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

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ABSTRACT
ATP-sensitive K$^+$ ($K_{\text{ATP}}$) channels play an important role in the regulation of smooth muscle membrane potential. To investigate the properties of $K_{\text{ATP}}$ 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$_4$(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$_{50}$) 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)2B and SUR1. The subunit profile together with pharmacological properties suggests that the $K_{\text{ATP}}$ 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 $K_{\text{ATP}}$ channels expressed in guinea pig bladder smooth muscle cells.

Potassium channels of the ATP-sensitive ($K_{\text{ATP}}$) family are inhibited by increases in intracellular ATP and activated by MgADP, thereby coupling cellular excitability and potassium fluxes to cell metabolism. $K_{\text{ATP}}$ 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 $K_{\text{ATP}}$ 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 $K_{\text{ATP}}$ channel expressed in pancreatic $\beta$ cells is a multimeric complex composed of an inward rectifying potassium channel, Kir6.2, and a sulfonylurea receptor (SUR) SUR1, with a stoichiometry of (SUR/Kir6.2)x4 (Clement et al., 1997; reviewed in Bryan and Aguilar-Bryan, 1997; Lorenz et al., 1998). Recent studies suggest that the site at which ATP interacts to mediate channel inhibition may reside within the Kir subunit, whereas the stimulatory effects of MgADP and KCOs may involve interactions with the SUR subunit (Gribble et al., 1997; Shyng et al., 1997; Tucker et al., 1997; Schwanstecher et al., 1998).

ABBREVIATIONS: KCO, potassium channel opener; $K_{\text{ATP}}$, ATP-sensitive K$^+$ channel; Kir, inwardly rectifying K$^+$ channel; SUR, sulfonylurea receptor; FLIPR, fluorescent imaging plate reader; RT, reverse transcription; PCR, polymerase chain reaction; DiBAC$_4$(3), bis-(1,3-dibutylbarbituric acid)trimethine oxonol.
In contrast to the well characterized pancreatic channels, the nature of K
\(_{\text{ATP}}\) channels present in other tissues is less well understood. The sulfonlurea receptor SUR2A has a lower affinity for glyburide than does SUR1 and combines with K\(_{\text{IR6.2}}\) to form channels that are activated by pinacidil but not diazoxide, properties typical of K\(_{\text{ATP}}\) 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 SUR subunits, it is currently thought that the molecular composition of the cardiac/skeletal muscle channel is SUR2A-K\(_{\text{IR6.2}}\), whereas SUR2B is thought to be one of the subunits constituting the smooth muscle-type K\(_{\text{ATP}}\) 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\(_{\text{IR6.2}}\) and K\(_{\text{IR6.1}}\) resembles that of smooth muscle K\(_{\text{ATP}}\) 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\(_{\text{ATP}}\) channels, leading to membrane hyperpolarization, reduction in cellular calcium entry, and inhibition of muscle contractility. Electrophysiological studies have demonstrated the presence of K\(_{\text{ATP}}\) 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\(_{\text{ATP}}\) 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\(_{\text{ATP}}\) 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\(^{2+}\)-free Krebs' solution (2.7 mM KCl, 1.5 mM KH\(_2\)PO\(_4\), 75 mM NaCl, 9.6 mM Na\(_2\)HPO\(_4\), 8 mM Na\(_2\)HPO\(_4\), 7H\(_2\)O, 2 mM MgSO\(_4\), 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 1300g 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 polypyrrole 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 95% air/10% CO\(_2\) 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 (Biomek, 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 indicate a 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\(^2\) flasks (1 \times 10\(^7\) 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 prepared 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- HC1, pH 8.4, 50 mM KC1), 2.5 mM MgCl\(_2\), 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 mM 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 22 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\(_{\text{ATP}}\) 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\(_{\text{IR6.2}}\) (rat, U73626; mouse, U73626; human, D50582), SUR1 (rat, L40624; human, L78207), and SUR2 (rat 2A, D83598; mouse 2A, D86037; human 2B, AF06138). 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\(_{\text{IR6.2}}\)) 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\(_{\text{ATP}}\) channels was measured by evaluating changes in membrane potential using the bishoxonol dye bis-(1,3-dibutylbarbituric acid/trimetidine oxonol [DiBAC\(_{4}(3)\)] (Molecular Probes, Eugene, OR) in a 96-well cell-based kinetic assay system, fluorescent imaging plate reader (FLIPR) (Schoeder and Neagle, 1996; Molecular Devices, Menlo Park, CA). Confuent 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\(_2\), 1 mM MgCl\(_2\), 5 mM glucose, pH 7.4, at 23°C) containing 5 μM DiBAC\(_{4}(3)\)
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 10× concentration of the test compound prepared in the assay buffer containing DiBAC4(3). Changes in fluorescence were monitored for 25 min by scanning 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 with 10 μM P1075 (N-cyano-N'-1, 1-dimethylpropyl)-N'-3-pyridylguanidine) P1075 (fluorescence units arbitrarily assigned as 100%), a potent activator of smooth muscle KATP channels (Quast et al., 1993). Generally, a second addition of 5 μM gliburide was made at the end of 25-min period to examine effects on activator-evoked responses. In studies in which sensitivities to sulfonylureas were evaluated, both openers and inhibitors were added simultaneously. In experiments assessing the effect of metabolic inhibitors, cells were incubated with assay buffer lacking glucose but containing 10 mM NaNO3, 20 mM NaHCO3, 11 mM dextrose, 4.7 mM KCl, 2.5 mM CaCl2, 1.5 mM MgSO4, 1.2 mM KH2PO4, 0.01 mM K3EDTA, equilibrated with 5% CO2/95% O2, pH 7.4 at 37°C. Propranolol (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, 0.5mA 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 gliburide for 30 min before generation of the activator dose-response curve. Individual tissues were exposed to only one concentration of the test antagonist.

