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Characterization of human urinary bladder K_{ATP} channels containing SUR2B splice variants expressed in L-cells

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Abstract

The molecular properties of the sulfonylurea receptor 2 (SUR2) subunits of K_{ATP} channels expressed in urinary bladder were assessed by polymerase chain reaction (PCR). This showed that SUR2B exon 17 − mRNA (72%) was predominant over the SUR2B exon 17+ splice variant (28%). The pharmacological properties of both of these isoforms stably expressed in mouse Ltk^-cells (L-cells) with K_{IR} 6.2 were determined by measuring changes in membrane potential responses evoked by K^+ channel openers using bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$) fluorescence. The rank order potency of a variety of structurally distinct K^+ channel openers was found to be the same in both stable cell lines and compared well with guinea pig bladder cells. The potency of these compounds in the SUR2B exon 17 − cells more closely resembled the potency measured in guinea pig bladder unlike the cell line containing the SUR2B exon 17+ subtype. Analysis of the displacement of [^{125}I]A-312110 binding with the same K^+ channel openers to the SUR2B exon 17- cells showed excellent correlation to those measured in guinea pig bladder. This study supports the notion that K_{ATP} channels containing SUR2B exon 17 − represent a major splice variant expressed in urinary bladder smooth muscle.

Keywords: K_{ATP} channel; SUR2B; Cell line, stable; Urinary bladder; [^{125}I]-A-312110; K^+ channel opener

1. Introduction

ATP-sensitive K^+ channels (K_{ATP}) are expressed in a wide variety of cells linking cellular excitability to the metabolic state of the cell (Ashcroft and Ashcroft, 1990). These channels have many diverse functions such as modulating insulin secretion in pancreatic cells, neurotransmitter release in neurons, cellular excitability in both skeletal and smooth muscle and playing a neuro-protective role in hypoxia-induced generalized seizure (Quayle et al., 1997; Coghlan et al., 2001; Yamada et al., 2001). K_{ATP} channels are regulated by the ratio of ATP/ADP present in the cell, being activated by increased intracellular ADP and inhibited by elevated ATP levels (Kakei et al., 1986; Dunne et al., 1988; Misler et al., 1986; Lederer and Nichols, 1989). The sensitivity of inhibition of the channel by ATP is also dependent on the concentration of phospholipids (phosphatidylinositol-4-phosphate and phosphatidylinositol-4, 5-bisphosphate) present in the cell membrane (Baukrowitz et al., 1998; Shyng et al., 2000).

Functional K_{ATP} channels have been shown to be hetero-octameric containing four sulfonylurea receptor (SUR) and four inward rectifying K^+ channel (K_{IR}) subunits (Clement et al., 1997; reviewed in Aguilar-Bryan et al., 1998). The K_{IR} 6.1 or 6.2 subunits mediate high affinity inhibition of the channel by ATP (Tucker et al., 1997; Tanabe et al., 1999). The SUR proteins contain the binding sites for K^+ channel openers, sulfonylureas and nucleotide binding folds (NBF) that are responsible for the hydrolysis of ATP to ADP (Aguilar-Bryan et al., 1998).

There are two SUR genes, SUR1 and SUR2 that have been shown to undergo alternative splicing to generate channels with diverse properties (Sakura et al., 1999; Gros et al., 2002; Hambrock et al., 2002; Inagaki et al., 1996;

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Chutkow et al., 1996). The SUR2 gene is composed of 39 exons and splicing has been shown to occur at the last exon (Inagaki et al., 1996; Isomoto et al., 1996) generating two splice variants, SUR2A and SUR2B that are differentially expressed and pharmacologically distinct. SUR2A expression is limited to cardiac and skeletal muscle while SUR2B is more widespread being found in every tissue examined in rodent (Isomoto et al., 1996; Chutkow et al., 1996) and human (Davis-Taber et al., 2000). Human, guinea pig and pig bladder have each been shown to express SUR2B and not SUR2A (Buckner et al., 2000; Gopalakrishnan et al., 1999). The biophysical and pharmacological properties of channels derived from these two splice variants may be differentiated by the sensitivity to certain K^+ channel openers. K_{ATP} channels composed of K_{IR} 6.2/SUR2A are insensitive to diazoxide, but are activated by pinacidil and cromakalim similar to cardiac K_{ATP} channels (Inagaki et al., 1996; Okuyama et al., 1998). In contrast, K_{IR} 6.2/SUR2B containing channels are activated by diazoxide, pinacidil, and cromakalim resembling smooth muscle K_{ATP} channels (Isomoto et al., 1996; Hambrock et al., 1999; Schwanstecher et al., 1998).

Additional splicing of the SUR2 gene occurring in the first nucleotide binding fold region encoded by exon 14 and/or exon 17 has also been reported (Chutkow et al., 1996, 1999). Expression studies with the exon 14 splice variant yielded a non-functional protein whereas the variant that lacks exon 17 generates K_{ATP} channels that are less sensitive to ATP than channels containing the full-length SUR2 subunit (Chutkow et al., 1999). In contrast, a splice variant of SUR1 that lacks exon 17 that has been described (Hambrock et al., 2002) forms functional K_{ATP} channels when coupled to K_{IR} 6.2 subunits, exhibits specific binding of glyburide, and has similar sensitivity to ATP compared to the wildtype SUR1 subunit. This indicates that exon 17 in these two related proteins has a different function with respect to ATP sensitivity. Previous studies from our laboratory examining the SUR2 transcript containing exon 17 has shown that it is the predominant SUR2 subtype expressed in a variety of tissues including smooth and skeletal muscle while SUR2 exon 17− constituted a smaller proportion (~ 35–40%) of the total SUR2 message (Davis-Taber et al., 2000). The focus of the present study was to determine the molecular properties of the SUR2 subunit of K_{ATP} channels predominantly expressed in urinary bladder smooth muscle and to relate these to the pharmacology of K^+ channel openers. Initial RNA analysis showed that, in contrast to intestinal and uterine smooth muscle, bladder smooth muscle predominantly expresses the SUR2B exon 17− splice variant. Stable cell lines expressing SUR2B exon 17+ or exon 17− together with human K_{IR} 6.2 were generated in the present study. Evaluation of the pharmacological profile of these recombinant K_{ATP} channels using known K^+ channel openers also supported the suggestion that the SUR2B exon17− subunit most closely resembled previously characterized native bladder K_{ATP} channels than those containing SUR2B exon17+.

