Characterization of the ATP-Sensitive Potassium Channels (K\textsubscript{ATP}) Expressed in Guinea Pig Bladder Smooth Muscle Cells

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Abstract

ATP-sensitive K\textsuperscript{+} (K\textsubscript{ATP}) channels play an important role in the regulation of smooth muscle membrane potential. To investigate the properties of K\textsubscript{ATP} channels in guinea pig urinary bladder smooth muscle cells, fluorescence-based assays were carried out with the membrane potential-sensitive probe bis-(1,3-dibutylbarbituric acid)trimethine oxonol [DiBAC\textsubscript{4}(3)]. The prototypical channel openers, including pinacidil, (-)-cromakalim, and diazoxide, elicited concentration-dependent decreases in membrane potential that were attenuated by glyburide. Similar responses were evoked by a reduction in intracellular ATP levels by metabolic inhibition. The observed rank order potency (EC\textsubscript{50}) for evoking membrane potential changes by potassium channel openers, P1075 (53 nM) > Bay X 9228 > (-)-cromakalim > ZD6169 > pinacidil > Bay X 9227 > ZM244085 > diazoxide (59 \mu M), showed a good correlation with that of bladder smooth muscle relaxation, as assessed by isolated tissue bath studies. The maximal efficacies of (-)-cromakalim, pinacidil, Bay X 9228, and ZD6169 were comparable with the response achieved by the reference activator P1075. Whole cell currents in bladder smooth muscle cells were increased in both inward and outward directions by P1075 and were reversed by glyburide to control levels. The molecular composition assessed by reverse transcriptase-polymerase chain reaction analysis using subunit-specific primers revealed the presence of mRNA for inward rectifying potassium channel (KIR6.2) and sulfonylurea receptors (SUR)\textsubscript{2B} and SUR1. The subunit profile together with pharmacological properties suggests that the K\textsubscript{ATP} channel in bladder smooth muscle cells could be composed of SUR2B associated with a single inward rectifier, KIR6.2. In summary, these studies have characterized the pharmacological profile using fluorescent imaging plate reader-based membrane potential techniques and provide evidence for the molecular identity of K\textsubscript{ATP} channels expressed in guinea pig bladder smooth muscle cells.

Potassium channels of the ATP-sensitive (K\textsubscript{ATP}) family are inhibited by increases in intracellular ATP and activated by MgADP, thereby coupling cellular excitability and potassium fluxes to cell metabolism. K\textsubscript{ATP} channels have been described in diverse cell types, including cardiomyocytes, pancreatic \( \beta \) cells, neurons, and smooth and skeletal muscle cells, where they play important physiological and pathophysiological roles (Noma, 1983; reviewed in Ashcroft and Ashcroft, 1990; Isomoto and Kurachi, 1997; Quayle et al., 1997). For example, activation of these channels protects cardiomyocytes from ischemia and relaxes smooth muscles in the vasculature, bladder, digestive tract, and uterus, whereas channel inhibition triggers events such as insulin secretion and transmitter release. These channels are inhibited by sulfonylurea analogs such as glyburide and activated by a structurally heterogeneous class of potassium channel openers (KCOs), including diazoxide, cromakalim, and pinacidil (Edwards and Weston, 1993; Gopalakrishnan et al., 1993).

Pharmacological evidence has suggested a diversity of K\textsubscript{ATP} channels among various tissues based on differing sensitivities to KCOs and sulfonylurea inhibitors, an observation consistent with the results of recent molecular cloning and heterologous expression studies (Inagaki et al., 1995, 1996; Isomoto et al., 1996). The K\textsubscript{ATP} channel expressed in pancreatic \( \beta \) cells is a multimeric complex composed of an inward rectifying potassium channel, KIR6.2, and a sulfonylurea receptor (SUR)\textsubscript{2B} (Inagaki et al., 1995, 1996; Isomoto et al., 1996). The K\textsubscript{ATP} channel expressed in bladder smooth muscle cells could be composed of SUR2B associated with a single inward rectifier, KIR6.2. In summary, these studies have characterized the pharmacological profile using fluorescent imaging plate reader-based membrane potential techniques and provide evidence for the molecular identity of K\textsubscript{ATP} channels expressed in guinea pig bladder smooth muscle cells.

