

# Functional involvement of sulphonylurea receptor (SUR) type 1 and 2B in the activity of pig urethral ATP-sensitive K<sup>+</sup> channels

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**1** We have investigated the possible roles of sulphonylurea receptor (SUR) type 1 and 2B in the activity of pig urethral ATP-sensitive K<sup>+</sup> channels (K<sub>ATP</sub> channels) by use of patch-clamp and reverse transcriptase–polymerase chain reaction (RT–PCR) techniques.

**2** In voltage-clamp experiments, not only diazoxide, a SUR1 and weak SUR2B activator, but also pinacidil, a selective SUR2 activator, caused an inward current at a holding potential of –50 mV in symmetrical 140 mM K<sup>+</sup> conditions.

**3** Gliclazide (≤1 μM), a selective SUR1 blocker, inhibited the 10 μM pinacidil-induced currents (K<sub>i</sub> = 177 μM) and the 500 μM diazoxide-induced currents (high-affinity site, K<sub>i1</sub> = 5 nM; low-affinity site, K<sub>i2</sub> = 108 μM) at –50 mV.

**4** Application of tolbutamide (≤100 μM) reversibly caused an inhibition of the 500 μM diazoxide-induced current at –50 mV.

**5** MCC-134, a SUR type-specific K<sub>ATP</sub> channel regulator (1–100 μM), produced a concentration-dependent inward K<sup>+</sup> current, which was suppressed by the application of glibenclamide at –50 mV. The amplitude of the MCC-134 (100 μM)-induced current was approximately 50% of that of the 100 μM pinacidil-induced currents.

**6** Using cell-attached configuration, MCC-134 activated a glibenclamide-sensitive K<sub>ATP</sub> channel which was also activated by pinacidil.

**7** RT–PCR analysis demonstrated the presence of SUR1 and SUR2B transcripts in pig urethra.

**8** These results indicate that both SUR1 and SUR2B subunits play a functional role in regulating the activity of pig urethral K<sub>ATP</sub> channels and that SUR1 contributes less than 25% to total K<sub>ATP</sub> currents.

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**Keywords:** ATP-sensitive K<sup>+</sup> channels; diazoxide; gliclazide; MCC-134; pinacidil; tolbutamide; smooth muscle; sulphonylurea receptor 1; sulphonylurea receptor 2B

**Abbreviations:** DMSO, dimethylsulphoxide; Kir, inwardly-rectifying K<sup>+</sup> channel; K<sub>ATP</sub> channels, ATP-sensitive K<sup>+</sup> channels; MCC-134, 1-[4-(1H-imidazol-1-yl)benzoyl]-N-methyl-cyclobutanecarbothioamide; NDP, nucleoside diphosphates; RT–PCR, reverse transcriptase–polymerase chain reaction; SUR, sulphonylurea receptor

## Introduction

Recent studies have revealed that ATP-sensitive K<sup>+</sup> channels (K<sub>ATP</sub> channels) are heteromeric complexes composed of at least two subunits; inwardly rectifying K<sup>+</sup> channels (Kir6.x) and sulphonylurea receptors (SURs) (Aguilar-Bryan *et al.*, 1995; Inagaki *et al.*, 1995). The SURs are responsible not only for sulphonylurea-sensitivity (such as glibenclamide, tolbutamide, etc.) but also for intracellular nucleoside diphosphate (NDP)-sensitivity. K<sub>ATP</sub> channel openers interact with the SUR subunits, and not with the Kir subunits of the K<sub>ATP</sub> channels (Schwanstecher *et al.*, 1998).

It has been reported that recombinant K<sub>ATP</sub> channels solely composed of homotetrameric subunits of SUR2B resemble those found in native vascular smooth muscle (Isomoto *et al.*, 1996; Yamada *et al.*, 1997). Although the molecular composition of SURs assessed by reverse transcriptase–polymerase chain reaction (RT–PCR) has revealed the presence of

mRNA transcripts for SUR1 and SUR2B in detrusor smooth muscle (guinea-pig, Gopalakrishnan *et al.*, 1999; pig and human, Buckner *et al.*, 2000), it was concluded that only SUR2B but not SUR1, is expressed as a SUR protein for regulating the activity of K<sub>ATP</sub> channels in the urinary bladder (Gopalakrishnan *et al.*, 1999). We believe that the significant differences in properties of K<sub>ATP</sub> channels between urinary bladder and urethra (such as different types of SUR subunits, etc.) hold out some hope for the development of tissue-selective K<sub>ATP</sub> channel openers for urge urinary incontinence (Teramoto *et al.*, 1997). To achieve this aim, it is essential to study the properties of SUR subunits in urethral K<sub>ATP</sub> channels in comparison with those in the urinary bladder.

Recently, Shindo *et al.* (2000), using functional expressing studies, have reported that MCC-134 is a full agonist for the SUR2B/Kir6.2 channel and a partial agonist for the SUR2A/Kir6.2 channel, whereas it inhibits the SUR1/Kir6.2, concluding that MCC-134 may be the prototype for new drugs acting selectively on distinct types of SURs.

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In the present experiments, the possible roles of SUR1 and SUR2B in the activity of K<sub>ATP</sub> channels in pig urethra were examined by the use of patch-clamp techniques. We have utilized diazoxide as an agonist for SUR1 and weak agonist for SUR2B, pinacidil as a selective agonist for SUR2, but not for SUR1, and MCC-134 as a novel K<sub>ATP</sub> channel modulator acting through distinct types of SURs. Gliclazide and tolbutamide were used as specific antagonists for SUR1. In addition, we have also performed RT-PCR analysis in order to examine whether or not mRNA for SUR1 and SUR2B are expressed in pig urethra. The present study thus provides evidence of the pharmacological and molecular identity of SUR1 and SUR2B subunits in pig urethral K<sub>ATP</sub> channels.

## 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 after treatment with papain and collagenase, as described previously (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 used within 2 h for experiments.

### Recording procedure

Patch-clamp experiments were performed at room temperature (21–23°C), as described previously (Teramoto *et al.*, 2001). 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 necessary. Capacitance noise was kept to a minimum by maintaining the test solution in the electrode as low as possible.