2. Materials and methods

2.1. Materials

Superscript II, TRIzol, Dulbecco's modified eagles media, pCRII TOPO and Lipofectamine Plus were purchased from Invitrogen (Carlsbad, CA). RNase Zap and Expand Long PCR template system and Complete™ Tablets were obtained from Ambion (Austin, TX) and Roche Applied Sciences (Indianapolis, IN), respectively. Chroma Spin + TE-100 columns were from Clontech (Palo Alto, CA). Quikchange mutagenesis kit and bis-(1,3-dibutylbarbituric acid)-trimethine oxonol ($DiBAC_4(3)$) were from Stratagene (La Jolla, CA) and Molecular Probes (OR). A-312110 was synthesized in-house as detailed in Davis-Taber et al., 2003. Labeled [^{125}I]A-312110 had a specific activity of approximately 2000 Ci/mmol (1 Ci = 3.7×10^{10} Bq) and was stored in ethanol at −20 °C protected from light. (−)-Cromakalim, P1075 [*N*-cyano-*N*′-(1,1-dimethylpropyl)-*N*″-3-pyridylguanidine], ZD6169 [*N*-(4-benzoyl phenyl)-3,3,3-trifluro-2-hydroxy-2-methylpropionamine], Bay X 9228 [((−)-*N*-(2-ethoxyphenyl)-*N*′-(1,2,3-trimethylpropyl)-2-nitroethene-1,1-diamine)] and A-151892 *N*-[2-(2,2,2-trifluoro-1-hydroxy-1-trifluoromethyl-ethyl)-naphthalen-1-yl] (Turner et al., 2003) were synthesized in-house or obtained from the Abbott compound library. Other compounds including glyburide and pinacidil were purchased from Research Biochemicals International/Sigma (St. Louis, MO). All other chemicals and inorganic salts were purchased from Sigma.

2.2. Isolation of total RNA from guinea pig bladder

Guinea pig bladders were rapidly removed following sacrifice and immediately frozen in liquid nitrogen prior to storage at −80 °C. To minimize the RNA degradation, all equipment was pretreated with RNase Zap and the bladder tissue was pulverized under liquid N_2 using a pestle and mortar in dry ice. The tissue was rapidly weighed and total RNA was isolated using the TRIzol procedure as detailed elsewhere (Davis-Taber et al., 2000). Briefly, 1 ml of TRIzol was added to 100 mg crushed bladder tissue and homogenized using the Tekmar polytron 3 × 30 s (13,000 rpm). Aliquots (1 ml) of the tissue–TRIzol mixture were incubated for 5 min at 15–30 °C. Chloroform (200 μl) was added and the mixture was shaken vigorously prior to incubation at room temperature for 2–3 min. The sample was subjected to centrifugation at 12,000 × *g* for 15 min at 4 °C. The aqueous clear phase was removed to a sterile tube and a second extraction with 200 μl of chloroform was performed. RNA was precipitated by the addition of 500 μl of

isopropanol and the samples were incubated for 10 min at 15–30 °C and then centrifuged at 12,000 × *g* for 10 min at 4 °C. The RNA pellet was washed with 75% (v/v) ethanol (1 ml) and sedimented by centrifugation at 7500 × *g* for 5 min at 4 °C. Ethanol was removed from the RNA and the sample was air-dried for 5–10 min. RNA was resuspended in diethylpyrocarbonate-treated water and the concentration was determined by absorption at A_{260}.

2.3. Reverse transcription-polymerase chain reaction analysis (RT-PCR)

Human total RNA was isolated as detailed previously (Davis-Taber et al., 2000). First-strand cDNA was synthesized from human and guinea pig bladder total RNA using Superscript II according to manufacturer's instructions. Briefly, the total RNA (2 μg) was primed with 200 ng of gene specific primers (Table 1) and incubated at 70 °C for 10 min. PCR buffer (20 mM Tris–HCl pH 8.4, 50 mM KCl), 2.5 mM $MgCl_2$, 1 μM dNTP and 10 mM dithiothreitol were added together and incubated for 5 min at 25 °C. The reverse transcription reaction was initiated by the addition of Superscript II RT (200 U) at 25 °C for 10 min and incubation at 42 °C for 50 min. Reactions were terminated by incubation at 70 °C for 15 min, prior to chilling on ice. RT-PCR was performed using 2 μl of cDNA in 50 μl reaction containing 0.4 μM each primer and the Expand Long PCR template system according to the manufacturer's instructions. The cycling conditions were 96 °C for 15 s, 55 °C for 30 s, 72 °C for 78 s for 40 cycles. An aliquot (30 μl) of the RT-PCR product was analyzed on a 1% agarose gel prepared in Tris–Borate–EDTA (TBE). The identity of each of the PCR products was confirmed by sequence analysis following subcloning into pCRII TOPO. Negative controls lacking reverse transcriptase during cDNA synthesis did not amplify any products. Oligonucleotide primers used for RT-PCR studies are listed in Table 1 or described previously (Davis-Taber et al., 2000).

2.4. Real-time Taqman polymerase chain reaction (Q-PCR)

mRNA for SUR2B exon 17+ and exon 17− were quantified based on normalization to a plasmid standard curve and to the human 28S ribosomal RNA gene (Genbank accession number M27830). The primer-probe set used to quantify the levels of human SUR2B exon 17+ and exon 17− message and the Q-PCR protocol were identical to that used in a previous study (Davis-Taber et al., 2000). Briefly, 6 μg of total RNA was reverse transcribed into cDNA and purified using Chroma Spin + TE-100 columns. Concentrations of cDNA samples were determined at A_{260}. Q-PCR was performed with 10 ng cDNA in a 25-μl reaction containing 1 × Taqman PCR Buffer (50 mM KCl, 10 mM Tris–HCl, 10 mM EDTA, 300 nM Passive Ref. 1, pH 8.3), 200 μM dATP, dCTP, dGTP, and dUTP, 5 mM $MgCl_2$, 0.625 U of AmpliTaq Gold DNA polymerase, 200 nM of each primer set, and 100 nM of a gene-specific detection probe. The cycling conditions included a hot start for 10 min at 95 °C followed by 40 cycles of 95 °C for 30 s and 60 °C for 1 min. All reactions were performed in ABI Prism 7700 and data was analyzed using a Sequence Detection System software 1.6.3. Plasmids containing each splice variant were used to verify that neither primer/probe set would detect the alternative SUR2 exon 17 variants. Calculation of the primer/probe set efficiencies using the detection software for Taqman showed that both SUR2 exon 17+ and exon 17− yielded similar annealing efficiencies. As a negative control, samples lacking reverse transcriptase during cDNA synthesis did not yield any products.