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Abbreviations: KCO, potassium channel opener; K\textsubscript{ATP}, ATP-sensitive K\textsuperscript{+} channel; KIR, inwardly rectifying K\textsuperscript{+} channel; SUR, sulfonylurea receptor; FLIPR, fluorescent imaging plate reader; RT, reverse transcription; PCR, polymerase chain reaction; DiBAC\textsubscript{4}(3), bis-(1,3-dibutylbarbituric acid)trimethine oxonol.
In contrast to the well characterized pancreatic channels, the nature of K\textsubscript{ATP} channels present in other tissues is less well understood. The sulfonylurea receptor SUR2A has a lower affinity for glyburide than does SUR1 and combines with K\textsubscript{IR}6.2 to form channels that are activated by pinacidil but not diazoxide, properties typical of K\textsubscript{ATP} channels in cardiac and skeletal muscle (Inagaki et al., 1996; Okuyama et al., 1998). From expression studies using rat or mouse SUR subunits, it is currently thought that the molecular composition of the cardiac/skeletal muscle channel is SUR2A-K\textsubscript{IR}6.2, whereas SUR2B is thought to be one of the subunits constituting the smooth muscle-type K\textsubscript{ATP} channels as inferred from pharmacological properties and tissue distribution. Studies by Kurachi and coworkers have shown that the pharmacology of the SUR2B subunit coexpressed with K\textsubscript{IR}6.2 and K\textsubscript{MR}6.1 resembles that of smooth muscle K\textsubscript{ATP} channels and smooth muscle nucleotide diphosphate-dependent channels, respectively (Isomoto et al., 1996; Yamada et al., 1997).

In smooth muscles, including those from the urinary bladder, KCOs activate K\textsubscript{ATP} channels, leading to membrane hyperpolarization, reduction in cellular calcium entry, and inhibition of muscle contractility. Electrophysiological studies have demonstrated the presence of K\textsubscript{ATP} channels in guinea pig urinary bladder, the modulation of which may regulate bladder contractility (Bonev and Nelson, 1993). The recent molecular identification of the K\textsubscript{ATP} channels has renewed interest in the development of selective channel openers for the treatment of smooth muscle dysfunction, including detrusor instability. Accordingly, the objective of the present study was to characterize the pharmacology of native K\textsubscript{ATP} channels expressed in cultured guinea pig bladder smooth muscle cells using fluorescence-based assays of membrane potential and to relate these to detrusor relaxation and molecular composition of these channels.

**Experimental Procedures**

**Cell Culture.** Urinary bladders were removed from anesthetized male guinea pigs (Hartley, Charles River, Wilmington, MA) weighing 250 to 300 g and placed in ice-cold Ca\textsuperscript{2+/-}-free Krebs' solution (2.7 mM KCl, 1.5 mM KH\textsubscript{2}PO\textsubscript{4}, 75 mM NaCl, 9.6 mM NaH\textsubscript{2}PO\textsubscript{4}, 8 mM Na\textsubscript{2}HPO\textsubscript{4}, 7H\textsubscript{2}O, 2 mM MgSO\textsubscript{4}, 5 mM glucose, 10 mM HEPES, pH 7.4). Cells were isolated by enzymatic dissociation as previously described (Klockner and Isenberg, 1985) with some modifications. The bladder was cut into small sections and incubated in 5 ml of the Krebs' solution containing 1 mg/ml collagenase (type VIII; Sigma Chemical Co., St. Louis, MO) and 0.2 mg/ml pronase (Calbiochem, La Jolla, CA) with continuous stirring at 37°C in a cell incubator for 30 min. The mixture was then centrifuged at 1300 g for 5 min, and the pellet resuspended in Dulbecco's PBS (Life Technologies, Gaithersburg, MD) and recentrifuged to remove residual enzyme. The cell pellet was resuspended in 5 ml of growth media (Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 units/ml penicillin, 100 units/ml streptomycin, and 0.25 mg/ml amphotericin B) and further dissociated by pipetting it repeatedly through a flame-polished Pasteur pipette and passing it through a polypropylene mesh membrane (Spectrum, Houston, TX). The cell density was adjusted to 100,000 cells/ml by resuspension in growth media and plated onto clear-bottomed black 96-well plates (Packard ViewPlate-96) for fluorescence studies at a density of 20,000 cells/well. Cells were maintained in a cell incubator with 90% air/10% CO\textsubscript{2} for 5 to 7 days.

**Cell Characterization.** Cells were labeled with smooth muscle-specific a-actin to determine the population of muscle-type cells in the preparation. For cytoskeletal staining, cells were removed from the culture flask after 5 days in culture and plated onto dual-chambered glass slides for 48 h in growth media. Cells were washed twice with Dulbecco's PBS, fixed in 70% methanol for 30 min, and blocked with 0.5% casein and 10% rabbit serum (Sigma Chemical Co.). The primary antibody was a monoclonal mouse anti-human a-smooth muscle actin raised against the amino-terminal region (Biomeda, Foster City, CA), and the secondary antibody was biotinylated rabbit anti-mouse. Horseradish peroxidase conjugate was used to detect the bound secondary antibody, and staining was enhanced using 5% copper sulfate. Positive staining for a-actin was observed in 60 to 70% of the cells cultured for 5 to 7 days, during which the cells were used for various studies. Although we have used anti-human a-smooth muscle actin, the positive staining data indicates cross-reactivity with the guinea pig protein. The remaining population of cells may include other cell types, such as those of urothelial origin. No specific staining was observed when the primary antibody was not included or when staining was performed under similar conditions using fibroblast lines such as COS-7.

