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Pharmacological and molecular analysis of ATP-sensitive K^+ channels in the pig and human detrusor

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Abstract

The pharmacological and molecular properties of ATP-sensitive K^+ channels present in pig detrusor smooth muscle were investigated. In isolated pig detrusor strips, ATP-sensitive K^+ channel openers inhibited contractions elicited by low frequency field-stimulation in a concentration-dependent manner. The inhibitory effects of P1075 [*N*-cyano-*N*′-(1,1-dimethylpropyl)-*N*″-3-pyridylguanidine] were attenuated by glyburide with a pA_2 value of 7.38 (slope = 1.08). The potency of the inhibitory effects of the K^+ channel openers on the field-stimulated contractions correlated well with those evoked by the muscarinic receptor agonist, carbachol ($r = 0.93$) and furthermore, to relaxation of the pre-contracted (25 mM potassium chloride, KCl) human detrusor ($r = 0.95$). Reverse transcriptase polymerase chain reaction (RT-PCR) analysis showed the presence of mRNA for sulfonylurea receptors SUR1 and SUR2B in both pig and human detrusor. Considering the similarities in the molecular and pharmacological profile of ATP-sensitive K^+ channels between the pig and the human detrusor, it is concluded that the pig detrusor may serve as a suitable in vitro model for the evaluation of novel K^+ channel openers with potential use in urological disorders in humans.

Keywords: K^+ channel opener; Detrusor smooth muscle; Smooth muscle relaxation; K^+ channels, ATP-sensitive; Sulfonylurea receptor

1. Introduction

Recent progress in the understanding of the molecular diversity and tissue localization of ATP-sensitive K^+ channels have provided a molecular correlate to earlier physiological and pharmacological studies demonstrating an important role in signal processing in many excitable and non-excitable tissues (Isomoto et al., 1997; Jan and Jan, 1997). ATP-sensitive K^+ channels function as key regulators of the resting membrane potential in cells. Opening of these channels results in hyperpolarization of the cell and a consequent decrease in cellular (hyper)excitability (Sakman and Trube, 1984). Therefore, ATP-sensitive K^+ channels can modulate contraction of smooth muscle (Quayle et al., 1997), insulin secretion (Dunne and Peterson, 1991), and synaptic transmission in the central and peripheral nervous system (Freedman and Lin, 1996). Advances in molecular biology have revealed a heterogeneity of ATP-sensitive K^+ channels encompassing different subtypes that display marked differences in localization and biophysical properties (Isomoto et al., 1997; Bryan and Aguilar-Bryan, 1999).

Bladder overactivity is a condition characterized by unwanted contractions of the urinary bladder that can be of neurogenic origin through spinal or supraspinal reflexes (De Groat, 1997) or of myogenic origin, (Brading, 1997). The overactive bladder is referred to as unstable when the etiology is non-neurogenic and as hyperreflexic when the etiology is neurogenic (Hampel et al., 1997). Activation of bladder smooth muscle ATP-sensitive K^+ channels can hyperpolarize the cell as well as function to decrease afferent signaling from the bladder to spinal/supraspinal sites. Thus, it is possible that activators of ATP-sensitive K^+ channels selective for the lower urinary tract may have beneficial effects on both the myogenic and neurogenic components of urge incontinence.

The pharmacology of K^+ channel openers has been investigated in guinea pig, and rat detrusor strips (Edwards

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et al., 1991; Zografos et al., 1992; Li et al., 1995, 1996; Gopalakrishnan et al., 1999) and in pig and human detrusor strips (Foster et al., 1989; Malmgren et al., 1990). These in vitro studies demonstrated that K^+ channel openers like (−)-cromakalim [(−)-trans-6-cyano-3,4-dihydro-2, 2-dimethyl-4-(2-oxo-1-pyrrolidyl)-2*H*-1-benzopyran-3-ol], pinacidil [(±)-*N*-cyano-*N′*-4-pyridinyl-*N″*-(1,2,2-trimethylpropyl)-quanidine], and ZD6169 [*N*-(4-benzoylphenyl)-3,3,3-trifluro-2-hydroxy-2-methylpropion amine], evoke a concentration-dependent relaxation of pre-contracted detrusor strips. The morphology of the urinary bladder of the Landrace pig has been reported to be similar in many ways to that of the human bladder (Crowe and Burnstock, 1989). Recent studies have shown that bladders from the pig may be a more appropriate model than bladders from rodent or canine to reproduce bladder overactivity observed in humans. The urinary bladder from both human and pig demonstrate post-junctional supersensitivity to acetylcholine, K^+ and desensitization to electrical stimulation after outflow obstruction. The urodynamic characteristics of the pig bladder are also similar to that of the human bladder as assessed by cystometry, and a model of detrusor instability similar to that found in humans has been described (Jorgensen et al., 1983; Sibley, 1985; Speakman et al., 1987; Chapple and Smith, 1994). However, a detailed analysis of the pharmacological properties of ATP-sensitive K^+ channels in the pig bladder and comparison of its molecular components has not been carried out. Accordingly, in the present study, the pharmacological properties and molecular composition of the ATP-sensitive K^+ channels present in pig and human bladder were evaluated by a combination of isolated tissue studies and RNA analysis. Our data demonstrate similarities in the molecular and pharmacological profile of ATP-sensitive K^+ channels and suggest that the pig detrusor may serve as a suitable in vitro model for the evaluation of K^+ channel openers with uroselectivity.