2.5. Transfection of K_{ATP} channel subunits into L-cells

The human K_{IR} 6.2 subunit (Genbank accession number D50582) was subcloned into a mammalian expression vector (pcDNA3.1-Neo) containing the antibiotic resistant cassette neomycin as previously described (Gopalakrishnan et al., 2000). The human SUR2B subunit (Gen-

Table 1
Oligonucleotides used in PCR studies

Subunit	Primer sequences (location)	Size (bp)	
gpSUR2 specific primer	R:5′ -CTATGGTGACAACCGTGCGA-3′ (4491–4510)[a]	NA	
hSUR2 specific primer	R:5′ -CTATTGTCACCACGGTCCGG-3′ (4491–4510)[a]	NA	
gSUR2 exon 17+/17−	F: 5′ -TTCGAATTCCAACAGGTCAG-3′ (2078–2097)	343	Exon 17+
	R: 5′ -CTCTCCAATTTCAGTTTGGTCTCC-3′ (2421–2398)	304	Exon 17−
SUR2	F: 5′ -TCCGCAAAAGCAGCGTTCTTATT-3′ (4391–4413)		
	R: 5′ -TCCATTTTCCTGAGCCAAGAGGCTT-3′ (4617–4593)	226	
K_{IR} 6.2	F: 5′ -ATGCTGTCCCGCAAGGGCATC-3′ (1–20)		
	R: 5′ -ATGAGGGTCTCAGCCCTGCG-3′ (525–545)	545	

The oligonucleotides that were used to gene specifically prime cDNA synthesis of guinea pig and human from bladder are given. [a]The nucleotide positions shown are relative to the human SUR2 sequence. The gSUR2 primer set was used to amplify SUR2 splice variants from guinea pig. The SUR2 and K_{IR} 6.2 oligonucleotides for RT-PCR confirmation of the stable cell line are given. In each case, F and R represent forward (upstream) and reverse (downstream) primers, respectively. The location in the gene is given, based upon the ORF together with the expected product sizes in the right-hand column.

bank accession number AF061289) in the mammalian expression vector pECE was obtained from Dr J. Bryan (Baylor College of Medicine, Houston, TX). The human SUR2B exon 17− splice variant was generated using the Quikchange mutagenesis kit deleting the 39 nucleotides of exon 17 from the full-length SUR2B-pECE construct (Davis-Taber et al., 2000). The stable cell line was generated by co-transfection of either SUR2B exon 17+ or SUR2B exon 17− pECE together with K_{IR} 6.2-pcDNA-Neo at a ratio of 100:1 (SUR2/K_{IR} 6.2) using the standard Lipofectamine Plus protocol according to the manufacturer's instructions into mouse fibroblast L-cells. Briefly, cells (500,000 cells/well in a 6-well tissue culture plate) were incubated with 1 μg of either SUR2B construct and 0.01 μg K_{IR} 6.2-Neo plasmids with Lipofectamine Plus reagent at 37 °C for 5 h in a humidified incubator at 5% CO_2. The DNA–lipid mixture was replaced with culture media in Dulbecco's modified eagles media containing 10% fetal bovine serum and the cells were propagated for an additional 2 days. Cells were split 1:100 and grown in culture media containing 550 μg/ml geneticin to select antibiotic-resistant clones and further propagated in media containing 500 μg/ml geneticin.

2.6. Characterization of stable cell lines expressing K_{ATP} channels

2.6.1. Molecular studies

The presence of K_{ATP} channel subunits in the stable cell lines was assessed by RT-PCR for both SUR2 and K_{IR} 6.2 from total RNA isolated from the cells. Total RNA was isolated from the stable cell lines using TRIzol as described above in Section 2.2 with the exception that instead of tissue, 1×10^6 cells/ml TRIzol were collected following dissociation from a T-75 flask. RT-PCR was performed using the oligonucleotides specific for K_{IR} 6.2 and SUR2B (Table 1) as detailed above. The identity of each of the products was sequence confirmed.

2.6.2. Functional studies

Positive clones for the SUR2B exon 17+/K_{IR} 6.2 and SUR2B exon 17−/K_{IR} 6.2 cell lines were also determined by assessing changes in membrane potential in a Fluorometric Imaging Plate Reader (FLIPR) assay using the cyanoguanidine K^+ channel opener, P1075 to activate the channels (see Section 2.7).

2.7. Membrane potential studies

Changes in membrane potential responses were assessed using the bis-oxonol dye $DiBAC_4(3)$ in the FLIPR as detailed previously (Gopalakrishnan et al., 1999). Confluent stably transfected SUR2B exon 17−/K_{IR} 6.2 cells were plated at a density of 40,000 cells/well in black, clear-bottomed 96-well plates and assays were carried out in buffer (20 mM HEPES, 120 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, pH 7.4) containing 5 μM $DiBAC_4(3)$ at 37 °C. Changes in fluorescence were monitored at excitation and emission wavelengths of 488 and 520 nm, respectively, for 25 min. Data was normalized to the response evoked by 10 μM P1075.

2.8. Cell membrane preparation and [^{125}I]A-312110 ligand binding

Cells stably expressing SUR2B exon 17−/K_{IR} 6.2 were removed from T-162 cm flasks or cell factories into ice-cold phosphate buffered saline containing protease inhibitors (Complete™ tablets) and homogenized using a Tekmar polytron at 13,500 × rpm for 2 × 30-s bursts. The homogenate was centrifuged at 20,000 × *g* for 10 min at 4 °C and the membrane pellets were resuspended in ice-cold assay buffer (139 mM NaCl, 5 mM KCl, 25 mM $MgCl_2$, 1.25 mM $CaCl_2$, and 20 mM HEPES; pH 7.4).

The binding of [^{125}I]A-312110 (specific activity 2000 Ci/mmol) was carried out as described previously (Davis-Taber et al., 2003). Briefly, membranes (1.2 mg/ml) were incubated in a total assay volume of 0.25 ml in assay buffer containing an ATP regeneration system (20 mM creatine phosphate, 50 U creatine phosphokinase and 1 mM Na_2ATP) with radioligand and unlabeled compounds (prepared as 10 × stocks in assay buffer) at 37 °C for 90 min. Specific radioligand binding was defined by subtracting the nonspecific binding (10 μM unlabeled A-312110) from the total binding. The binding assay was terminated by rapid vacuum filtration through GF/B filters, washed twice with ice-cold 50 mM Tris–HCl (pH 7.2) and radioactivity bound to the filters was assessed by γ-counting at an efficiency of 80%.