**RNA Preparation and Reverse Transcription-Polymerase Chain Reaction Analysis (RT-PCR).** For RNA isolation, guinea pig bladder cells grown in T162-cm\textsuperscript{2} flasks (1 x 10\textsuperscript{7} cells) were rinsed twice with Dulbecco's PBS, gently trypsinized (0.25% trypsin, 1 mM EDTA; Life Technologies), pelleted by centrifugation, and stored at -80°C until used. Total RNA from guinea pig bladder smooth muscle cells was isolated using TRIzol reagent according to the manufacturer's instructions (Life Technologies). First-strand synthesis of cDNA using random hexamers was performed as follows: An 1- to 2-μg aliquot of DNase I-treated total RNA isolated from cells or 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\textsubscript{2}, 1 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 at 70°C for 15 min, before chilling on ice. PCR was performed using 2 to 4 μl of cDNA in 50-μl reaction containing 0.4 μM concentration of each primer, 200 mM concentration of each deoxynucleoside-5'-triphosphate, and 2.5 units of Taq polymerase (Perkin Elmer, Norwalk, CT). The cycling conditions were 95°C for 24 s, 55°C for 22 s, and 72°C for 78 s for 40 cycles. An aliquot (30 μl) of the RT-PCR product was analyzed on a 10% Tris-borate-EDTA polyacrylamide gel.

Because no sequence information is available about K\textsubscript{ATP} channel subunits in the guinea pig, generic subunit-specific primers were designed based on information from rat, mouse, and human sequences (Table 1). The locations of the primers indicated are based on the subunit sequence information obtained from GenBank: K\textsubscript{MR}6.2 (rat, U73626; mouse, U73626; human, D50582); SUR1 (rat, L40624; human, L78207); and SUR2 (rat 2A, D83598; mouse 2A, D86097; mouse 2B, D86038; human 2A, AF061323; human 2B, AF061324). 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 using corresponding templates (SUR1 and K\textsubscript{IR}6.2) 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.

**Membrane Potential Assay.** Functional activity of K\textsubscript{ATP} channels was measured by evaluating changes in membrane potential using the bissoxonol dye bis-(1,3-dibutylbarbituric acid)trimethine oxonol [DiBAC\textsubscript{4}(3); Molecular Probes, Eugene, OR] in a 96-well cell-based kinetic assay system, fluorescent imaging plate reader (FLIPR) (Schroeder and Neagle, 1996; Molecular Devices, Menlo Park, CA). Confluent cells cultured (typically 5–7 days) in black clear-bottomed 96-well plates were rinsed twice with 200 μl of assay buffer (20 mM HEPES, 120 mM NaCl, 2 mM KCl, 2 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 5 mM glucose, pH 7.4, at 23°C) containing 5 μM DiBAC\textsubscript{4}(3)
and incubated with 180 μl of buffer in a cell incubator for 30 min to ensure dye distribution across the cell membrane. Assays were carried out at 37°C and were initiated by the addition of 20 μl of a 10X concentration of the test compound prepared in the assay buffer containing DiBAC4(3). Changes in fluorescence were monitored for 25 min by sampling every 30 s from 96 wells simultaneously at excitation and emission wavelengths of 488 and 520 nm, respectively. Responses were corrected for any background changes in fluorescence, and data were normalized to the response observed by adding 0.4 ml of buffer containing varying concentrations of 

$$^{3}H$$P1075. Unlabeled P1075 (10 μM) was used to define nonspecific binding. Radioligand Binding.

**Table 1**

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Primer Sequence</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K&lt;sub&gt;ir&lt;/sub&gt;6.2</td>
<td>F: 5'-GGCTCCTAGTGCACCTGCACCA-3' (810-830 bp)</td>
<td>316</td>
</tr>
<tr>
<td>SURI</td>
<td>R: 5'-CCAGACACGCAAATCACCTGATTTG-3' (1126-1105 bp)</td>
<td>355</td>
</tr>
<tr>
<td>SUR2A/B</td>
<td>F: 5'-GCTGAGAATAGTGGTAATACTCT-3' (4278-4300 bp)</td>
<td>2A: 451</td>
</tr>
<tr>
<td></td>
<td>R: 5'-CGGATGCGTATTTCAAAAAAAT-3' (4590-4569 bp)</td>
<td>2B: 312</td>
</tr>
</tbody>
</table>

Oligonucleotide positions are indicated considering the ATG codon at position 1. F and R, forward (upstream) and reverse (downstream) primers, respectively.

