposed in the absence (control; just before the application of each concentration of glibenclamide) and presence of glibenclamide (0.5 and 5 μM). Continuous lines in the histograms are theoretical curves fitted with the Gaussian distribution, by the least-squares method. 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. (c, d) Application of ZD6169 (10 or 100 μM) reversibly inhibited the channel openings of the 100 μM levcromakalim-induced K^+ channels.

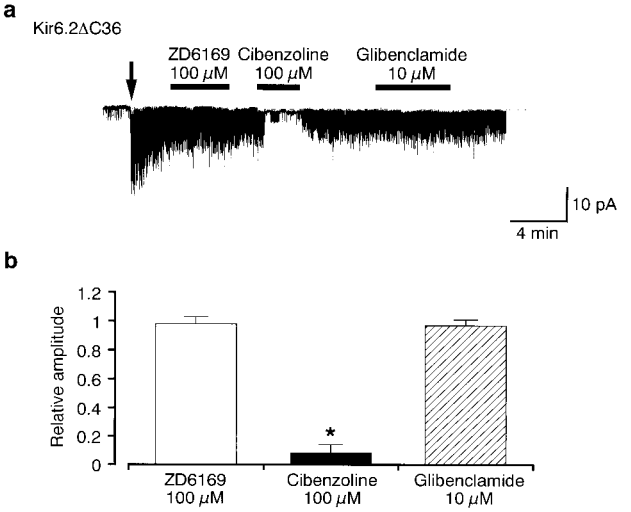

Figure 8 Effects of ZD6169 (100 μM) on channel activity of Kir6.2ΔC36. (a) Current trace of the effects of 100 μM ZD6169 on channel activity of Kir6.2ΔC36 in inside-out configuration at a holding potential of −50 mV. The arrow indicates the time when the inside-out patch was excised from the cell. The dashed line indicates the current when the channel is not open. (b) Effects of 100 μM ZD6169, 100 μM cibenzoline and 10 μM glibenclamide on channel activity of Kir6.2ΔC36 at −50 mV. To obtain the mean patch current, the integral of open channel currents during the 30 s before application of each drug was divided by time for integration, measuring from the current level when the channel was not open. Each column shows the relative mean patch current (mean value + s.d.) when the mean patch current of Kir6.2ΔC36 was normalized as 1.0 ($n=4$).

development of tissue-selective K_{ATP} channel openers for urge urinary incontinence (Teramoto *et al*., 1997a).

In vivo pharmacological studies have established that ZD6169 demonstrates a urinary bladder-selectivity after oral administration in the anaesthetized dog (Howe *et al*., 1995). However, since bell-shaped concentration response relationships for ZD6169-induced K_{ATP} currents have been demonstrated in a wide variety of tissues with a similar potency (guinea-pig urinary bladder, Hu & Kim, 1997; cat heart, Jow & Numann, 1999; pig urethra, the present study), it is unlikely that ZD6169 is selective for K_{ATP} channel in a detrusor smooth muscle.

There is also some question as to the main channel in the bladder that is targeted by ZD6169. In the guinea-pig, the ZD6169-induced relaxation was not significantly affected by charybdotoxin (Li *et al*., 1995), while Hu & Kim (1997) showed that ZD6169 activates maxi-K^+ channels.

On the other hand, in human bladder, ZD6169 (1–100 μM) clearly increased ^{42}K efflux in a concentration-dependent manner (Trivedi *et al*., 1995). In the present experiments, we have been able to demonstrate with single-channel recordings that ZD6169 selectively activates glibenclamide-sensitive K_{ATP} channels in pig urethra. Furthermore, we have clearly demonstrated that ZD6169 (100 μM) had an inhibitory effect on the activity of the maxi K^+ channels. These results strongly suggest that the K^+ channel that is opened by ZD6169 in pig urethra is the K_{ATP} channel which can be activated by application of other types of K_{ATP} channel openers, such as nicorandil, pinacidil and levcromakalim (Teramoto & Brading, 1997).