**Materials.** Diazoxide, pinacidil, gliburide, glipizide, 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)hexahydropyrano-1,8 acridinedione), Bay X 9228 ((−)-N-(2-ethoxyphenyl)-N’-(1,2,3-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. Triggle (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 Biomedia ( Foster City, CA). Biotinylated rabbit antinumouse, 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(A)+ RNAs 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 EC50 (or IC50) values as appropriate. Wherever plateau responses were not attained, the EC50 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

**TABLE 1**

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Primer Sequence</th>
<th>Size (bp)</th>
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<tbody>
<tr>
<td>Kir6.2</td>
<td>F:5′-GGCTCTAGTACGTGCACCA-3′ (810–830 bp)</td>
<td>316</td>
</tr>
<tr>
<td></td>
<td>R:5′-CCAGACCCACACTGGGCTGGC-3′ (1126–1105 bp)</td>
<td>355</td>
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<tr>
<td>SUR1</td>
<td>F:5′-TACCTGACTTGTGGGTACAC-3′ (2358–2378 bp)</td>
<td>355</td>
</tr>
<tr>
<td></td>
<td>R:5′-TACCTGACTTGTGGGTACAC-3′ (2713–2698 bp)</td>
<td>355</td>
</tr>
<tr>
<td>SUR2A/B</td>
<td>F:5′-GCTAGAAGATATGGCTAACAC-3′ (4278–4300 bp)</td>
<td>2A: 451</td>
</tr>
<tr>
<td></td>
<td>R:5′-CGGATGCTGCTATTCGAAAATAA-3′ (4590–4569 bp)</td>
<td>2B: 312</td>
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</tbody>
</table>