**Materials.** Diazoxide, pinacidil, glyburide, tolbutamide, glibizide, 2-deoxy-D-glucose, and oligomycin were purchased from RBI (Natick, MA) or Sigma Chemical Co. (St. Louis, MO). (−)-Cromakalim, P1075, ZD-6169 (S)-N-(4-benzoylphenyl)-3,3,3-trifluoro-2-hydroxy-2-methyl-propionamide, ZM244085 (9-(3-cyanophenyl)hexahydro-1,8 acridinedione), Bay X 9228 ((+)-N-(2-ethoxyphenyl)-N'-(2,3,3-trimethylpropyl)-2-nitroethene-1, 1-diamine), and Bay X 9227 ((−)-N'-(2-ethoxyphenyl)-N'-(1,2,3-trimethylpropyl)-2-nitroethene-1, 1-diamine) were synthesized at Abbott Laboratories (Abbott Park, IL). (−)-Cromakalim was obtained from Dr. D. J. Trigg (Buffalo, NY). Stock solutions of compounds were prepared in 100% dimethyl sulfoxide and diluted in buffer before use. DiBAC4(3) was purchased from Molecular Probes. Black 96-well plates with clear bottoms were purchased from Packard Instruments (Meriden, CT). All cell culture products were purchased from Life Technologies (Gaithersburg, MD). The monoclonal mouse anti-human α-smooth muscle actin was purchased from Biomek (Foster City, CA). Biotinylated rabbit antibody to mouse, horseradish peroxidase conjugate, and staining reagents were from Dako (Carpinteria, CA). The RNA isolation and SuperScript II cDNA synthesis kits were purchased from Life Technologies. Rat and human poly(AD) RNA were obtained from Clontech (Palo Alto, CA). Tq polymerase was from Perkin Elmer (Norwalk, CT). The 10% Tris-borate-EDTA polyacrylamide gels were purchased from Bio-Rad (Hercules, CA).

**Data Analysis.** The maximal steady-state levels in fluorescence were normalized to the response evoked by a reference potassium channel activator, P1075 (10 μM). Sigmoid curves were fitted to concentration-response data by nonlinear regression analysis (Prism; GraphPad, San Diego, CA) to obtain EC<sub>50</sub> (or IC<sub>50</sub>) values as appropriate. Wherever plateau responses were not attained, the EC<sub>50</sub> values were estimated. Data are expressed as mean ± S.E.M.

**Results**

**RNA Analysis.** RT-PCR analysis of total cellular RNA isolated from urinary bladder cells grown in culture for 5 days through minor modifications of protocols previously described (Hoffman et al., 1993; Quast et al., 1993). Briefly, culture medium was aspirated from the wells, and the cells were washed once with assay buffer (20 mM HEPES, 120 mM NaCl, 2 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5 mM glucose, pH 7.4 at 25°C). Binding was initiated by adding 0.4 ml of buffer containing varying concentrations of [H]P1075. Unlabeled P1075 (10 μM) was used to define nonspecific binding. Reactions were terminated after 90 min of incubation in a cell incubator with 10% CO<sub>2</sub> by aspirating the supernatant. Free [H]P1075 was removed by washing the cell monolayer three times with ice-cold assay buffer. The cells were harvested with 0.4 ml of 0.1 N NaOH, neutralized with 0.1 N HCl, and then transferred to scintillation vials, and radioactivity was assessed by liquid scintillation counting.

**Isolated Tissue Relaxation.** Urinary bladders were removed and immediately placed in Krebs-Ringer bicarbonate solution (120 mM NaCl, 20 mM NaHCO<sub>3</sub>, 11 mM dextrose, 4.7 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.5 mM MgSO<sub>4</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 0.01 mM K<sub>2</sub>EDTA, equilibrated with 5% CO<sub>2</sub>–95% O<sub>2</sub> at pH 7.4 at 37°C). Propanolol (4 μM) was included to block β-adrenoceptors. The trigonal and dome portions were discarded, and strips 3 to 5 mm wide and 10 mm long were prepared from the remaining tissue by cutting in a circular fashion. One end of the strip was fixed to a stationary glass rod, and the other was attached to a Grass FT03 transducer at a basal preload of 1.0g. This preload proved to be the best condition for a steady-state baseline and reproducible responses to field stimulation. Two parallel platinum electrodes were included in the stationary rod to provide field stimulation (parameters: 0.05 Hz, 0.5 mA at 20 V). Tissues were allowed to equilibrate for at least 60 min before the assay. Cumulative concentration-response curves were generated for each tissue, and each tissue was exposed to only one test compound. For evaluation of sulfonylurea sensitivity, tissues were exposed to glyburide for 30 min before generation of the activator dose-response curve. Individual tissues were exposed to only one concentration of the test antagonist.

| Primer positions and expected product sizes of K<sub>ATP</sub> channel subunits |
|----------------------|------------------|---|
| Subunit | Primer Sequence | Size (bp) |
| K<sub>ir</sub>6.2 | F: 5'-GGCTCCTAGTGCACCTGCACCA-3' (810-830 bp) | 316 |
| | R: 5'-CCAGACACGCAAATCACCTGATTTG-3' (1126-1105 bp) | 355 |
| SURI | F: 5'-GCTGAGAATAGTGGTAATACTCT-3' (4278-4300 bp) | 2A: 451 |
| | R: 5'-CGGATGCGTATTTCAAAAAAAT-3' (4590-4569 bp) | 2B: 312 |