2.9. Data analysis

For analysis of the FLIPR data, background changes in fluorescence were corrected and the data was normalized to the response evoked by 10 μM P1075 (fluorescence units were arbitrarily assigned as 100%) included in the same assay plate. Concentration–response curves were generated by nonlinear least square regression analysis of the data using GraphPad Prism. The ligand affinity (equilibrium dissociation constant or K_d) and maximal receptor density (B_{max}) were analyzed by nonlinear regression of the saturation binding data using Microsoft Excel and GraphPad Prism data analyzing packages. The K_i values were calculated from the concentration inhibition curve by the method of Cheng and Prusoff (1973). Correlation coefficients were determined from linear regression analysis (GraphPad Prism). Significant differences were assessed at $P < 0.05$ level. All values are expressed as mean ± S.E.M.

3. Results

3.1. RT-PCR analysis of SUR2 splice variants in bladder

To investigate the presence of the SUR2B exon 17 − splice variant in human bladder, oligonucleotide primers that flanked the exon 17 region of the human gene as shown in the schematic (Fig. 1A) were designed (Davis-Taber et al., 2000) and used to amplify PCR products from cDNA that was reverse transcribed from bladder total RNA using a SUR2-specific oligonucleotide for priming the cDNA synthesis. This resulted in amplification of a product of the expected size for SUR2 that included exon 17 or SUR2 exon 17+ (328 bp), in addition to the 39 nucleotides shorter SUR2 exon 17 − splice variant (289 bp) (Fig. 1B) that is lacking exon 17. The identity of these two products was confirmed by DNA sequencing. To determine if this splice variant also exists in bladder tissue from other species, total RNA was freshly isolated from guinea pig bladder and reverse transcribed into cDNA using gene specific primers. Since limited sequence information on the nucleotide sequence of the SUR2 gene from guinea pig was known (Hambrock et al., 2002), a set of generic oligonucleotide primers were designed that lay outside the exon 17 region of the gene using published data from rat, human and mouse SUR2 in Genbank (Table 1). These primers were used to amplify PCR products of the expected size for SUR2B exon 17+ (343 bp) and SUR2B exon 17 − (304 bp) splice variants (Fig. 1B) from guinea pig bladder. Both of these products were sequenced to confirm their identity as part of