### Drugs and solutions

For whole-cell recording (conventional whole-cell and nystatin-perforated patch), the following solutions were used: 140 mM K<sup>+</sup> solution containing (mM): Na<sup>+</sup> 5, K<sup>+</sup> 140, Mg<sup>2+</sup> 1.2, Ca<sup>2+</sup> 2, glucose 5, Cl<sup>-</sup> 151.4, HEPES 10, titrated to pH 7.35–7.40 with Tris base; high potassium pipette solution containing (mM): K<sup>+</sup> 140, Cl<sup>-</sup> 140, glucose 5, EGTA 5, and HEPES 10/Tris (pH 7.35–7.40). The perforated-patch technique with nystatin was also occasionally used to record whole-cell currents (Teramoto & Brading, 1996). In short, nystatin was freshly dissolved in acidified methanol (1 N HCl to about pH 2) and the pH was readjusted to 7.4 with Tris base. This stock solution (10 mg ml<sup>-1</sup>) was diluted in the pipette solution at a final concentration of 50 μg ml<sup>-1</sup> just before use. Whole-cell recording was performed with a pipette that was first dipped in normal pipette solution (nystatin-free) and then back-filled with nystatin-containing pipette solution. This resulted in chemical perforation of the membrane. For cell-attached recordings, the pipette and bath solution were high potassium solutions (mM): K<sup>+</sup> 140, Cl<sup>-</sup> 140, EGTA 5, glucose 5, HEPES 10/Tris (pH 7.35–7.40). 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<sup>-1</sup>. MCC-134 (Mitsubishi Pharma Corporation, Tokyo, Japan), gliclazide (Dainippon Pharmaceuticals Co., Ltd, Osaka, Japan), and glibenclamide were prepared daily as 100 mM stock solutions in dimethylsulphoxide (DMSO). Other drugs were obtained from Sigma Chemical (Sigma Chemical K.K., Tokyo, Japan). The maximum final concentration of DMSO in the bath solution was 0.3% for patch-clamp experiments. These concentrations were shown not to affect K<sup>+</sup> channels.

### Data analysis

The whole-cell current data were low-pass filtered at 500 Hz by an eight pole Bessel filter, sampled at 25 ms intervals (continuous traces) or 1 ms (ramp current) and analysed on a computer (PowerMac G3, Tokyo, Japan) by the 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 by a computer with a digitalized interval of 80 μs using 'PAT' program (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*<sub>open</sub>) were measured at -50 mV for 1 min.

$$NP_o = \left( \sum_{j=1}^N t_j j \right) / T$$

where *t<sub>j</sub>* 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*<sub>open</sub> was relatively high. Data points were fitted using the method of least-squares. Data are expressed as mean with the standard deviation (s.d.).

### Equation for curve fitting of K<sub>ATP</sub> channel opener-induced currents

The curve for K<sub>ATP</sub> channel opener-induced current was drawn by fitting the equation using the least-squares method:

$$\text{Relative value} = A / \{1 + (K/D)n_H\}$$

where *A*, *K*, *D* and *n<sub>H</sub>* are maximum relative current induced by K<sub>ATP</sub> channel opener, apparent dissociation constant, concentration of K<sub>ATP</sub> channel opener (μM) and the Hill coefficient, respectively.

### Equation for curve fitting of the effects of gliclazide on K<sub>ATP</sub> channel opener-induced currents

The mean amplitude of each K<sub>ATP</sub> opener-induced current at the holding potential of -50 mV just before application of gliclazide (30 s duration) was normalized as 1.0. The curve was drawn by fitting the equation using the least-squares method:

$$I/I_c = xy$$

where *I* is the mean amplitude of the K<sub>ATP</sub> opener-induced current in the presence of gliclazide, *I<sub>c</sub>* is the mean amplitude of the K<sub>ATP</sub> opener-induced current in control (in the absence

of gliclazide),  $x$  is a term describing the high affinity site, and  $y$  a term describing the low affinity site.

$$x = L + \{(1 - L)/(1 + ([Glic]/K_{i1})^{h1})\}$$

$$y = 1/(1 + ([Glic]/K_{i2})^{h2})$$

where [Glic] is the concentration of gliclazide ( $\mu\text{M}$ ),  $K_{i1}$ ,  $K_{i2}$  are the gliclazide concentrations at which inhibition is half maximum at high- and low-affinity sites, respectively;  $h1$ ,  $h2$  are the Hill coefficients for the high- and low-affinity sites, respectively; and  $L$  is the fractional current amplitude remaining when the high-affinity sites are fully occupied. When only a single site is present, the equation reduces to  $I/I_c = x$ .

### Statistics

Statistical analyses were performed with a two-paired *t*-test (two-factor with replication). Changes were considered significant at  $P < 0.05$ .

### RNA preparation and RT-PCR analysis

For RNA isolation, total RNA from pig urethral smooth muscle was isolated using the TRIzol reagent according to the manufacturer's instructions (RNeasy Mini Kit, QIAGEN K.K., Tokyo, Japan). First-strand synthesis of cDNA using random hexamers was carried out as follows: DNase I-treated total RNA (0.5–1.0  $\mu\text{g}$ ) isolated from tissues was incubated with random hexamers at 70°C for 10 min and then with PCR buffer (20 mM Tris/HCl, pH 8.4, 50 mM KCl), 2.5 mM MgCl<sub>2</sub>, 0.5 mM deoxynucleoside-5'-triphosphate, and 10 mM dithiothreitol at 25°C for 5 min. RT-PCR was initiated by the addition of Superscript II RT (200 U) at 25°C for 10 min followed by incubation at 42°C for 50 min. The reaction was terminated by incubation with RNase H at 70°C for 15 min, before chilling on ice. PCR was performed using 1  $\mu\text{l}$  of cDNA in a 12.5  $\mu\text{l}$  reaction containing 0.4  $\mu\text{M}$  concentration of each primer, 200  $\mu\text{M}$  concentration of each deoxynucleoside-5'-triphosphate, and 0.5 U of *Taq* polymerase (Takara Co. Ltd, Kyoto, Japan). The cycling conditions were 94°C for 25 s, 54°C for 25 s, and 72°C for 25 s for 40 cycles. An aliquot (10  $\mu\text{l}$ ) of the RT-PCR product was analysed on a 3% Tris-borate-EDTA polyacrylamide gel. Since no sequence information is available for K<sub>ATP</sub> channel subunits in pig, generic subunit-specific primers were designed based on information from rat, mouse, and human sequences. The locations of the primers indicated are based on the subunit sequence information obtained from GenBank: SUR1 (human, L78207), and SUR2 (rat 2A, D83598; mouse 2A, D86037; mouse 2B, D86038; human 2A, AF061323; human 2B, AF061324). The sequences of the primers were as follows: for SUR1 (forward) 5'-GCGTGCAAAAGCTAAGCGAG-3' and (reverse) 5'-GAC-GCTTGC GGTTCAACAAC-3' (corresponding to nucleotide positions 1817–1836 and 1951–1933, respectively); for SUR2A/B (forward) 5'-GCTGAAGAATATGGTCAAATC-TC-3' and (reverse) 5'-CGGAGTGTTCGTATTCCAAAATA-3' (corresponding to nucleotide positions, 4278–4300 and 4590–4569, respectively). Control reactions were carried out where samples in the absence of reverse transcriptase were amplified to ensure that the detected product was not the result of possible DNA contamination and by use of corresponding

templates as positive controls to ensure that the primers were annealing successfully. These primers gave products of the expected sizes that were confirmed by DNA sequence analysis.