2. Materials and methods

2.1. Tissue preparation

Female Landrace pigs weighing 9–25 kg were used. All studies were carried out in accordance with guidelines outlined by the Animal Welfare Act, the Association for Assessment and Accreditation of Laboratory Animals (AAALAC) and the Institutional Animal Care and Use Committee of Abbott Laboratories.

Pigs were sacrificed with an intraperitoneal injection of pentobarbital (Somlethol®) at a lethal dose of 150–200 mg/kg. The entire urinary bladder was removed and placed in Krebs–Ringer bicarbonate solution of the composition, (mM): 120 NaCl, 20 $NaHCO_3$, 11 dextrose, 4.7 KCl, 2.5 $CaCl_2$, 1.5 $MgSO_4$, 1.2 KH_2PO_4, equilibrated with 5% CO_2: 95% O_2 (pH = 7.4 at 37°C). (±)-Propranolol, 4.0 μM, was included in all assays to ensure β-adrenoceptor blockade (Grimes et al., 1987; Constantine et al., 1982). The bladder was sectioned into three pieces discarding the top dome portion and the lower trigonal area. The remaining tissue (detrusor) was sliced horizontally into approximately 4 × 10-mm strips. The mucosal layer was removed and discarded. Strips were mounted in 10-ml tissue baths maintained at 37°C with one end fixed to a stationary rod and the other to a Grass FT03 transducer at a basal pre-load of 1.0 g. The stationary rods contained parallel platinum electrodes that fit on each side of the tissue strips.

Tissues were rinsed at 10-min intervals and allowed to equilibrate for at least 60 min before being exposed to 80-mM potassium chloride (KCl). This initial exposure to KCl caused maximum contraction of the smooth muscle strips and improved the reproducibility of the response to subsequent stimulation by exogenous agents. The tissues were stimulated with a Grass model S88 stimulator at a frequency of 0.05 Hz, duration of 0.5 ms, 20 V, and current < 150 mA, except in cases where indicated. The voltage was adjusted to be supramaximal in terms of the evoked twitch. A voltage–current amplifier circuit was placed between the stimulator and the electrodes to maintain constant stimulation parameters. The electrical stimulus was set to deliver square wave pulses for the duration of the protocol. These parameters produced uniform, single shock twitches that returned to baseline levels (1.0 g) between each stimulus. These twitches were completely abolished by 100-nM tetrodotoxin. Tissue sensitivity was initially assessed by generating a cumulative inhibitory–response curve to P1075, [*N*-cyano-*N′*-(1,1-dimethylpropyl)-*N″*-3-pyridylguanidine], a pinacidil analog that has been shown to potently stimulate $^{86}Rb^+$ efflux and to relax contractions in the rat aorta (Quast et al., 1993). After a 75-min rinsing period, a second concentration–response curve was generated for the test compound. The concentration-dependent reduction in the peak amplitude (measured in grams) was used for calculating the EC_{50} values. In all cases, 10-μM glyburide was added at the conclusion of each concentration–response curve to clarify the interpretation that the effects were mediated via ATP-sensitive K^+ channels. In cases where glyburide sensitivity was further evaluated by Schild analysis, tissues were pre-treated for a 30-min period. For the generation of frequency–response curves, the tissues were stimulated at 0.05, 0.50, 1.0, 2.0, 4.0, 8.0, and 16.0 Hz for 60 s in order to achieve maximum contraction, then rinsed and exposed to P1075 for a 15-min period prior to repeat stimulation. Tissues were washed and the sequence repeated at 15-min intervals with increasing concentrations of the compound.

For comparative experiments using carbachol, the protocol was non-cumulative with rinse cycles between each concentration of K^+ channel opener. The tissues were pre-treated with the K^+ channel opener for 15 min, exposed to a fixed concentration of agonist (100 nM, equipo-

tent to that produced by 0.05 Hz field stimulation) until maximum tension developed and rinsed for 15 min, repeating the cycle for each concentration of K^+ channel opener.