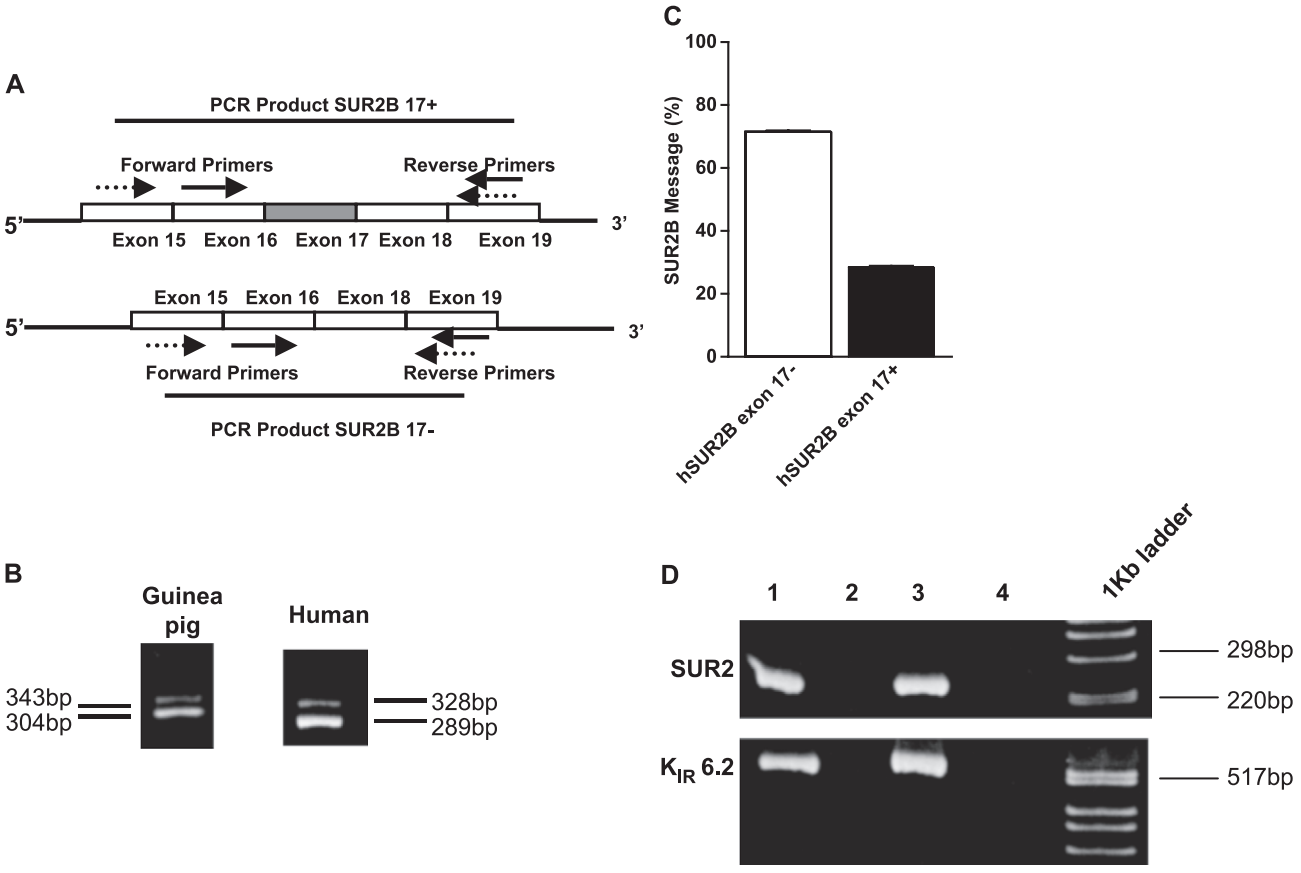

Fig. 1. Molecular characterization of K_{ATP} channels expressed in bladder and recombinant cell lines. (A) Schematic representation showing the region of the SUR2 gene that encompasses exon 17 (exons 15–19). The solid arrows indicates the position of the oligonucleotide primers that were used to amplify the human SUR2 gene PCR product, while the dotted arrows specify the positions of the generic oligonucleotide primers that were used to amplify the guinea pig PCR products. (B) Total RNA was isolated from human and guinea pig bladder and cDNA was synthesized using gene-specific primers (Table 1). RT-PCR was performed using oligonucleotide primers for human (Davis-Taber et al., 2000) and guinea pig (see Table 1) that encompass the exon 17 region of the SUR2 gene and following 40 cycles of PCR the products were analyzed on a 1% agarose gel and stained with ethidium bromide. The expected PCR product sizes are as shown and the identity of each fragment was confirmed by sequencing. (C) Real-time PCR was performed on total RNA samples using oligonucleotide primers for human (Davis-Taber et al., 2000). The data is represented as the relative amounts of both of the splice variants of SUR2B compared to total amount of SUR2B expressed in this tissue ($n = 3$). (D) RT-PCR analysis of the expression of SUR2 and K_{IR} 6.2 in SUR2B exon 17+/K_{IR} 6.2 and SUR2B exon 17 − / K_{IR} 6.2 stable cell lines. Total RNA from both cell lines and the host L-cells was isolated and using oligonucleotide primers to SUR2 and K_{IR} 6.2, PCR products of the expected size were amplified following 40 cycles. The products were resolved on a 1% agarose gel and visualized with ethidium bromide. Top gel shows SUR2 PCR products (expected size 226 bp) and bottom gel indicates the K_{IR} 6.2 PCR products (expected size 630 bp) with the following samples lane 1, SUR2B exon 17+/K_{IR} 6.2 stable cell line, lanes 2 and 4; host L-cells; lane 3 SUR2B exon 17 − /K_{IR} 6.2 stable cell line. Ladder (1 Kb) was run in each case as indicated and the identity of all products was confirmed by DNA sequencing.

the SUR2 gene in guinea pig. More recently, sequence information within the region selected in this study was reported for guinea pig SUR2 (Hambrock et al., 2002), and comparison of the nucleotide sequence was an exact match between these two PCR products, although the product we obtained was 30 nucleotides longer. Furthermore, comparison of the nucleotide sequence of SUR2 from guinea pig and human bladder showed that within the nucleotide sequence of the PCR product (2078 to 2421 bp), there was a 91.5% identity between these two species which translated to a 99% identity at the amino-acid level (single conserved substitution) (data not shown).

3.2. Q-PCR of the SUR2B exon 17+ and exon 17− splice variants

While RT-PCR identified the presence of both SUR2B splice variants in human and guinea pig bladder, the relative levels of both of these subtypes was not known. Previous studies had quantified the levels of SUR2 exon 17+ and exon 17 − in a variety of other human tissues (Davis-Taber et al., 2000), and using the same Taqman primer-probe set as described in that study the relative levels of both splice variants were investigated in human bladder. In contrast to the previous study, it was found that the splice variant lacking exon 17 was expressed predominantly in human bladder over the exon 17+ variant by 3-fold comparing the total message for SUR2 (Fig. 1C).

3.3. Generation and selection of the stable cell lines expressing of SUR2B exon 17+/K_{IR} 6.2 and SUR2B exon 17−/K_{IR} 6.2 K_{ATP} channels

To characterize the pharmacology of K_{ATP} channels containing K_{IR} 6.2 and either SUR2B exon17+ or exon17 − , these subunits were stably transfected into mouse fibroblast L-cells. To determine if there was endogenous expression of either SUR2B or K_{IR} 6.2 in the host cells, RT-PCR was performed with oligonucleotides specific for the mouse subunits. No PCR products were amplified from the L-cell total RNA though the oligonucleotides were shown to successfully amplify mouse SUR2 and K_{IR} 6.2

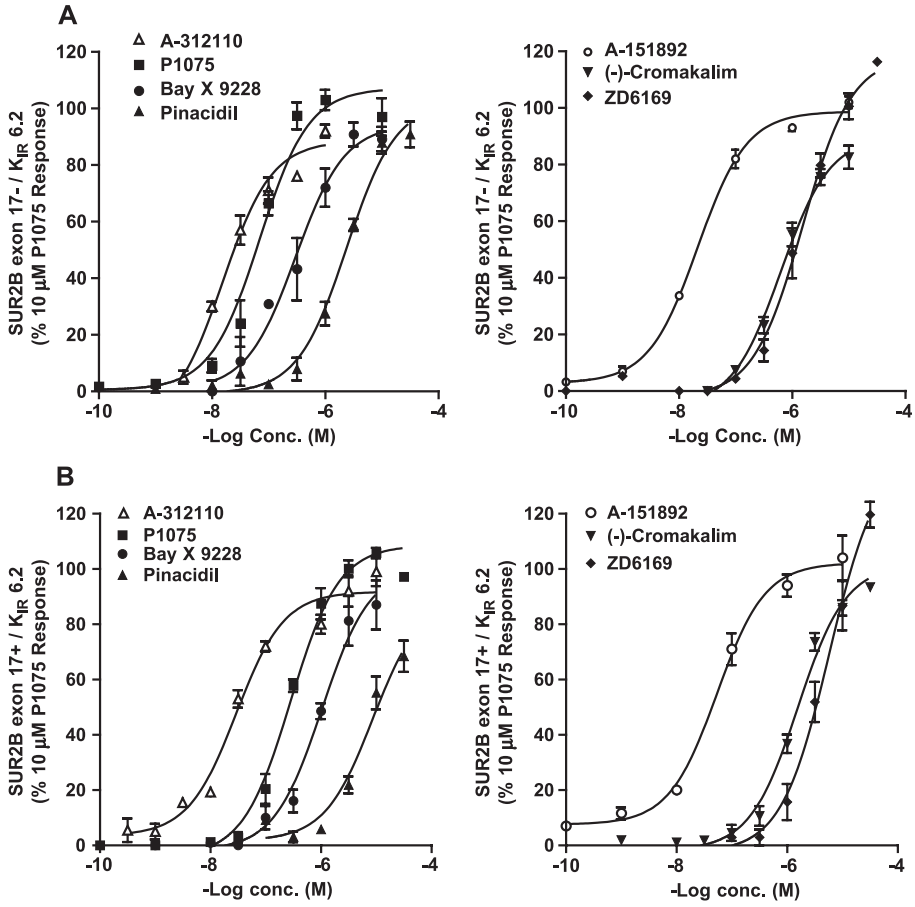

Fig. 2. Assessment of the effect of K^+ channel openers on membrane potential responses in the human SUR2B exon 17 − /K_{IR} 6.2 and SUR2B exon 17+/K_{IR} 6.2 stably transfected cell lines. Activation of K_{ATP} channels containing (A) SUR2B exon 17 − /K_{IR} 6.2 or (B) SUR2B exon 17+/K_{IR} 6.2 by K^+ channel openers. Concentration response of membrane potential changes evoked by A-312110, P1075, Bay X 9228, pinacidil, A-151892, (−)-cromakalim and ZD6169. All values are normalized and expressed as a percentage of response to 10 µM P1075 and each data point represents the mean ± S.E.M. of three to seven determinations. The mean EC_{50} values are given in Table 2.

from bladder RNA (data not shown). The SUR2B constructs were obtained from J Bryan in the pECE mammalian expression vector that does not have an antibiotic selection required for stable cell line generation. Many attempts were made to subclone both of these constructs from the SV40-pECE vector into other CMV-containing vectors such as pcDNA3.1-Neo and pCI-Neo. Unfortunately, these efforts were unsuccessful and in each case repeated rearrangements, introduction of transposons and deletions of the construct DNA resulted. Therefore to generate the stable cell line, transfection of different ratios of DNA of the SUR2B constructs with K_{IR} 6.2 was performed. It was found that the ratio of 100:1 for SUR2 plasmid over K_{IR} 6.2 gave the two cell lines that showed both subunits to be present molecularly and functionally gave the best activity in the clonal cell lines though 1:1 and 10:1 were also performed but were unsuccessful.