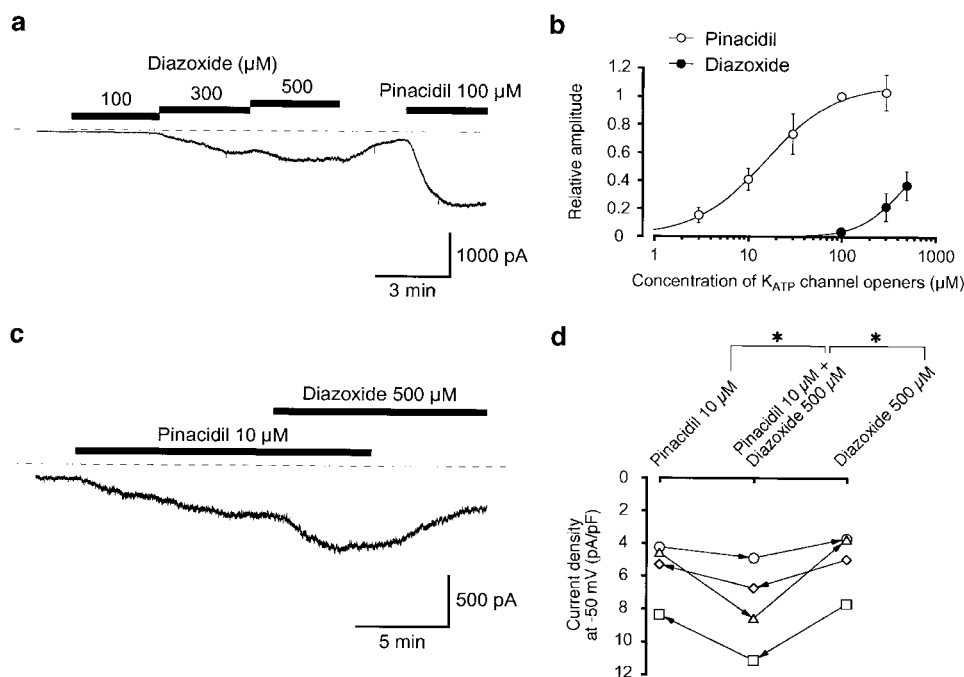
## Results

### Comparison between the diazoxide-induced inward current and the pinacidil-induced inward current in pig urethral myocytes by use of nystatin-perforated patch

In order to investigate pharmacological properties of K<sub>ATP</sub> channels in pig urethra, two distinct types of well-known K<sub>ATP</sub> channel openers (diazoxide, a SUR1 activator; pinacidil, a selective SUR2 activator; reviewed by Babenko *et al.*, 1998; Fujita & Kurachi, 2000) were utilized by use of the nystatin-perforated patch. After the establishment of the nystatin-perforated patch, approximately 15 min later, cumulative application of diazoxide (100–500  $\mu\text{M}$ ) caused an inward current in a concentration-dependent manner at a holding potential of –50 mV (bath solution, 140 mM K<sup>+</sup> solution; pipette solution, 140 mM KCl solution containing 5 mM EGTA; that is, symmetrical 140 mM K<sup>+</sup> conditions, Figure 1). After diazoxide was removed, the current recovered to the control level. Subsequently, application of pinacidil (100  $\mu\text{M}$ ) caused an inward current. Figure 1b shows the relation between the concentrations of each K<sub>ATP</sub> channel opener (diazoxide and pinacidil) and the peak amplitude of each K<sub>ATP</sub> channel opener-induced inward currents when the peak amplitude of the 100  $\mu\text{M}$  pinacidil-induced current was taken as one. As shown in Figure 1c, additional application of diazoxide (500  $\mu\text{M}$ ) further enhanced the 10  $\mu\text{M}$  pinacidil-induced currents. Similarly, pinacidil caused an additional effect on the diazoxide-induced current (Figure 1d).

### Differential sensitivity of K<sub>ATP</sub> channel opener-induced membrane currents to gliclazide in pig urethra

In order to further investigate whether the SUR1 subunit may regulate the activity of K<sub>ATP</sub> channels in pig urethra, gliclazide, a specific SUR1 blocker (Gribble & Ashcroft, 1999), was used. The concentration of each K<sub>ATP</sub> channel opener, which caused a similar potency for the current activation on the membrane currents was chosen (10  $\mu\text{M}$  pinacidil and 500  $\mu\text{M}$  diazoxide). As shown in Figure 2a, application of 10  $\mu\text{M}$  gliclazide, a selective SUR1 blocker, caused a small inhibitory effect on the pinacidil (10  $\mu\text{M}$ )-induced current ( $0.94 \pm 0.07$ ,  $n = 4$ ). Further application of gliclazide (100–1000  $\mu\text{M}$ ) inhibited the pinacidil-induced current at –50 mV (Figure 2c, 10  $\mu\text{M}$  pinacidil,  $K_i = 177 \mu\text{M}$ ; 100  $\mu\text{M}$  pinacidil,  $K_i = 285 \mu\text{M}$ ). In contrast, 10 nM gliclazide reduced the 500  $\mu\text{M}$  diazoxide-induced current amplitude by approximately 18% ( $0.82 \pm 0.09$ ,  $n = 5$ ,  $P < 0.01$ ) and higher concentrations of gliclazide inhibited the diazoxide-induced current in a concentration-dependent manner, demonstrating two inhibitory sites: a high-affinity site ( $K_{i1} = 5 \text{ nM}$ ) and a low-affinity site ( $K_{i2} = 108 \mu\text{M}$ ) in Figure 2c. Furthermore, as shown in Figure 3a, the application of tolbutamide, a SUR1 inhibitor, caused an inhibition of the 500  $\mu\text{M}$  diazoxide-induced current at –50 mV, demonstrating a concentration-dependent manner (Figure 3c). However, lower concentrations of tolbutamide (30–100  $\mu\text{M}$ ) had little effect on