Electrophysiology. Whole-cell currents from guinea pig bladder smooth muscle cells were measured using the conventional tight-seal whole-cell clamp method (Hamill et al., 1981). Measurements were carried out within 48 h after cell dissociation, during which the cells appear elongated and contractile. The intracellular pipette solution contained 107 mM KCl, 1.2 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 10 mM EGTA, 5 mM HEPES, and 0.1 mM ATP (pH 7.2 with KOH; total K<sup>+ </sup> ~ 140 mM). The bath solution contained 40 mM KCl, 100 mM NaCl, 2.6 mM CaCl<sub>2</sub>, 1.2 mM MgCl<sub>2</sub>, and 5 mM HEPES (pH 7.4 with NaOH). The microelectrodes had a resistance of 2 to 5 MΩ. After a tight seal was formed, the membrane was ruptured, and the capacitance transient was integrated online to estimate cell capacitance. Uncompensated series resistance was typically 3 to 10 MΩ. The whole-cell currents were amplified using Axopatch-200B amplifier (Axon Instruments, Foster City, CA) and low pass filtered at 5 kHz (3 dB, four-pole Bessel filter) before digitization by Digidata 1200B at a sampling rate of 10 kHz. Data were analyzed using pClamp 6.0 (Axon Instruments).

**Radioisotopes.** [H]P1075 binding was performed on intact bladder smooth muscle cells grown on 12-well dishes for 5 to 6 days through minor modifications of protocols previously described (Hoffman et al., 1993; Quast et al., 1993). Briefly, culture medium was aspirated from the wells, and the cells were washed once with assay buffer (20 mM HEPES, 120 mM NaCl, 2 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5 mM glucose, pH 7.4 at 25°C). Binding was initiated by adding 0.4 ml of buffer containing varying concentrations of [H]P1075. Unlabeled P1075 (10 μM) was used to define nonspecific binding. Reactions were terminated after 90 min of incubation in a cell incubator with 10% CO<sub>2</sub> by aspirating the supernatant. Free [H]P1075 was removed by washing the cell monolayer three times with ice-cold assay buffer. The cells were harvested with 0.4 ml of 0.1 N NaOH, neutralized with 0.1 N HCl, and then transferred to scintillation vials, and radioactivity was assessed by liquid scintillation counting.

**RT-PCR analysis of total cellular RNA isolated from urinary bladder cells grown in culture for 5 days**
days showed the expected fragment sizes for K\text{IR}6.2 (316 bp), K\text{UR}1 (355 bp), and K\text{UR}2B (312 bp) (Fig. 1). Each of these products was confirmed by DNA sequence analysis. Notably, the primers that were designed to identify K\text{UR}2B would also detect the presence of K\text{UR}2A message based on the genomic structure of the K\text{UR}2 gene. If both K\text{UR}2A and K\text{UR}2B were expressed, two products of 451 and 312 bp, respectively, would have been amplified. However, in this case, no 451-bp product corresponding to K\text{UR}2A was detected, indicating that the K\text{UR}2B variant alone is expressed in guinea pig bladder cells. In addition, negative controls lacking the reverse transcriptase during the cDNA synthesis step did not yield products.

Membrane Potential Studies. Functional activities of $K_{\text{ATP}}$ channels were measured by evaluating changes in fluorescence responses using DiBAC$_4$(3), an anionic potentiometric probe that partitions between cells and extracellular solution in a membrane potential-dependent manner (Epps et al., 1994). With increasing membrane potential (e.g., depolarization by 50 mM K$^+$), the probe further partitions into the cell, resulting in an increase in fluorescence due to dye interaction with intracellular lipids and proteins, whereas hyperpolarization evokes a decrease in fluorescence.

Effects of Metabolic Inhibition. A notable feature of $K_{\text{ATP}}$ channels is their sensitivity to intracellular levels of ATP (Noma, 1983). To decrease the cellular ATP content, bladder smooth muscle cells were treated with metabolic inhibitors 2-deoxy-D-glucose (10 mM) and oligomycin (1 $\mu$g/ml), agents that have previously been shown to reduce intracellular ATP levels by $\geq$10-fold (Schmid-Antomarchi et al., 1987; Bonev and Nelson, 1993). The addition of metabolic inhibitors resulted in a significant decrease in the fluorescence response that was attenuated by the addition of 5 $\mu$M glyburide (Fig. 2).