To establish that both subunits were present in the single stable cell line clone for each cell line, molecular analysis of the total RNA by RT-PCR for both human SUR2B and K_{IR} 6.2 constructs (Fig. 1D) was performed. This provided strong evidence that both subunits together were stably expressed in each of these single clonal cell types particularly since we had established no endogenous expression of mouse SUR2 or K_{IR} 6.2 in these host L-cells.

The above molecular experiments were performed on cells derived from single clones though it could be argued that there is a possibility that more than one cell was present during the clone selection process. This may result in positive RT-PCR data though each subunit could be expressed in different cells. Therefore, we performed functional studies measuring changes in membrane potential in both of these cell lines with $DiBAC_4(3)$ using FLIPR. The rationale being that K_{IR} 6.2 does not form functional channels unless co-expressed with SUR subunits. In addition, many K^+ channel openers such as P1075 activate the channel through binding to the SUR2 subunit. In both cell lines P1075 activated channels that were reversed by the K_{ATP} channel blocker glyburide. This data confirmed that the two cell lines co-expressed the SUR2 subunit together with K_{IR} 6.2. Assessment of the host L-cells gave no signal when treated with P1075 followed by glyburide in the FLIPR assay (data not shown).

3.4. Pharmacological characterization of K_{ATP} channels containing SUR2B exon 17+ or SUR2B exon 17− and K_{IR} 6.2

Modulation of K_{ATP} channels leads to changes in membrane potential and these can be measured as corresponding changes in fluorescence of the membrane potential sensitive dye $DiBAC_4(3)$ in the FLIPR assay as previously described (Gopalakrishnan et al., 1999). Addition of P1075 evoked a concentration-dependent change in membrane potential ($EC_{50} = 65 \pm 23$ nM; $n = 7$) indicated by a decrease in fluorescence (Fig. 2A). This response was reversed by the addition of glyburide (5 μM), indicating that the change in fluorescence was due to opening the K_{ATP} channels. To further characterize the K_{ATP} channels composed of SUR2B exon 17+ or exon 17− and K_{IR} 6.2, the activity of K^+ channel openers from different structural classes was also evaluated (Fig. 2A and B, respectively). P1075 (cyanoguanidine), (−)-cromakalim (benzopyran), Bay X 9228 (nitroethene), pinacidil (cyanoguanidine), ZD6169 (tertiary carbonyl), A-151892 (naphthylamide) and A-312110 (dihydropyridine) each evoked concentration-dependent changes in membrane potential in both stable cell lines with the same rank order potency as that measured in native guinea pig bladder cells (Gopalakrishnan et al., 1999; Turner et al., 2003; Davis-Taber et al., 2003). The EC_{50} values for these K^+ channel openers in both cell lines are given in Table 2. It was found that each of the compounds tested were all significantly more potent in the SUR2B exon 17−/K_{IR} 6.2 cell line over the SUR2B exon 17+/K_{IR} 6.2 cells (Figs. 2 and 3A), though the rank order potency was the same in both cell lines. Comparison of the EC_{50} values for the K^+ channel openers against the SUR2B exon 17−/K_{IR} 6.2 cell line and those previously measured in native guinea pig bladder cells (Figs. 2A and 3B) (Gopalakrishnan et al., 1999; Davis-Taber et al., 2003; Turner et al., 2003) showed excellent correlation close to unity. In contrast, each of the values for the SUR2B exon 17+/K_{IR} 6.2 cell line are shifted to a 3-fold lower potency for each K^+ channel opener assessed when compared to guinea pig bladder (Fig. 3C) though the correlation coefficient was good ($r^2 = 0.89$). This data suggests that the pharmacology of the K^+ channel openers assessed in native guinea pig bladder cells is more closely resembling that measured in the recombinant stable cell line expressing SUR2B exon 17−/K_{IR} 6.2 over that containing the exon 17+ isoform.

Table 2
Functional characterization of stable cell lines expressing recombinant K_{ATP} channels

	Membrane potential assay		$[^{125}I]$A-312110 binding assay
	SUR2B exon 17−/K_{IR} 6.2 (−log EC_{50} ± S.E.M.) M	SUR2B exon 17+/K_{IR} 6.2 (−log EC_{50} ± S.E.M.) M	SUR2B exon 17−/K_{IR} 6.2 (−log K_i ± S.E.M.) M
A-312110	7.8 ± 0.15	7.5 ± 0.11	8.2 ± 0.03
A-151892	7.7 ± 0.07	7.3 ± 0.08	7.4 ± 0.01
P1075	7.2 ± 0.1	6.5 ± 0.06	8.3 ± 0.01
Bay × 9228	6.5 ± 0.12	6.0 ± 0.1	8.1 ± 0.28
(−)-Cromakalim	6.2 ± 0.07	5.8 ± 0.08	6.9 ± 0.06
ZD6169	5.8 ± 0.08	5.2 ± 0.07	5.7 ± 0.09
Pinacidil	5.6 ± 0.07	5.1 ± 0.2	7.1 ± 0.03

Potencies of K^+ channel openers to evoke membrane potential responses in the cell line stably expressing SUR2B exon 17− or SUR2B exon 17+ together with K_{IR} 6.2. Displacement of the binding of $[^{125}I]$A-312110 to cell membranes expressing SUR2B exon 17−/K_{IR} 6.2. Values represent three to seven separate determinations in duplicate.