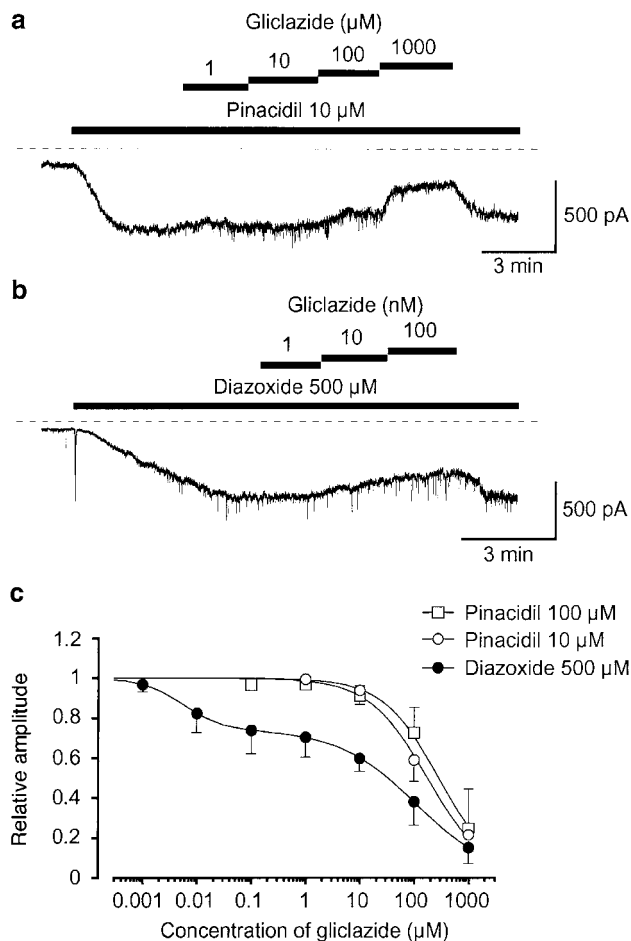
**Figure 1** Comparison of the potency of diazoxide and pinacidil in activation of K<sub>ATP</sub> membrane currents at -50 mV in pig urethra. Nystatin-perforated patch configuration; the bath contained a 140 mM K<sup>+</sup> solution and pipette 140 mM KCl containing 5 mM EGTA (i.e., symmetrical 140 mM K<sup>+</sup> conditions). (a) Diazoxide (100 – 500 μM) caused an inward membrane current in a concentration-dependent manner. On removal of diazoxide, the current recovered to the control level. Subsequent application of pinacidil (100 μM) caused an inward current. The dashed line indicates zero current level. (b) The relation between the concentrations of K<sub>ATP</sub> channel openers and the relative peak amplitude of K<sub>ATP</sub> channel opener-induced membrane currents in pig urethra. Note that the peak current amplitude was measured from the level before application of K<sub>ATP</sub> channel openers and normalized with respect to the 100 μM pinacidil-induced current from the same dispersed myocytes. The following values were used for the curve fitting:  $A = 1.1$ ,  $K = 15 \mu\text{M}$ ,  $n_H = 1.2$ . The line for the diazoxide-induced current was drawn by eye. Each symbol indicates the mean of 3–10 observations with  $\pm$ s.d. shown by vertical lines. (c) Diazoxide (500 μM) enhanced the 10 μM pinacidil-induced current. The dashed line indicates zero current level. (d) The effects of diazoxide and pinacidil were additive. The different symbols represent four separated experiments. The arrow between points indicates the order of application of the K<sub>ATP</sub> channel openers (diazoxide and pinacidil) onto the same dispersed cell. Asterisks indicate a statistically significant difference, demonstrated using a paired *t*-test ( $P < 0.05$ ).

the 10 μM pinacidil-induced current. As shown in Figures 2 and 3, occasionally, a small amount of spontaneous transient inward currents were observed at -50 mV. The spontaneous transient inward currents were selectively inhibited by additional application of 100 nM iberiotoxin (data not shown).

#### Relations between peak amplitude of MCC-134-induced currents and concentrations of MCC-134

In order to further investigate whether the SUR1 subunit may regulate the activity of K<sub>ATP</sub> channels in pig urethra, MCC-134, a novel K<sub>ATP</sub> channel modulator acting through distinct types of SURs (Shindo *et al.*, 2000), was used. When the concentration of MCC-134 was increased cumulatively (from 10 to 100 μM), the peak amplitude of the MCC-134-induced basal membrane current at -50 mV increased in a concentration-dependent manner (Figure 4a). On removal of MCC-134, subsequent application of 100 μM pinacidil caused a larger amplitude inward current, which was suppressed by 5 μM glibenclamide. Further studies were carried out on the effects of the two different types of drugs (MCC-134 and pinacidil) using triangular ramp potential pulses (see inset in Figure 4a). Three of these were applied from -120 to +80 mV, obtaining the current-voltage relations in the absence and presence of

each drug. Figure 4b shows the averaged membrane currents during the falling phase of the ramp pulses under the various experimental conditions. The MCC-134-induced membrane current was obtained by subtracting the averaged control current from the membrane current in the presence of 100 μM MCC-134 and demonstrated an inwardly rectifying property at positive potentials (Figure 4c). The pinacidil-induced membrane current was obtained by subtracting the averaged control current from the membrane current in the presence of pinacidil, and showed a similar inwardly rectifying property at positive potentials (Figure 4c). Figure 4d shows the relation between the concentrations of MCC-134 and the peak amplitude of the MCC-134-induced inward currents when the peak amplitude of the 100 μM pinacidil-induced current was taken as one. The MCC-134-induced inward current was suppressed by additional application of 5 μM glibenclamide (Figure 5a). However, a low concentration of gliclazide (1 μM), a selective SUR1 blocker, had no effect on the 100 μM MCC-134-induced inward current ( $0.99 \pm 0.03$ ,  $n = 4$ ) when the peak amplitude of the MCC-134-induced current was normalized as one just before application of 100 μM MCC-134 (i.e., control). In each condition, current-voltage relations were obtained by applying four ramp pulses (from -120 to +80 mV for 1 s duration, see inset in Figure 5a) every 15 s at -50 mV (Figure 5b). Figure 5c demonstrates the net membrane current