Effects of KCOs. The addition of $K_{\text{ATP}}$ channel openers, including pinacidil analog P1075, ($-$)-cromakalim, and diazoxide, elicited concentration-dependent decreases in fluorescence responses (Fig. 3). The addition of glyburide (5 $\mu$M) or barium (3 mM; data not shown) attenuated the responses evoked by P1075. To further verify the nature of fluorescence changes, experiments were carried out by depolarizing the cells by increasing extracellular K$^+$ concentration. As shown in Fig. 4, the P1075 (10 $\mu$M)-evoked responses were clearly dependent on the membrane potential with fluorescence responses diminishing at higher K$^+$ (>30 mM) concentrations.

To further characterize the pharmacological properties of $K_{\text{ATP}}$ channels, other known KCOs were evaluated for their effects on membrane potential (Fig. 5). Concentration-dependent decreases in responses were observed with the 1,4-dihydropyridine ZM244085, the tertiary carbinol ZD6169 and the nitroethene analog Bay X 9228. The maximal responses of ($-$)-cromakalim (101%), pinacidil (81%), Bay X 9228 (87%), and ZD6169 (113%) were comparable with the response of 10 $\mu$M P1075 (100%). The responses showed stereoselectivity with the nitroethene analog Bay X 9228 and the benzopyran ($-$)-cromakalim ~30- to 50-fold more potent than their corresponding enantiomers (Fig. 5B). The rank order potencies (EC$_{50}$) for the openers were P1075 (53 nM) ~ Bay X 9228 > ($-$)-cromakalim ~ ZD6169 ~ pinacidil > Bay X 9227 ~ ZM244085 > ($+$)-cromakalim = diazoxide (59 $\mu$M).

Sensitivity to Inhibitors. The sulfonyleurea analogs glyburide and glipizide inhibited fluorescence changes evoked by the potassium channel activator P1075. Glyburide inhibited responses evoked by P1075 (3 $\mu$M) with an IC$_{50}$ value of 0.90 ± 0.28 $\mu$M (n = 4). Glyburide alone had no significant effect on baseline fluorescence (data not shown). As shown in

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**Fig. 1.** RT-PCR analysis of $K_{\text{ATP}}$ channel subunits in guinea pig bladder smooth muscle cells. Total RNA were isolated from guinea pig bladder smooth muscle cells and reactions performed as described in Experimental Procedures. Shown is a representative gel in which lanes 1, 2, and 3 represent reaction products corresponding to $K_{\text{IR}}6.2$ (316 bp), $K_{\text{UR}}1$ (355 bp), and $K_{\text{UR}}2B$ (312 bp), respectively.

**Fig. 2.** Activation of $K_{\text{ATP}}$ channels in guinea pig bladder smooth muscle cells evoked by reduction of intracellular ATP levels. Changes in DiBAC$_4$(3) fluorescence responses were measured as described in Experimental Procedures after the application of 2-deoxy-D-glucose (10 mM) and oligomycin (1 $\mu$g/ml). Also shown is the attenuation of responses by the addition of glyburide (5 $\mu$M).

**Fig. 3.** Changes in fluorescence responses in guinea pig bladder smooth muscle cells in response to prototypical $K_{\text{ATP}}$ channel openers ($-$)-cromakalim, pinacidil analog, P1075, and diazoxide. Shown are changes in fluorescence responses to varying concentrations of (A) ($-$)-cromakalim (0.03, 0.3, 1.0, 3.0, and 30 $\mu$M), (B) P1075 (0.001, 0.01, 0.03, 0.1, 1.0, and 10 $\mu$M), and (C) diazoxide (1, 3, 10, 30, and 100 $\mu$M). The y-axis represents changes in arbitrary fluorescent units. Fluorescence sampling was done every 30 s. Representative traces are shown and are mean values from duplicate wells. Compounds were added after a baseline period of 5 min using a 96-well pipettor. After 25 min of data collection, glyburide (5 $\mu$M) was added.
Fig. 6. Inhibition of fluorescence responses evoked by K<sub>ATP</sub> channel activation by sulfonylurea analogs in guinea pig bladder smooth muscle cells. Shown are the concentration dependencies of inhibition by glyburide and glipizide in the presence of P1075 (3 μM). Values are normalized and expressed as a percentage of the response evoked by P1075.

**Radioligand Binding.** Additional support for the presence of K<sub>ATP</sub> channels in guinea pig bladder smooth muscle cells is derived from radioligand binding experiments using [3H]P1075. Recent studies have shown that this radioligand binds to the SUR2B subunit of the K<sub>ATP</sub> channel in the presence of ATP (Schwanstecher et al., 1998). Consistent with the presence of SUR2B mRNA and KCO-evoked membrane potential responses, specific and saturable binding of [3H]P1075 was observed in intact cells within the radioligand concentration range examined. Scatchard analysis of the data yielded a B<sub>max</sub> value of 48 ± 14 fmol/mg protein and a K<sub>D</sub> value of 6.4 ± 0.9 nM (n = 4; Fig. 7). This affinity of [3H]P1075 is comparable with those values reported previously in intact cells or in cells transfected with the SUR2B subunit (Quast et al., 1993; Schwanstecher et al., 1998).