Human bladders were obtained from the Anatomic Gift Foundation (Phoenix, AZ). The detrusor smooth muscle strips were pre-contracted with 25 mM KCl and dose–response curves performed in a cumulative fashion employing a single response curve per tissue. Studies were carried out in accordance with guidelines set forth by the National Cancer Institute, 1987 (Guidelines for Handling Human Tissues and Body Fluids Used in Research).

2.2. RNA preparation and reverse transcriptase polymerase chain reaction (RT-PCR) analysis

Total RNA from Landrace pig and human detrusor tissues were isolated using Trizol reagent according to the manufacturer's instructions (Gibco BRL, Gaithersburg, MD). First strand synthesis of cDNA using random hexamers was prepared as follows. An aliquot (1–2 μg) of DNA-ase I treated total RNA isolated from detrusor and heart from both pig and human 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 $MgCl_2$, 1 mM dNTP and 10 mM dithiothreitol at 25°C for 5 min. 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. The reaction was terminated by incubation at 70°C for 15 min, prior to chilling on ice. RT-PCR was performed using 2–4 μl of cDNA in 50 μl reaction containing 0.4 μM each primer, 200 μM each dNTP, 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, 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 (TBE) polyacrylamide gel.

Since no sequence information is known about the ATP-sensitive K^+ channel subunits in pig, subunit specific species generic primers were designed based upon information known from rat, mouse, and human. Polymerase chain reaction using these generic primers gave products of the expected sizes whose identity was confirmed by DNA sequence analysis. The sequence of the primers used are shown in Table 1 and were designed based upon the subunit sequence information obtained from GenBank; Kir 6.1 (rat [D42145] and human [D50312]); Kir 6.2 (rat [U73626], mouse [U73626] and human [D50582]); SUR1 (rat [L40624] and human [L78207]) and SUR2 (rat [2A: D83598] and mouse [2A: D86037; 2B D86038]). Control reactions were performed where samples in the absence of reverse transcriptase were subjected to PCR as above, as negative controls to ensure that the detected products were not the result of possible DNA contamination and by using corresponding subunit templates from human (SUR1, Kir 6.2 and Kir 6.1) as positive controls to ensure that the primers were annealing specifically. In cases where the expected bands were not detected, total RNA derived from sources known to contain the appropriate mRNAs such as pig heart RNA (Kir 6.1 and SUR2A) were employed as additional positive controls.

2.3. Drugs and chemicals

Glyburide [5-Chloro-*N*-[2-[4-[[[(cyclohexylamino)-carbonyl]amino]-sufonyl]phenyl]ethyl] -2- methoxyben-zamide], diazoxide [7-chloro-3-methyl-2*H*-1,2,4-benzothiadiazine 1,1-dioxide], pinacidil [(±)-*N*-cyano-*N′*-4-pyridinyl-*N″*-(1,2,2-trimethylpropyl)-quanidine], nifedipine [1 ,4 -dihydro-2, 6-dimethyl-4- (2-nitro-phenyl)-3, 5-pyridinedicarboxylic acid dimethyl ester], tetrodotoxin [Octahydro-12-(hydroxymethyl)-2-imino-5,9:7,10a-dimethano-10aH-[1,3]dioxocino[6,5-d]pyrimidine-4,7,10,11,12-pentol]

Table 1
Oligonucleotide primers for RT-PCR analysis

K_{ATP} channel subunit	Species	Primer sequence (location)	Size (bp)
Kir 6.1	Human	F: 5′-GACGGAGAGGCAGGTGAGAG-3′ (−53–34 bp)	737
		R: 5′-CTGGATGCGCACAGAGGCAC-3′ (684–665 bp)	
	Generic	F: 5′-CATCGCAGCGGAGAACCTGCGCA-3′ (48–70 bp)	
		R: 5′-CATTGGTCACACAGACAGCGGA-3′ (370–349 bp)	322
Kir 6.2	Human	F: 5′-ATGCTGTCCCGCAAGGGCATC-3′ (1–21 bp)	636
		R: 5′-GCTGATGATCATGCTCTTGC-3′ (636–617 bp)	
	Generic	F: 5′-GGCTCCTAGTGACCTGCACCA-3′ (810–830 bp)	316
		R: 5′-CCACAGCCACACTGCGCTTGCG-3′ (1126–1105 bp)	
SUR1	Human	F: 5′-GCGTGCAAAAGCTAAGCGAG-3′ (1817–1836 bp)	134
		R: 5′-GACGCTTGCGGTTCACAAC-3′ (1951–1933 bp)	
	Generic	F: 5′-CAGAAACCATGGCTGCTAAA-3′ (2320–2339 bp)	
		R: 5′-TACTGTAGCTTGTGGGTCAC-3′ (2675–2656 bp)	355
SUR2A/B	Generic	F: 5′-GCTGAAGAATATGGTCAAATCTC-3′ (4278–4300 bp)	A: 451
		R: 5′-CGGAGTGTCGTATTCCAAAATA-3′ (4590–4569 bp)	B: 312