Primer positions and expected product sizes of KATP channel subunits. Oligonucleotide positions are indicated considering the ATG codon at position 1. F and R, forward (upstream) and reverse (downstream) primers, respectively.
days showed the expected fragment sizes for K_\text{IR6.2} (316 bp), SUR1 (355 bp), and SUR2B (312 bp) (Fig. 1). Each of these products was confirmed by DNA sequence analysis. Notably, the primers that were designed to identify SUR2B would also detect the presence of SUR2A message based on the genomic structure of the SUR2 gene. If both SUR2A and SUR2B were expressed, two products of 451 and 312 bp, respectively, would have been amplified. However, in this case, no 451-bp product corresponding to SUR2A was detected, indicating that the SUR2B 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\textsubscript{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\textsuperscript{+}), 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 M), agents that have previously been shown to reduce intracellular ATP levels by \geq 10-fold (Schmid-Antomarchi et al., 1987; Boney 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\textsuperscript{+} 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\textsuperscript{+} (>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 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\textsubscript{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 sulfonylurea 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\textsubscript{50} value of 0.90 \pm 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{IR6.2} (316 bp), SUR 1 (355 bp), and SUR2B (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\textsubscript{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 M). 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 (A) (-)-cromakalim, (B) P1075 (0.001, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, and 30 \mu M), (B) P1075 (0.001, 0.01, 0.03, 0.1, 0.3, 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.
Complete inhibition of activator response was observed with 10 μM glyburide. When a second addition of glyburide was performed at 25 min as depicted in Fig. 3, the responses were only partially reversed that may be attributed to the concentration of glyburide used or alternatively due to the subsequent sampling duration. Glipizide was 50-fold weaker than glyburide in inhibiting P1075-evoked responses (IC50 = 46.8 ± 11.3 μM; n = 3; Fig. 6).

Radioligand Binding. Additional support for the presence of KATP 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 KATP 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 Bmax value of 48 ± 14 fmol/mg protein and a KD 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 KATP 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+ 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+ 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 EC_{50} 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 EC_{50} 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 PA value of 6.94 ± 0.39 (slope = 0.99 ± 0.11; r = 0.88; n = 8). The rank order potency (EC_{50}) 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 K_{ATP} channels in the detrusor (Boney 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 K_{ATP} channels expressed in cultured guinea pig urinary bladder cells by fluorescence-based membrane potential techniques. It was found that K_{ATP} channels could be activated by metabolic inhibition or by diverse KCOs with a rank order potency consistent with those typical of smooth muscle K_{ATP} channels. Furthermore, the subunit profile together with the observed pharmacological properties suggest that the K_{ATP} 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 K_{ATP} Channels.** Previous studies have examined K_{ATP} channels in bladder smooth muscle, by tissue relaxation subsequent to mild contractile stimulus, by assessing sulfonylurea-sensitive channel conductance after metabolic inhibition.

### TABLE 2

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<th>Compound</th>
<th>Membrane Potential</th>
<th>Detrusor Relaxation</th>
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<tr>
<td></td>
<td>EC_{50} M</td>
<td>Efficacy</td>
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<tr>
<td>P1075</td>
<td>5.3 ± 0.30 × 10^{-5}</td>
<td>107.7 ± 6.6</td>
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<td>Bay X 9228</td>
<td>4.7 ± 0.70 × 10^{-8}</td>
<td>86.4 ± 14.9</td>
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<td>(-)-Cromakalim</td>
<td>4.0 ± 0.14 × 10^{-7}</td>
<td>111.3 ± 5.5</td>
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<tr>
<td>ZD6169</td>
<td>2.7 ± 0.32 × 10^{-7}</td>
<td>117.5 ± 8.6</td>
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<tr>
<td>Pinacidil</td>
<td>6.4 ± 0.52 × 10^{-7}</td>
<td>79.9 ± 4.5</td>
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<td>(+)-Cromakalim</td>
<td>2.5 ± 0.03 × 10^{-5}</td>
<td>64.6 ± 0.8</td>
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<td>ZM244085</td>
<td>6.1 ± 0.87 × 10^{-6}</td>
<td>63.3 ± 3.7</td>
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<tr>
<td>Bay X 9227</td>
<td>2.2 ± 0.15 × 10^{-6}</td>
<td>61.7 ± 0.87</td>
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<td>Diazoxide</td>
<td>5.9 ± 0.32 × 10^{-5}</td>
<td>60.5 ± 8.2</td>
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</table>