**Whole-Cell Patch-Clamp Measurements.** Direct evidence for the presence of K<sub>ATP</sub> channels in guinea pig bladder smooth muscle cells was derived from whole-cell patch-clamp recording studies. Cells were held at a holding potential of −80 mV, and recordings were made in the presence of extracellular and intracellular K<sup>+</sup> concentrations of 140 and 60 mM, respectively. In agreement with previous results (Bonev and Nelson, 1993), the application of P1075 (10 μM) increased whole-cell inward currents, which were significantly inhibited by the addition of 5 μM glyburide (Fig. 8). The currents reversed at −20 mV, which agrees with equilibrium potential for K<sup>+</sup> ions (data not shown).

**Tissue Relaxation.** Several KCOs were evaluated for their effects on field-stimulated contractions of guinea pig detrusor. Low-frequency stimulation (0.1 Hz, 0.5 ms at 20 V) elicited a stable contractile response in detrusor strips with a...
tension response of 155 ± 9.9 cg (n = 52). (-)-Cromakalim, P1075, pinacidil, diazoxide, ZM244085, ZD6169, and Bay X 9228 all suppressed field-stimulated twitch responses in a concentration-dependent fashion. The EC50 values together with the efficacies of these compounds are summarized in Table 2. P1075 and Bay X 9228 were the most potent of the compounds examined with EC50 values of 1.2 × 10^-7 and 1.7 × 10^-7 M, respectively. Bay X 9227 was ~30-fold less potent and only ~40% as efficacious compared with its enantiomer Bay X 9228. Consistent with previous observations (Winquist et al., 1989), glyburide inhibited the relaxant effects of the potassium channel activator pinacidil in an apparently competitive manner with a pA2 value of 6.94 ± 0.39 (slope = 0.99 ± 0.11; r = 0.88; n = 8). The rank order potency (EC50) values of KCOs observed for the relaxation of low-frequency stimulated guinea pig detrusor strips, P1075 – Bay X 9228 > (-)-cromakalim ~ ZD6169 ~ pinacidil > (+)-cromakalim ≥ diazoxide, showed an excellent correlation (r = 0.96) with the potencies to evoke membrane potential changes (Fig. 9).

**Discussion**

Different types of K+ channels (voltage gated, calcium-activated, and ATP sensitive) have been shown to modulate spontaneous electrical and mechanical activity in the detrusor smooth muscle (Brading et al., 1996). Although previous electrophysiological studies have demonstrated the presence of KATP channels in the detrusor (Bonev and Nelson, 1993), the pharmacological properties and the molecular composition of these channels have not been examined in detail. In this study, we characterized the pharmacology of KATP channels expressed in cultured guinea pig urinary bladder cells by fluorescence-based membrane potential techniques. It was found that KATP channels could be activated by metabolic inhibition or by diverse KCOs with a rank order potency consistent with those typical of smooth muscle KATP channels. Furthermore, the subunit profile together with the observed pharmacological properties suggest that the KATP channels expressed in guinea pig bladder cells are likely to be composed of SUR2B coassociated with a single inward rectifier, Kir6.2.

**FLIPR-Based Membrane Potential Assessment of KATP Channels.** Previous studies have examined KATP channels in bladder smooth muscle, by tissue relaxation subsequent to mild contractile stimulus, by assessing sulfonylurea-sensitive channel conductance after metabolic inhibition.
or after potassium channel activation by KCoF or by the internal perfusion of ATP-free solutions and by assessing membrane potential changes by microelectrode techniques or by using potentiometric dyes. The latter technique, more recently developed in a 96-well microplate format (FLIPR) using the bisoxonol dye DiBAC4(3), enables studies on KATP channels in a rapid and high-throughput manner (Schoeder and Neagle, 1996). Epps et al. (1994) studied the interaction of this bisoxonol dye with A10 smooth muscle cells and have shown a significant increase in the fluorescence intensity in response to the addition of KCl, which increases the membrane potential and, furthermore, a linear relationship between the change in fluorescence and change in the cell membrane potential.

The results of the present study confirm and extend previous observations that KATP channels are present in bladder smooth muscle cells and can be activated either by reduction in intracellular ATP levels subsequent to blockade of glycolysis or by KCoF (Quayle et al., 1997). Metabolic inhibition or KCoF-evoked membrane potential responses were sensitive to sulfonylurea inhibitors as well as to the potassium channel blocker barium. KCoF-mediated membrane potential changes showed a clear dependence on extracellular K+ concentrations; moreover, the potencies of KCoF in the membrane potential assay showed a good correlation with the EC50 values observed for relaxation of detrusor strips, indicating that the measured changes in membrane potential in cultured cells closely relate to tissue relaxation evoked by these compounds. Evidence for the presence of functional KATP channels in bladder smooth muscle cells was also derived from whole-cell patch-clamp studies showing that P1075 increased whole-cell membrane currents in both inward and outward directions that were inhibited by glyburide. Moreover, intact cell binding with [3H]P1075 revealed a KI value similar to those previously reported in smooth muscle (Quast et al., 1993) and consistent with recent studies by Schwanstecher et al. (1998) in which activator binding was detected in COS cells transiently expressing the hSUR2B subunit. Collectively, our studies demonstrate that the use of guinea pig bladder smooth muscle cells in culture in FLIPR-based membrane potential assays serves as a valuable and high-throughput model system for studying the pharmacology of KATP channels.