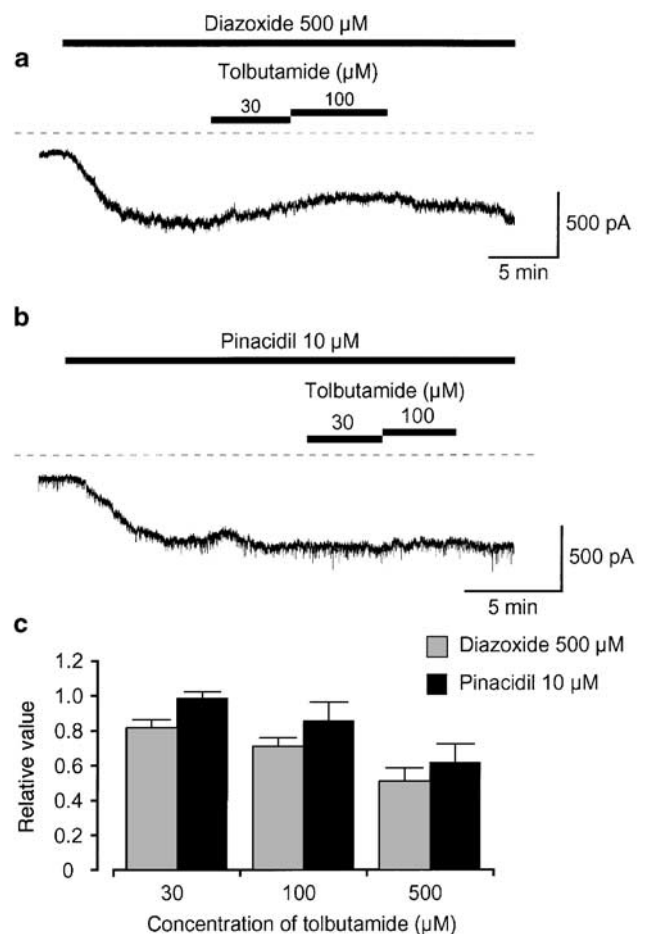


**Figure 2** Inhibitory effects of gliclazide on (a) 10  $\mu\text{M}$  pinacidil- and (b) 500  $\mu\text{M}$  diazoxide- induced inward membrane currents at  $-50\text{ mV}$  in symmetrical 140 mM  $\text{K}^+$  conditions by use of nystatin-perforated patch configuration. The dashed line indicates zero current. (c) Relation between relative inhibition of the peak amplitude of each  $\text{K}_{\text{ATP}}$  channel opener (pinacidil (10 and 100  $\mu\text{M}$ ) and 500  $\mu\text{M}$  diazoxide)-induced current and the concentration of gliclazide. The following values were used for the curve fitting: 10  $\mu\text{M}$  pinacidil,  $K_i = 177\ \mu\text{M}$ ,  $h = 0.8$ ; 100  $\mu\text{M}$  pinacidil,  $K_i = 285\ \mu\text{M}$ ,  $h = 0.8$ ; diazoxide,  $K_{i1} = 5\ \text{nM}$ ,  $h_1 = 1.2$ ;  $K_{i2} = 108\ \mu\text{M}$ ,  $h_2 = 0.7$ ,  $L = 0.74$ . Each symbol indicates the mean of 3–8 observations with s.d. shown by vertical lines.

obtained by subtraction of the two mean ramp currents recorded before and during application of 5  $\mu\text{M}$  glibenclamide in the presence of 100  $\mu\text{M}$  MCC-134, demonstrating an inward rectification at positive potentials.

#### MCC-134 activates a glibenclamide-sensitive $\text{K}_{\text{ATP}}$ channel in pig urethra

Single-channel recordings were performed by use of cell-attached configuration. To minimize the activity of maxi  $\text{K}^+$  channels, experiments were performed at  $-50\text{ mV}$ . In Figure 6a, application of 1  $\mu\text{M}$  MCC-134 evoked opening of a 2.1 pA  $\text{K}^+$  channel. In the presence of 100  $\mu\text{M}$  MCC-134, current–voltage relations were obtained by changing the holding membrane potential from  $-120$  to  $-10\text{ mV}$  in 10 mV increments. The results indicate that the conductance of the

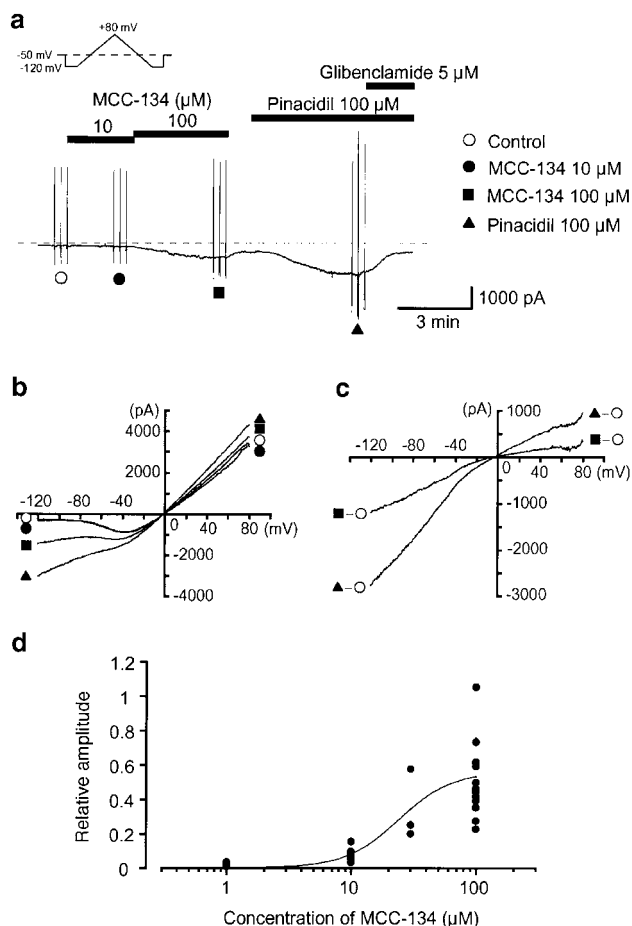


**Figure 3** The effects of tolbutamide (30, 100  $\mu\text{M}$ ) on (a) 500  $\mu\text{M}$  diazoxide- and (b) 10  $\mu\text{M}$  pinacidil- induced inward membrane currents at  $-50\text{ mV}$  in symmetrical 140 mM  $\text{K}^+$  conditions by use of nystatin-perforated patch configuration. The dashed line indicates zero current. (c) Tolbutamide (30–500  $\mu\text{M}$ ) inhibited the 500  $\mu\text{M}$  diazoxide- and the 10  $\mu\text{M}$  pinacidil-induced inward membrane current at  $-50\text{ mV}$  in a concentration-dependent manner. Each column indicates the mean 4–5 observations with +s.d.

channel is approximately 43 pS ( $43 \pm 1\ \text{pS}$ ,  $n = 8$ ) with an inwardly rectifying property at positive membrane potentials (data not shown). Subsequent application of pinacidil (100  $\mu\text{M}$ ) caused opening of the same amplitude  $\text{K}^+$  channel (Figure 6a). When the  $NP_o$  value in the presence of 100  $\mu\text{M}$  pinacidil was normalized as 1.0, the relative  $NP_o$  value in the presence of MCC-134 in the same membrane patches was  $0.03 \pm 0.01$  (10  $\mu\text{M}$ ,  $n = 4$ ) and  $0.28 \pm 0.16$  (100  $\mu\text{M}$ ,  $n = 4$ ) (Figure 6c). The channel opening was reversibly inhibited by 10  $\mu\text{M}$  glibenclamide (data not shown).

#### Molecular expression of SURs in pig urethra

In order to investigate the molecular component of SURs in pig urethral  $\text{K}_{\text{ATP}}$  channels, RT–PCR studies were performed. The specific primers for amplification of each SUR mRNA were designed to produce a cDNA fragment. As shown in Figure 7, both SUR1 (134 bp) and SUR2B (312 bp) mRNA were expressed in rat cDNA and pig urethra with the expected fragment size for SUR1 or SUR2B.