N.D., not determined.
or after potassium channel activation by KCOs 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 K\textsubscript{ATP} channels in a rapid and high-throughput manner (Schoedel 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 K\textsubscript{ATP} 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 KCOs (Quayle et al., 1997). Metabolic inhibition or KCO-evoked membrane potential responses were sensitive to sulfonylurea inhibitors as well as to the potassium channel blocker barium. KCO-mediated membrane potential changes showed a clear dependence on extracellular K\textsuperscript{+} concentrations; moreover, the potencies of KCOs in the membrane potential assay showed a good correlation with the EC\textsubscript{50} 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 K\textsubscript{ATP} 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 \textsuperscript{3}H]glyburide revealed a K\textsubscript{d} 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 K\textsubscript{ATP} channels.

**Pharmacologic Characteristics of K\textsubscript{ATP} 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 K\textsubscript{ATP}, currents, to hyperpolarize, and to relax a wide variety of smooth muscles at concentrations ranging from 1 to 10 \mu M (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 (Hunnicutt et al., 1994). The 1,4-dihydropyridine ZM244085 has previously been shown, by microelectrode techniques, to activate K\textsubscript{ATP} 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\textsuperscript{+}-stimulated guinea pig detrusor (4 \mu M) by Li et al. (1996). ZD6169 is a tertiary carbinal 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\textsuperscript{+}-stimulated guinea pig detrusor strips (EC\textsubscript{50} = 1.6 \mu M) and to increase whole-cell glyburide-sensitive K\textsuperscript{+} currents in detrusor smooth muscle cells (Grant et al., 1994).

**Molecular Analysis of K\textsubscript{ATP} Channels.** RT-PCR studies demonstrated the presence of the inward rectifier K\textsubscript{IR}6.2 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 K\textsubscript{ATP} 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 \mu M; 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 K\textsubscript{ATP} channels composed of SUR1-K\textsubscript{IR}6.2 subunits (IC\textsubscript{50} = 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 K\textsubscript{IR}6.2. Although SUR1 message was detected by PCR analysis, \textsuperscript{3}H]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 \textsuperscript{3}H]glyburide binding (K\textsubscript{d} ~ 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 K\textsubscript{ATP} 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-cm\textsuperscript{2} 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 K\textsubscript{IR} and SUR subunit combinations (Isomoto and Kurachi, 1997). Studies by Yamada et al. (1997) have shown that the SUR2B-K\textsubscript{IR}6.1 may underlie a low conductance K\textsubscript{ATP} 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 K\textsubscript{ATP} channels composed of SUR2B-
K_{ATP}6.2 contribute to the pharmacological properties in bladder smooth muscle cells, the existence of other K_{ATP} channel subunit combinations cannot be eliminated. For example, a glyburide-sensitive current with conductance properties markedly different from those of the prototypical K_{ATP} channel has been described in pig urethral myocytes (Teramoto et al., 1997). In summary, our results demonstrate that the use of cultured guinea pig bladder smooth muscle cells in FLIPR-based membrane potential assays serves as a valuable model system to assess the pharmacology of K_{ATP} channels and has provided preliminary evidence for the molecular identity of these channels. Although there has been considerable interest in the development of K_{ATP} channel modulators for urological indications, it is now recognized that the beneficial effects of compounds available thus far, quite generally, parallel their undesirable cardiovascular effects. Accordingly, novel agents that reduce excitability of the bladder smooth muscle to maintain normal micturition, but with limited cardiovascular side effects, may have potential use in the management of urge incontinence. Recent studies have shown that KCOs such as (−)-cromakalim and YM-934 can effectively relax both normal and hyperreflexic human bladder smooth muscle, suggesting no appreciable alteration in K_{ATP} channel function under hyperreflexic conditions (Martin et al., 1997). The present study, together with previous in vitro studies using guinea pig, pig, and human urinary bladder, clearly demonstrate the presence of K_{ATP} channels (Foster et al., 1989a, 1989b), of which the selective modulation by KCOs could, in principle, suppress unstable bladder contractions, thereby providing potential use for such agents in the treatment of bladder instability.

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References


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