**Pharmacologic Characteristics of KATP Channels.** The rank order potencies for activation observed in membrane potential studies are consistent with properties typical of smooth muscle channels that are activated by cromakalim, pinacidil, and diazoxide. For example, (-)-cromakalim has been shown to activate KATP currents, to hyperpolarize, and to relax a wide variety of smooth muscles at concentrations ranging from 1 to 10 μM (Quast and Cook, 1989; Quayle et al., 1995). The potency of the nitroethene Bay X 9228 to evoke membrane potential responses and relaxation of bladder compares well with those reported previously by Hoffman et al. (1993) to relax vascular strips. Bay X 9227, on the other hand, was ≥30-fold weaker and less efficacious in both membrane potential and relaxation measurements, although a previous study with this compound has claimed glyburide-insensitive activity at much lower concentrations (Hunicutt et al., 1994). The 1,4-dihydropyridine ZM244085 has previously been shown, by microelectrode techniques, to activate KATP channels (Li et al., 1996). In our study, this compound evoked glyburide-sensitive responses with potencies similar to those reported for the relaxation of low K+-stimulated guinea pig detrusor (4 μM) by Li et al. (1996). ZD6169 is a tertiary carbimil with reported in vivo bladder selectivity identified as a development compound for the treatment of urge incontinence (Howe et al., 1995). The potency value for activation of membrane potential responses by this compound is comparable with the concentrations required to relax 15 mM K+-stimulated guinea pig detrusor strips (EC50 = 1.6 μM) and to increase whole-cell glyburide-sensitive K+ currents in detrusor smooth muscle cells (Grant et al., 1994).

**Molecular Analysis of KATP Channels.** RT-PCR studies demonstrated the presence of the inward rectifier KIR6.2 as well as the sulfonylurea receptors SUR1 and SUR2B in cultured bladder smooth muscle cells. Although these studies have addressed only the expression of mRNA transcripts, it is tempting to speculate that KATP channels in the bladder may be composed of SUR2B-containing subunits on the basis of the following observations. Although diazoxide is known to activate both SUR1- and SUR2B-containing channels with similar potencies (50–200 μM; Ashcroft and Ashcroft, 1990), the potency of glyburide to inhibit activator-evoked responses in bladder smooth muscle cells is ~1000-fold lower than its potency for inhibition of KATP channels composed of SUR1-KIR6.2 subunits (IC50 = 1–10 nM; Inagaki et al., 1995, 1996). This supports the contention that the predominant combination that mediates activator interactions in the bladder smooth muscle cells could be composed of SUR2B and KIR6.2. Although SUR1 message was detected by PCR analysis, [3H]glyburide binding to guinea pig bladder membranes failed to reveal any high-affinity saturable binding sites within the concentration range examined (0.01–6.0 nM), although under similar conditions, high-affinity [3H]glyburide binding (Kd ~ 0.1 nM) was detected in RINm5F and brain membranes (data not shown) consistent with previous reports (Schmid-Antomarchi et al., 1987; Gopalakrishnan et al., 1991). This suggests that either SUR1 protein may not be expressed at significant levels or if it is expressed at all, it is barely detectable by the current methods of analyses. A necessary confirmation of the molecular identity of subunits constituting functional KATP channels could arise from the analysis of protein levels by the use of antibodies or by antisense studies. It should be noted, however, that in the present study, the cell population used in RT-PCR studies is slightly different from those used in membrane potential measurements in terms of their growth conditions (i.e., T162 cm² flasks versus 96-well plates, respectively). Cells sustained under these different culture environments would not be expected to exhibit a similar differentiation pattern, which may alter cell composition (smooth muscle versus non-smooth muscle cells) and expression of mRNA and/or surface protein.

Recent studies have shown that multiple channel types with defined physiological and pharmacological properties can be constituted by the heteromeric assembly of various KIR and SUR subunit combinations (Isomoto and Kurachi, 1997). Studies by Yamada et al. (1997) have shown that the SUR2B-KIR6.1 may underlie a low conductance KATP channel previously described in certain vascular smooth muscle cells based on conductance similarities, activation by pinacidil and nicorandil and by UDP, and the lack of spontaneous channel activation (Zhang and Bolton, 1996). Although our studies suggest that KATP channels composed of SUR2B-