**Figure 4** Concentration-dependent effects of MCC-134 on the membrane currents at  $-50$  mV in pig urethra. Whole-cell recording, the bath contained a  $140$  mM K<sup>+</sup> solution and pipette  $140$  mM KCl containing  $5$  mM EGTA (i.e., symmetrical  $140$  mM K<sup>+</sup> conditions). (a) MCC-134 caused an inward membrane current in a concentration-dependent manner. After MCC-134 was removed, the current recovered to the control level. Pinacidil induced an inward current, which was suppressed by  $5$   $\mu$ M glibenclamide. The dashed line indicates zero current level. (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 three ramps in each condition. (c) The MCC-134-induced membrane current was obtained by subtraction of the two mean ramp currents recorded before and during application of  $100$   $\mu$ M MCC-134. The pinacidil-induced current was obtained from the membrane currents in the absence and presence of  $100$   $\mu$ M pinacidil. (d) The relation between the concentrations of MCC-134 and the relative peak amplitude of the MCC-134-induced membrane currents. The peak current amplitude was measured from the level in the presence of  $5$   $\mu$ M glibenclamide and normalized with respect to the  $100$   $\mu$ M pinacidil-induced current from the same dispersed cells. The curve was drawn by fitting the equation using the least-squares method at concentrations between  $1$  and  $100$   $\mu$ M. The following values were used for the curve fitting:  $A = 0.57$ ,  $K = 23$   $\mu$ M,  $n_H = 1.6$ . Each symbol indicates the relative amplitude at each concentration of MCC-134 ( $1$   $\mu$ M,  $0.04 \pm 0.03$ ,  $n = 5$ ;  $10$   $\mu$ M,  $0.11 \pm 0.07$ ,  $n = 6$ ;  $30$   $\mu$ M,  $0.34 \pm 0.20$ ,  $n = 3$ ;  $100$   $\mu$ M,  $0.52 \pm 0.27$ ,  $n = 10$ ).

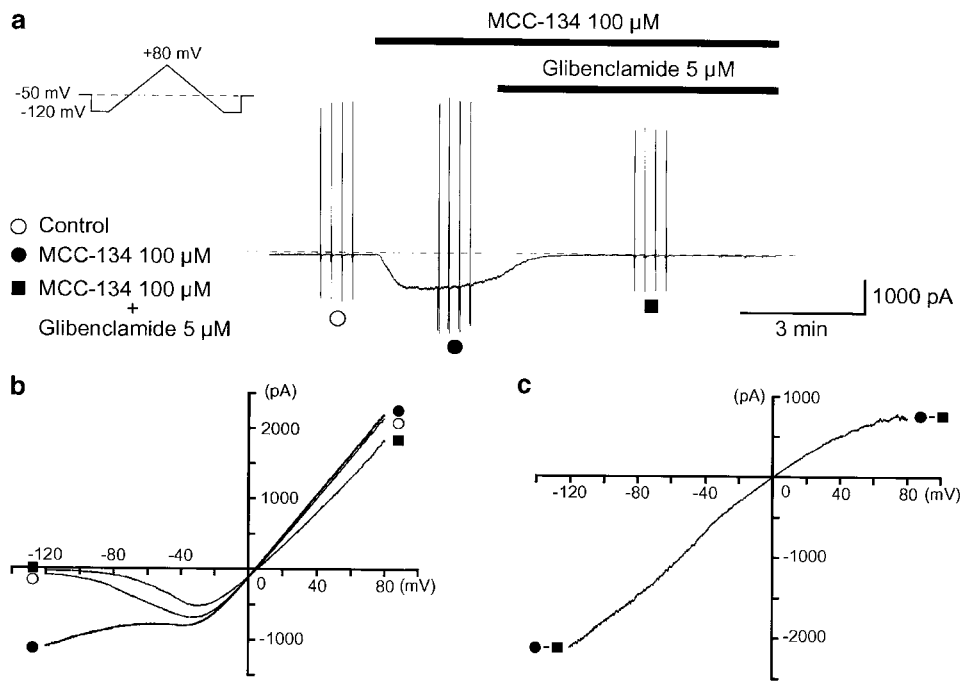
## Discussion

It is commonly assumed that SURs are responsible for differential pharmacological effects, depending on either the types of K<sub>ATP</sub> channel openers used or three subtypes of SURs

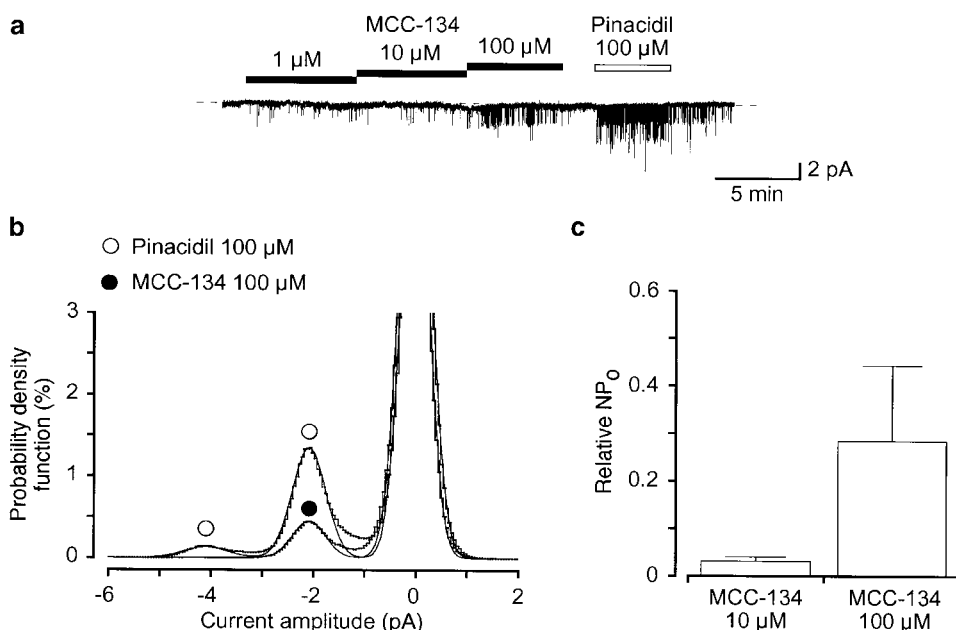
(SUR1, SUR2A, and SUR2B) present. In functional expression studies, pharmacological and electrophysiological studies have reported that SUR1/Kir6.2 represents the pancreatic  $\beta$ -cell K<sub>ATP</sub> channel, that SUR2A/Kir6.2 is thought to represent the cardiac K<sub>ATP</sub> channel, whereas SUR2B/Kir6.1 represents the smooth muscle-type K<sub>ATP</sub> channel (reviewed by Babenko *et al.*, 1998; Fujita & Kurachi, 2000). Previous work has shown that the SUR1/Kir6.2 channel is activated by diazoxide ( $EC_{50}$ ,  $50$   $\mu$ M) but not by pinacidil ( $\leq 1$  mM); the SUR2A/Kir6.2 channel is strongly activated by pinacidil ( $EC_{50}$ ,  $10$   $\mu$ M) but not responsive to diazoxide ( $\leq 200$   $\mu$ M); and the SUR2B/Kir6.2 channel is activated by both agents (Babenko *et al.*, 1998; Fujita & Kurachi, 2000).