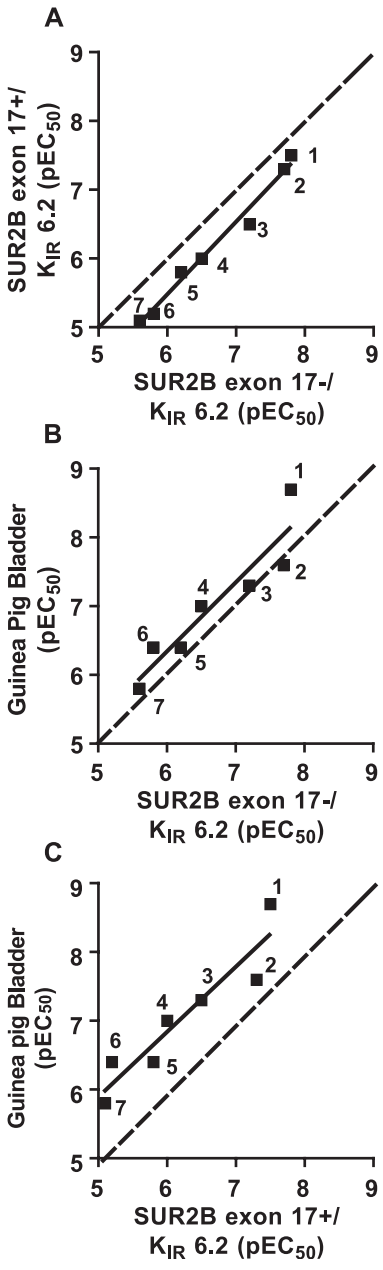

Fig. 3. Correlation analysis of the activities (− log EC_{50}) of structurally distinct K^+ channel openers in recombinant and native cell lines as determined using changes in membrane potential in FLIPR. In each case on the correlation plots, the K^+ channel openers represented are (1) A-312110; (2) A-151892; (3) P1075; (4) Bay X 9228; (5) (−)-cromakalim; (6) ZD6169 and (7) pinacidil. Comparison of the EC_{50} values of K^+ channel openers between (A) the two stable cell lines expressing either SUR2B exon 17+ or SUR2B exon 17 − together with K_{IR} 6.2 ($r^2 = 0.98$). (B) K_{ATP} channels expressed in native guinea pig bladder cells and the SUR2B exon 17 − /K_{IR} 6.2 ($r^2 = 0.87$) and (C) K_{ATP} channels expressed in native guinea pig bladder cells and the SUR2B exon 17+/K_{IR} 6.2 ($r^2 = 0.88$). The data for the guinea pig bladder cells is taken from Gopalakrishnan et al. (1999). In each case, the solid line represents linear regression through the data points and the dashed line corresponds to unity.

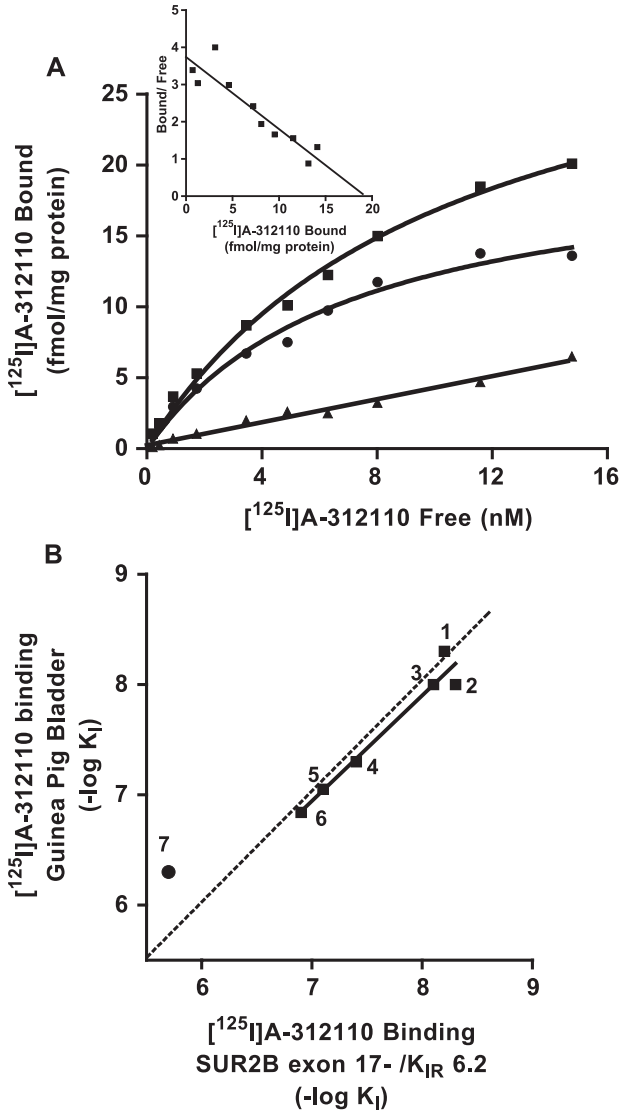

Fig. 4. Saturation binding of [^{125}I]A-312110 to SUR2B exon 17 − /K_{IR} 6.2 cell membranes. (A) A representative plot indicating increases in total (■), specific (●) and nonspecific (▲) binding with increasing radioligand concentrations. Specific binding was defined by the inclusion of 10 μM unlabeled A-312110. Inset. Scatchard analysis of the specific binding data gave a mean $K_d = 3.5 \pm 1.2$ nM and a $B_{max} = 18 \pm 0.6$ fmol/mg of protein. (B) Correlation of the affinities of K^+ channel openers to displace [^{125}I]A-312110 to guinea pig bladder membranes (− log K_i) (data taken from Davis-Taber et al., 2003) and the stable cell line (Table 2) is shown. In each case on the correlation plots the K^+ channel openers represented are (1) A-312110; (2) P1075; (3) Bay X 9228; (4) A-151892; (5) pinacidil; (6) (−)-cromakalim and (7) ZD6169. The solid line represents linear regression through the data points and the dashed line corresponds to unity. Correlation coefficient ($r^2 = 0.96$) derived from the comparison of inhibition constants (ZD6169 is not included in the calculations of correlation coefficient).

3.5. [^{125}I]A-312110 binding to the SUR2B exon 17−/K_{IR} 6.2 cell lines

Since the SUR2B exon17 − /K_{IR} 6.2 cell line has been shown both molecularly and pharmacologically to resemble channels expressed in bladder tissue over the SUR2B ex-

on17+/K_{IR} 6.2 subtype, binding studies with the recently identified dihydropyridine [^{125}I]A-312110 were performed using this cell line only. Saturation analysis with increasing concentrations of [^{125}I]A-312110 (0.2–15 nM) in the absence and presence of unlabeled ligand to membranes isolated from the SUR2B exon 17 − /K_{IR} 6.2 stable cell line showed that this ligand bound in a saturable manner with a $K_d = 3.52 \pm 1.2$ nM ($n = 3$) and a $B_{max} = 18 \pm 0.6$ fmol/mg protein ($n = 3$) (Fig. 4A). This binding affinity is comparable to that measured in native guinea pig bladder (Davis-Taber et al., 2003). Binding of [^{125}I]A-312110 to K_{ATP} channels in stably transfected cell lines is dependent upon ATP (data not shown) in a manner similar to that found in bladder and cardiac preparations (Loffler-Walz and Quast, 1998; Davis-Taber et al., 2003).