Bell-shaped action of ZD6169 on the activity of K_{ATP} channels in pig urethra

Bell-shaped concentration-response relationships for ZD6169 have been reported in guinea-pig urinary bladder (Hu & Kim, 1997) and cat ventricular myocytes (Jow & Numann, 1999) by use of conventional whole-cell recordings, reaching maximum effects at 5 and 10 μM, respectively. In the present experiments, we have found a bell-shaped relationship between ZD6169 concentration and ZD6169-induced membrane hyperpolarization (Figure 1b) and current (Figure 4b) in whole-cell recordings of pig urethral myocytes, reaching maximum at a concentration of 10–20 μM. Furthermore, we have also noticed using the

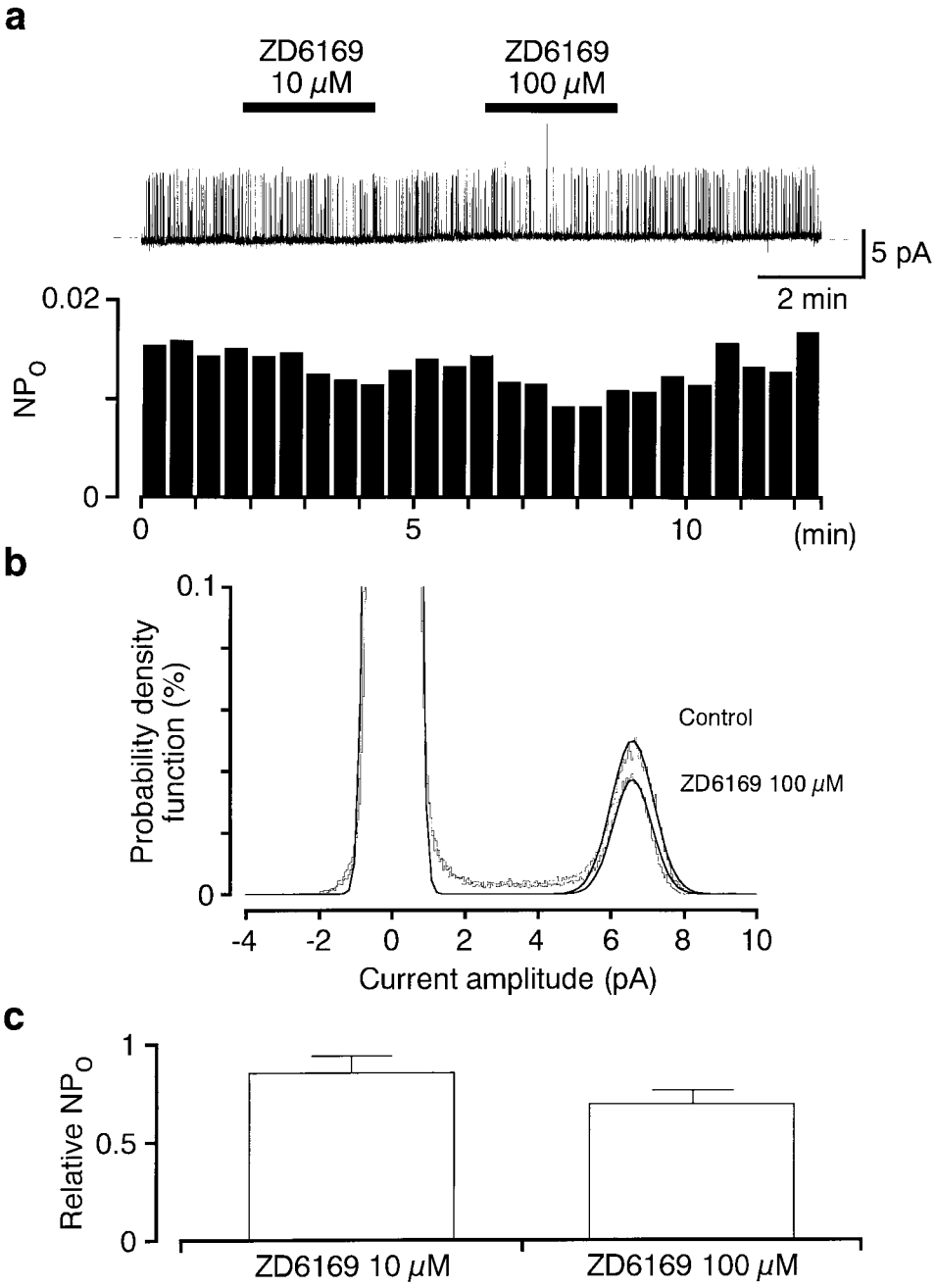

Figure 9 Effects of ZD6169 (10 and 100 μM) on the 6.7 pA K^+ channel at +30 mV in pig urethra. (a) Current trace of the effects of each concentration of ZD6169 (3 min duration) on the activity of maxi K^+ channels in inside-out configuration (symmetrical 140 mM K^+ conditions), with the NP_o values shown below, calculated for every 30 s segment of the record. The dashed line indicates the current base line when the channel is not open. (b) The all-point amplitude histogram for 100 μM ZD6169, obtained during the last 2 min of a 2.5 min application. The histograms in the absence (control; just before the application of ZD6169) or presence of ZD6169 (100 μM) are superimposed. Continuous lines in the histograms are theoretical curves fitted with the Gaussian distribution by the least-squares method. The abscissa shows the amplitude of the current (pA) and the ordinate shows the percentage value of the probability density function (%) for the recording period (2 min). Histograms were obtained from the results shown in (a). (c) Each column shows the relative NP_o of the maxi K^+ channels (mean value+s.d.) when the channel activity was taken as one just before application of each concentration of ZD6169 ($n=6$).