Our present observations regarding the actions of diazoxide, pinacidil, gliclazide, tolbutamide, and MCC-134 on the pig urethral K<sub>ATP</sub> channels can be summarized as follows: (1) Diazoxide, a SUR1 activator, and pinacidil, a selective SUR2 activator, induce glibenclamide-sensitive inward K<sup>+</sup> currents. Similarly, by use of tension measurements, we have demonstrated that cumulative application of pinacidil and diazoxide produced a concentration-dependent relaxation of the resting urethral tone, which was suppressed by additional application of  $1$   $\mu$ M glibenclamide (pinacidil,  $EC_{50} = 1.6$   $\mu$ M; diazoxide,  $EC_{50} = 71$   $\mu$ M; Teramoto & Ito, 1999). (2) Gliclazide, a selective antagonist for SUR1, reversibly inhibits the diazoxide- but not the pinacidil-induced currents at low concentrations (between  $10$  nM and  $1$   $\mu$ M). At higher concentrations ( $\geq 10$   $\mu$ M), this agent also suppressed pinacidil-induced currents. (3) Low concentrations of tolbutamide ( $30$ – $100$   $\mu$ M), a selective SUR1 inhibitor, inhibited the  $500$   $\mu$ M diazoxide-induced current and the  $10$   $\mu$ M pinacidil-induced current with a different potency. (4) The inhibitory effects on not only the  $500$   $\mu$ M diazoxide-induced current but also the  $10$   $\mu$ M pinacidil-induced current were reversible. Low concentrations of tolbutamide ( $\leq 100$   $\mu$ M) inhibit the activity of SUR1/Kir6.2 not but SUR2A/Kir6.2 and inhibition of SUR1/Kir6.2 by tolbutamide ( $\leq 100$   $\mu$ M) is readily reversible on washout, indicating that the reversibility of the inhibition by tolbutamide is a good clue to identify SUR subtypes (Gribble *et al.*, 1998). However, application of  $500$   $\mu$ M tolbutamide also caused a reversible inhibition of the SUR2B/Kir6.2 current activated by  $200$   $\mu$ M diazoxide (Isomoto *et al.*, 1996). Thus, we can only exclude the possibility that SUR2A subunits are in pig urethral K<sub>ATP</sub> channels. (5) MCC-134 induces a concentration-dependent glibenclamide-sensitive inward K<sup>+</sup> current and activates glibenclamide-sensitive K<sub>ATP</sub> channels, as assessed by single-channel recordings. These results strongly suggest that SUR1 as well as SUR2B play physiological roles in the regulation of the muscle tone in pig urethra.

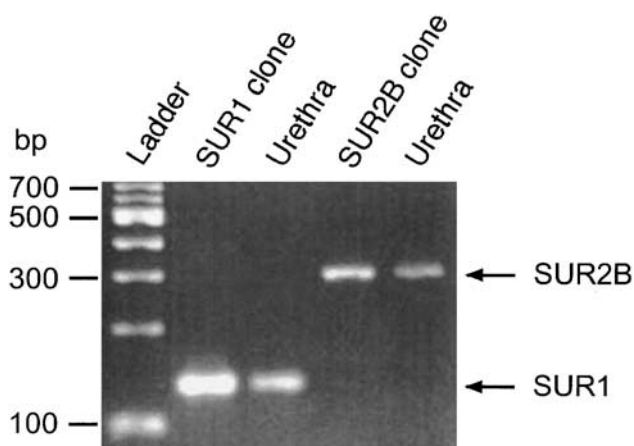
In the present RT–PCR studies, we have been able to demonstrate the presence of SUR1 as well as SUR2B in pig urethral smooth muscle cells although these studies have addressed only the expression of mRNA transcripts. Similarly, both SUR1 and SUR2B are widely expressed in the detrusor smooth muscle of several species (guinea-pig, Gopalakrishnan *et al.*, 1999; pig and human, Buckner *et al.*, 2000) as well as rat pulmonary vascular smooth muscle (Cui *et al.*, 2002) as determined by RT–PCR analysis. However, in urinary bladder, it is concluded that SUR1 protein is not expressed at significant levels, since there was much less sensitivity to diazoxide in comparison to that of pancreatic  $\beta$ -cells and no high-affinity binding site for [<sup>3</sup>H]glyburide (Gopalakrishnan



**Figure 5** Effects of glibenclamide on the 100  $\mu\text{M}$  MCC-134-induced membrane current at  $-50$  mV in a conventional whole-cell recording (the bath contained a 140 mM K<sup>+</sup> solution and pipette 140 mM KCl containing 5 mM EGTA, i.e., symmetrical 140 mM K<sup>+</sup> conditions). (a) Inhibitory effects of 5  $\mu\text{M}$  glibenclamide on the 100  $\mu\text{M}$  MCC-134-induced inward current. The vertical deflections indicate triangle ramp potential pulses (see inset) applied every 15 s from  $-120$  to 80 mV for 1 s after a 300 ms conditioning pulse. The dashed line indicates the zero current level. (b) The mean ramp membrane currents (the negative-going limb of the ramp pulse) on an expanded time scale in each condition. Symbols as in (a). (c) Difference between the mean MCC-134-induced membrane current in the presence and absence of 5  $\mu\text{M}$  glibenclamide. Net membrane current (glibenclamide-sensitive current) was obtained by subtraction of the two ramp membrane currents recorded before and during application of 5  $\mu\text{M}$  glibenclamide when 100  $\mu\text{M}$  MCC-134 was present in the bath.



**Figure 6** The MCC-134-induced opening of K<sup>+</sup> channels recorded with cell-attached configuration at a holding membrane potential of  $-50$  mV. (a) Comparison of the properties of the MCC-134 (1 – 100  $\mu\text{M}$ ) activated K<sup>+</sup> channels and the 100  $\mu\text{M}$  pinacidil induced K<sup>+</sup> channels in the same membrane patch at  $-50$  mV. The dashed line indicates the current when the channel is not open. (b) All-point amplitude histograms in the presence of 100  $\mu\text{M}$  MCC-134 and 100  $\mu\text{M}$  pinacidil (obtained during 1 min). Continuous lines in the histograms are theoretical curves fitted with the Gaussian distribution, by the least-squares method. The abscissa scale is the amplitude of the current (pA) and the ordinate scale is the percentage value of the probability density function (%) for the recording period (1 min). (c) Each column shows the relative level of the activity of the MCC-134-induced K<sub>ATP</sub> channel (mean value with +s.d.) when the mean amplitude of the pinacidil-induced channel activity was normalized as one ( $n = 4$ ).