To assess the SUR2B exon 17 − /K_{IR} 6.2 stable cell line further, displacement of the radioligand binding by K^+ channel openers was evaluated. In the presence of 1 mM ATP, [^{125}I]A-312110 binding was displaced by increasing concentrations of unlabeled A-312110 (0.01 pM–1 μM) with a K_i value of 4.8 ± 0.7 nM ($n = 3$) (Table 2). The K_i values of the K^+ channel openers displacing [^{125}I]A-312110 were determined in the SUR2B exon 17 − /K_{IR} 6.2 cell line (Table 2) and compared well with those calculated from native K_{ATP} channels in guinea pig bladder membranes (Fig. 4B) (Davis-Taber et al., 2003).

4. Discussion

In the present study, the nature of the SUR2 subunit of K_{ATP} channels expressed in urinary bladder was investigated. It was found that in addition to the full length SUR2B exon 17+ subunit, a splice variant was also present that lacked exon 17. Analysis of the relative levels of message by Q-PCR showed that the SUR2 exon 17 − splice variant was predominant in human bladder over the full-length message (72% versus 28% total SUR2 message). Evaluation of stable cell lines co-expressing SUR2B exon 17+ or exon 17 − with K_{IR} 6.2 revealed a pharmacological profile of K^+ channel openers similar to that derived from K_{ATP} channels in native guinea pig bladder and indicated the same rank order of potency across a number of structurally different K^+ channel openers. However, the relative potency of these agents in the SUR2B exon 17 − cell line compared more closely with that measured for the same compounds in guinea pig bladder.

In an earlier study examining different tissues, it was found that the SUR2B exon 17+ splice variant was the predominant subtype (Davis-Taber et al., 2000). In brain, heart and kidney this variant constituted >95% of the total SUR2 message present, whereas in skeletal and smooth (uterus or small intestine) muscle, ~ 60% of the message was the exon 17+ variant. Herein, the SUR2B exon 17 − splice variant is found to be the major subtype expressed in urinary bladder smooth muscle which indicates that the K_{ATP} channels expressed in this tissue have a different molecular composition to those expressed in other tissues including other smooth muscles. Analysis of the amino acids that are found in the nucleotide binding fold 1 region of human and guinea pig showed that the sequence was very highly conserved. Within a region of 110 amino acids encompassing nucleotide binding fold 1, only a single amino acid was found to be different (conserved substitution) between these two species. Recent studies have shown that the full-length sequence of SUR2B is highly conserved between guinea pig and human (>97% identity at the amino acid level) (Hambrock et al., 2002) which is in contrast to the 60% identity that is found for SUR1 in guinea pig and human.

In rat and human smooth muscle tissues, K_{ATP} channels have been shown to be composed of K_{IR} 6.2 or K_{IR} 6.1 and SUR2B (Isomoto et al., 1996; Davis-Taber et al., 2000). In particular, small intestine and uterus smooth muscle from rodent and human were also shown to express both subtypes of the SUR2B subunit, with and without exon 17 (Chutkow et al., 1999; Davis-Taber et al., 2000). The functional properties of this novel splice variant were investigated by electrophysiology and based on the position of exon 17 in nucleotide binding fold 1, the sensitivity to ATP was evaluated. Co-expression of mouse K_{IR} 6.2 together with mouse SUR2A/B exon 17 − splice variants transiently in Cos-7 cells generated channels that show modest differences (2-fold decrease) in ATP sensitivity over the wildtype SUR2A/B subunit in whole cell patch clamp studies (Chutkow et al., 1999). In contrast, a recent study assessing the SUR1 exon 17 − splice variant showed identical ATP sensitivity compared to channels containing the full-length subunit (Hambrock et al., 2002). While the Walker A and B domains of nucleotide binding fold 1 of SUR1 and 2 are well conserved, the linker region that is encoded by exon 17 in both subunits share only 4 out of 13 amino acids which may be responsible for the functional differences seen.

To determine if these two splice variants are pharmacologically distinct, stable cell lines expressing either SUR2B exon 17 − or SUR2B exon 17+ together with K_{IR} 6.2, were generated. Both cell lines were characterized by molecular and pharmacological methods that confirmed both subunits were stably expressed despite the fact that the SUR2B subunits were not under antibiotic selection. The pharmacological characteristics of these different K_{ATP} channels were evaluated using structurally distinct K^+ channel openers in FLIPR assays with the membrane potential sensitive dye $DiBAC_4(3)$. It was found that channels containing either SUR2B exon 17 − or SUR2B exon 17+ subunits with K_{IR} 6.2 shared the same rank order potency with structurally distinct K^+ channel openers as those measured for guinea pig bladder. However, the relative potencies of the predominant splice variant in bladder more closely resemble those measured in native guinea pig bladder cells under similar assay conditions (Gopalakrishnan et al., 1999; Turner et al., 2003; Davis-Taber et al., 2003).

The SUR2B exon 17 − /K_{IR} 6.2 cell line was further assessed in binding studies with the dihydropyridine radioligand, [^{125}I]A-312110 which gave a K_d value of 3.52 nM, which is comparable to that previously reported in bladder membranes (4.9 nM) (Davis-Taber et al., 2003). Likewise, comparison of the rank order potencies of inhibition of binding by K^+ channel openers from diverse chemical series, showed an excellent correlation between guinea pig bladder and transfected cell membranes, with the exception of the compound ZD6169. The reason for this discrepancy is unknown but may be due to the structural class. To address this issue further would require analysis of additional members of the tertiary carbonyl series which is outside the scope of the present study.

In conclusion, the molecular composition of K_{ATP} channels expressed in urinary bladder smooth muscle with respect to the SUR2B subunit was evaluated. Comparison of the pharmacological properties of the splice variant SUR2B exon 17 − together with K_{IR} 6.2 heterologously expressed in mouse fibroblast L-cells with K_{ATP} channels natively expressed in guinea pig bladder showed excellent correlation in functional studies. Additionally, the high affinity binding of [^{125}I]A-312110 that compares well with native bladder K_{ATP} channels also supports the notion that SUR2B exon 17 − is a major component in this tissue. Collectively, this data suggests that the SUR2B exon 17 − splice variant most closely resembles native K_{ATP} channels in bladder smooth muscle and thereby provides a useful tool for studying K_{ATP} channels and therapeutic agents that modulate these channels with the potential for the treatment of bladder disorders such as overactivity and lower urinary tract symptoms.

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