cell-attached configuration that there is a bell-shaped relationship between ZD6169 concentration and ZD6169-induced K_{ATP} channel activity, with maximum activation at a concentration of approximately 30 μM (Figure 6b). There is thus little difference in the concentration of ZD6169 needed to induce maximum effects on the membrane potential, current and channel activity, despite the different recording techniques employed.

Interestingly, although application of 100 μM ZD6169 initially induced channel openings, this was followed by a gradual decrease in the activity of the K_{ATP} channels. Furthermore, application of 100 μM ZD6169 reversibly

suppressed the 100 μM levcromakalim-induced K^+ current as effectively as did 5 μM glibenclamide in whole-cell recordings. Similar inhibitory effects of ZD6169 on the 100 μM levcromakalim-induced K_{ATP} channel activity were observed in cell-attached configuration. These results show that ZD6169 possesses a dual effect on the K_{ATP} channels, depending on its concentration and time course. They also show that ZD6169 may transiently open K_{ATP} channels at concentrations which block the channels in a steady state and that the inhibitory effect of ZD6169 can be seen at lower concentrations when the K_{ATP} channel had been activated. During the transient activating phase, when 100 μM ZD6169 was applied, application of voltage ramps showed that the membrane current was actually inhibited at potentials more positive than -30 mV; later, application of the drug inhibited the membrane currents over the whole range of membrane potentials. Similar inhibitory effects of 100 μM ZD6169 on the membrane currents were observed even in the presence of 5 μM glibenclamide (unpublished observations, N. Teramoto). We have also demonstrated that ZD6169 (100 μM) inhibits the activity of maxi K^+ channels in pig urethra. Therefore, it is reasonable to assume that the additional inhibitory effects of ZD6169 on membrane currents may be through this route.