**Figure 7** RT-PCR detection of SUR1 and SUR2B. RT-PCR was performed as described in Methods and size markers were used to indicate the size of the amplified fragments. RT-PCR yielded visible amounts of SUR1 (134 bp fragment) and SUR2B (312 bp fragment) in mRNAs from rat cDNA and pig urethra.

*et al.*, 1999; Buckner *et al.*, 2000). Thus, until our study, no functional study has yet been undertaken in smooth muscle K<sub>ATP</sub> channels to establish the involvement of SUR1 subunits.

In the present experiments, gliclazide ( $\leq 1 \mu\text{M}$ ), a selective sulphonylurea drug for SUR1, reversibly inhibited the diazoxide-induced K<sub>ATP</sub> current, but not the pinacidil- or the MCC-134-induced urethral K<sub>ATP</sub> current. When the concentration of gliclazide is less than  $1 \mu\text{M}$ , it is believed that gliclazide possesses selective inhibitory effects on SUR1, but not SUR2A or SUR2B, in recombinant K<sub>ATP</sub> channels and that only much higher concentrations of gliclazide ( $\geq 10 \mu\text{M}$ ) directly block the channel pore region in K<sub>ATP</sub> channels (Gribble & Ashcroft, 2000). Therefore, it is reasonable to assume that, in pig urethral K<sub>ATP</sub> channels, gliclazide specifically inhibits SUR1 at relatively lower concentrations ( $\leq 1 \mu\text{M}$ ) and interacts with the channel pore at higher concentrations ( $\geq 10 \mu\text{M}$ ), demonstrating two affinity sites (the high- and low-affinity sites). However, gliclazide ( $\geq 10 \mu\text{M}$ ) suppressed pinacidil (10 and  $100 \mu\text{M}$ )-induced current by an interaction with a single affinity site. Similarly, in rat mesenteric artery, the pinacidil-induced current was also blocked by gliclazide at a single site (Lawrence *et al.*, 2001). These results suggest that pinacidil and MCC-134 may selectively activate SUR2B, but not SUR1 in pig urethra, coinciding with observations in recombinant K<sub>ATP</sub> channels (pinacidil, Fujita & Kurachi, 2001; MCC-134, Shindo *et al.*, 2000). In pig urethra, the peak amplitude of the  $100 \mu\text{M}$  MCC-134-induced K<sup>+</sup> currents at  $-50 \text{ mV}$  was significantly smaller than that induced by pinacidil ( $100 \mu\text{M}$ ) in the same dispersed cells, demonstrating that MCC-134 is less potent than pinacidil in activating K<sub>ATP</sub> channels. Similarly, using the same membrane patches, the activity of the MCC-134-induced K<sub>ATP</sub> channels was much less than that of the pinacidil-induced K<sub>ATP</sub> channels in single-channel recordings. In contrast, the peak amplitude of the MCC-134 ( $100 \mu\text{M}$ )-induced channel current at recombinant SUR2B/Kir6.2 channels was much larger than that of the  $100 \mu\text{M}$  pinacidil-induced K<sup>+</sup> current (Shindo *et al.*, 2000). We are not certain why these significant discrepancies exist between the native urethral K<sub>ATP</sub> channels and the

recombinant K<sub>ATP</sub> channels regarding the effects of MCC-134. However, we suggest the possibility that MCC-134 may bind to both SUR1 (antagonistic action) and SUR2B (agonistic action) in urethral K<sub>ATP</sub> channels, since the peak amplitude of the MCC-134-induced current was much smaller than that of the pinacidil-induced current because of its dual action.

In the present experiments, application of diazoxide enhanced the amplitude of the pinacidil-induced current at  $-50 \text{ mV}$ , demonstrating an additional effect. Similarly, pinacidil caused a further increase in the diazoxide-induced current. These results also suggest the coexistence of SUR1 and SUR2B in pig urethral K<sub>ATP</sub> channels. Yokoshiki *et al.* (1999) have reported that a small population of K<sub>ATP</sub> channels in neonatal rat ventricular myocytes is constructed by a combination of SUR1 and SUR2A in antisense studies, suggesting the existence of heterotetrameric combinations. However, Giblin *et al.* (2002) have recently demonstrated biochemical and electrophysiological evidence that SUR1 and SUR2 do not interact to form heteromultimers in K<sub>ATP</sub> channels. Since the sensitivity of gliclazide in pig urethral K<sub>ATP</sub> channels reflected the reported pharmacological properties of SUR1, we suggest that SUR1 and SUR2B do not combine, but rather form homomultimeric subunits in pig urethral K<sub>ATP</sub> channels. However, since the gliclazide-sensitive component was less than approximately 25% in the diazoxide-induced inward current, we suggest that SUR subunits in pig urethral K<sub>ATP</sub> channels may be composed mainly of SUR2B and that those containing SUR1 play a minor but important role in regulating channel activity of urethral K<sub>ATP</sub> channels.

In the present experiments, MCC-134 activated a 2.1 pA glibenclamide-sensitive K<sup>+</sup> channel (i.e. K<sub>ATP</sub> channel) at  $-50 \text{ mV}$ , demonstrating the size of the channel conductance is 43 pS at negative potentials. The same K<sub>ATP</sub> channels were activated by other types of K<sub>ATP</sub> channel openers (levromakalim, Teramoto & Brading, 1996; nicorandil, Teramoto & Brading, 1997; pinacidil, Teramoto *et al.*, 2000; ZD6169, Teramoto *et al.*, 2001) in pig urethra. The channel conductance of this K<sub>ATP</sub> channel is intermediate between those of Kir6.1 and Kir6.2 (Babenko *et al.*, 1998). In functional expression experiments, Cui *et al.* (2001) have recently reported that heteromultimerization readily occurs between Kir6.1 and Kir6.2, producing functional glibenclamide-sensitive K<sub>ATP</sub> channels. Cui *et al.* (2001) also suggest a possibility that K<sub>ATP</sub> channels in pig urethra may possess a heteromeric Kir6.1/Kir6.2 structure. Further study is certainly necessary in order to elucidate the interaction of Kir subunits in pig urethral K<sub>ATP</sub> channels.

In conclusion, we have been able to demonstrate that SUR1 and SUR2B play a functional role in the activity of K<sub>ATP</sub> channels in pig urethral myocytes using several pharmacological and molecular approaches.

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