Interaction of ZD6169 with K_{ATP} channels in pig urethra

It is generally established that K_{ATP} channels are composed of at least two distinct subunits: a sulphonylurea receptor (SUR) and an inwardly rectifying K^+ channel (Kir) subunit (reviewed by Aguilar-Bryan & Bryan, 1999). Recently, we have shown that Kir6.2 subunit may be present in pig urethra by use of RT–PCR analysis (Teramoto *et al*., 2000). It has been reported that mRNA encoding a truncated form of Kir6.2 in which the last 36 amino acids of the C-terminus had been deleted (i.e. Kir6.2ΔC36) could elicit significant currents even in the absence of SURs when injected into *Xenopus* oocytes (Tucker *et al*., 1997). This makes it possible to investigate the direct effects of certain drugs on the pore forming Kir6.2 subunits (Proks & Ashcroft, 1997). In the present experiments, we have demonstrated that application of ZD6169 had no significant effect on the channel activity of Kir6.2ΔC36 expressed in COS7 cells although cibenzoline, a potent pore blocker of Kir6.2 (Mukai *et al*., 1998), reversibly suppressed the channel activity in the same excised membrane patches. Furthermore, application of glibenclamide has no significant effect on the activity of Kir6.2ΔC36 channels suggesting that there may be little native SURs on the surface of COS7 cells membrane. These results strongly suggest that ZD6169 itself may not modulate the pore forming Kir6.2 subunits of K_{ATP} channels.

Jow & Numann (1999) proposed a new hypothesis regarding the bell-shaped concentration-response relationships for ZD6169, suggesting that ZD6169 acts as an agonist at low concentrations by displacing ATP but as a partial antagonist at higher concentrations by blocking the channel possibly by mimicking the effects of ATP ('ATP displacing hypothesis'). Studies on the Kir6.2ΔC36 channels (Tucker *et al*., 1997) suggest that the ATP binding site(s) may be located on Kir6.2 in K_{ATP} channels. In the present experiments, however, the activity of Kir6.2ΔC36 channels was not significantly inhibited by application of 100 μM ZD6169 even in the absence of ATP. Similarly, applying 100 μM ZD6169 had no significant effect on Kir6.2ΔC36 channel activity in cell-attached configuration (data not shown). Thus, it is somewhat difficult to explain the inhibitory effects of 100 μM ZD6169 on the activity of K_{ATP} channels based on the 'ATP displacing hypothesis'. Recent molecular biological studies demonstrate that the binding sites for both sulphonylureas (glibenclamide etc.) and K_{ATP} channel openers (diazoxide, pinacidil, cromakalim, P1075 etc.) reside on SURs although the location of these sites within the receptor remains to be elucidated (Aguilar-Bryan & Bryan, 1999). Binding studies have confirmed that ZD6169 binds to the same site as ^{3}H-P1075 and other types of K_{ATP} channel openers in guinea-pig urinary bladder at low concentrations of ZD6169 (Trivedi *et al*., 1995). It therefore seems probable that ZD6169 like other types of K_{ATP} channel openers binds to SURs. One interpretation could be that there may be at least two different binding sites for ZD6169 in SURs; a high affinity binding site and a low affinity binding site ('two binding sites hypothesis'). At lower concentrations of ZD6169, ZD6169 may bind mainly to the high affinity binding site which may activate K_{ATP} channels although a small amount of ZD6169 may also bind to the low affinity site which may block K_{ATP} channels or antagonize the function of the high affinity site, depending on pharmacokinetics and the bioavailability of SUR. On the other hand, when higher concentrations of ZD6169 are applied, ZD6169 may initially bind to the high affinity site causing transient activation, and eventually bind to the low affinity binding site blocking the channels. On removal of high concentrations of ZD6169 ($\geqslant$100 μM), ZD6169 may readily be removed from the low affinity blocking site, allowing reactivation of the K_{ATP} channels through the more persistent binding to the high affinity activating site. This 'two binding sites hypothesis' is consistent with the results of our present data.

We are grateful to AstraZeneca Pharmaceuticals for the generous gift of ZD6169. We also thank SmithKline Beecham Pharmaceuticals for the generous gift of levcromakalim. This work was supported by the grants-in-aid from the Clinical Research Foundation (Fukuoka, Japan), awarded to Noriyoshi Teramoto.

References

AGUILAR-BRYAN, L. & BRYAN, J. (1999). Molecular Biology of adenosine triphosphate-sensitive potassium channels. *Endocrine Rev.,* **20,** 101–135.

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.

HOWE, B.B., HALTERMAN, T.J., YOCHIM, C.L., DO, M.L., PETTINGER, S.J., STOW, R.B., OHNMACHT, C.J., RUSSELL, K., EMPFIELD, J.R., TRAINOR, D.A., BROWN, F.J. & KAU, S.T. (1995). ZENECA ZD6169: A novel K_{ATP} channel opener with *in vivo* selectivity for urinary bladder. *J. Pharmacol. Exp. Ther.,* **274,** 884–890.

HU, S. & KIM, H.S. (1997). Modulation of ATP-sensitive and large-conductance Ca^{++}-activated K^+ channels by Zeneca ZD6169 in guinea pig bladder smooth muscle cells. *J. Pharmacol. Exp. Ther.,* **280,** 38–45.

JOW, B. & NUMANN, R. (1999). The effects of ZD6169 on the ATP-dependent K^+ current (IK_{ATP}) in isolated cat ventricular myocytes. *Eur. J. Pharmacol.,* **383,** 197–202.

LI, J.H., YASAY, G.D., ZOGRAFOS, P., KAU, S.T., OHNMACHT, C.J., RUSSELL, K., EMPFIELD, J.R., BROWN, F.J., TRAINOR, D.A., BONEV, A.D., HEPPNER, T.J. & NELSON, M.T. (1995). Zeneca ZD6169 and its analogs from a novel series of anilide tertiary carbinols: in vitro K_{ATP} channel opening activity in bladder detrusor. *Pharmacology,* **51,** 33–42.

MUKAI, E., ISHIDA, H., HORIE, M., NOMA, A., SEINO, Y. & TAKANO, M. (1998). The antiarrhythmic agent cibenzoline inhibits K_{ATP} channels by binding to Kir6.2. *Biochem. Biophys. Res. Commun.,* **251,** 477–481.

PROKS, P. & ASHCROFT, F.M. (1997). Phentolamine block of K_{ATP} channels is mediated by Kir6.2. *Proc. Natl. Acad. Sci. U.S.A.,* **94,** 11716–11720.

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.,* **481P,** 56P.

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.

TERAMOTO, N. & BRADING, A.F. (1997). Nicorandil activates glibenclamide-sensitive K^+ channels in smooth muscle cells of pig proximal urethra. *J. Pharmacol. Exp. Ther.,* **280,** 483–491.

TERAMOTO, N., CREED, K.E. & BRADING, A.F. (1997a). Activity of glibenclamide-sensitive K^+ channels under unstimulated conditions in smooth muscle cells of pig proximal urethra. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **356,** 418–424.

TERAMOTO, N., MCMURRAY, G. & BRADING, A.F. (1997b). Effects of levcromakalim and nucleoside diphosphates on glibenclamide-sensitive K^+ channels in pig urethral myocytes. *Br. J. Pharmacol.,* **120,** 1229–1240.

TERAMOTO, N., YUNOKI, T., TANAKA, K., TAKANO, M., MASAKI, I., YONEMITSU, Y., SUEISHI, K. & ITO, Y. (2000). The effects of caffeine on ATP-sensitive K^+ channels in smooth muscle cells from pig urethra. *Br. J. Pharmacol.,* **131,** 505–513.

TRIVEDI, S., STETZ, S.L., POTTER-LEE, L., MCCONVILLE, M., LI, J.H., EMPFIELD, J., OHNMACHT, C.J., RUSSELL, K., BROWN, F.J., TRAINOR, D.A. & KAU, S.T. (1995). K-channel opening activity of ZD6169 and its analogs: effect on ^{86}Rb efflux and ^{3}H-P1075 binding in bladder smooth muscle. *Pharmacol.,* **50,** 388–397.

TUCKER, S.J., GRIBBLE, F.M., ZHAO, C., TRAPP, S. & ASHCROFT, F.M. (1997). Truncation of Kir6.2 produces ATP-sensitive K^+ channels in the absence of the sulphonylurea receptor. *Nature,* **387,** 179–183.

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

(Received October 10, 2000
Revised February 19, 2001
Accepted February 19, 2001)