Oligonucleotide primers that were used for RT-PCR studies are shown with positions in the sequence indicated at the end of each primer sequence; position 1 represents the A from the ATG start codon. In the case of species generic primers, sequence information from rat, mouse and human were used to design primers that reacted with pig cDNA since no ATP-sensitive K^+ channel sequence information is known in this species.

were obtained from Research Biochemicals International (Natick, MA) and minoxidil sulfate [6-(1-Piperidinyl)-2,4-pyrimidinediamine 3-oxide] from ICN Biochemicals, (Aurora, OH). Other compounds including P1075 [*N*-cyano-*N′*-(1,1-dimethylpropyl)-*N″*-3-pyridylguanidine], ZD6169 [*N*-(4-benzoylphenyl)-3,3,3-trifluro-2-hydroxy-2-methylpropion amine], (−)-cromakalim [(−)-*trans*-6-cyano-3,4-dihydro-2,2-dimethyl-4-(2-oxo-1-pyrrolidyl)-2-*H*-1benzopyran-3-ol], ZM244085 [9-(3-cyanophenyl)-3,4,6,7,9,10-hexahydro-1,8-(2*H*,5*H*)-acridinedione], Bay X-9227 [((−)-*N*-(2-ethoxyphenyl)-*N′*-(1,2,3-trimethylpropyl)-2-nitroethene-1,1-diamine)], Bay X-9228 (enantiomer of Bay X-9227) were synthesized in-house. Stock solutions were prepared fresh daily in water or in DMSO. All other chemicals including (±)-propranolol HCl, isoproterenol HCl, and methyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO). Reagents for RNA isolation and RT-PCR analysis were obtained from Gibco BRL and Perkin Elmer (Norwalk, CT).

2.4. Data analysis

Concentration–response curves were analyzed using a four-parameter curve fitting routine, described previously (Zielinski and Buckner, 1998) similar to ''ALLFIT'' (DeLean et al., 1980). The maximum peak amplitude response was used for analysis. Results were expressed as g of tension and as a percentage of maximum response. Potencies were expressed as the geometric mean of the EC_{50} values ± S.E.M. Differences in EC_{50} values were calculated by analysis of variance (ANOVA), followed by Fisher's probabilistic least significant difference (PLSD) test for significance. Schild analysis was used to determine the antagonist potency and are expressed as pA_2 (Schild, 1947).

3. Results

3.1. Characterization of field-stimulated responses in the pig detrusor

The pig detrusor responded to field stimulation in a frequency-dependent manner with a maximal contractile response of 26.50 ± 2.53 g at 8.0 Hz. Low frequency stimulus (0.05 Hz) produced a continuous transient twitch response (5.58 ± 0.48 g) that returned to baseline values between each pulse. However, stimulus frequencies 1.0 Hz or higher evoked a fused response that reached a maximum within 1 min and then slowly decayed. Since our objective was to determine stimulation parameters that would provide steady-state conditions from which to compare the effects of K^+ channel openers, low frequency stimulation was adopted in subsequent experiments. We did confirm that at lower frequencies the detrusor was more sensitive to α,β-methylene ATP desensitization whereas higher frequencies were more sensitive to atropine blockade and that a combination of both agents completely abolished the twitch as previously reported (Masuda et al., 1995).

P1075, [*N*-cyano-*N′*-(1,1-dimethylpropyl)-*N″*-3-pyridylguanidine], a structural analog of pinacidil, completely inhibited field-stimulated twitch responses in the pig detrusor over the frequency range 0.05–4.0 Hz with similar EC_{50} values (56.8 ± 15.9 to 69.7 ± 5.4 nM), $n \geq 5$, with little or no effect on basal tension. At frequencies ≥ 8.0 Hz, the maximum inhibition, and potency of P1075 began to decline, EC_{50} value at 8 Hz was 682 ± 146 nM, efficacy 65 ± 6.4%, $p < 0.0001$, $n \geq 5$ as compared to data from the 0.05 Hz group. Low-frequency stimulus (0.05 Hz) was chosen for subsequent experiments as it provided steady and consistent responses permitting the generation of two separate concentration–response curves, one with the reference compound followed by the test agent in the same tissue strip.

In an initial set of experiments, we evaluated the nature of electrically evoked contractions as it has been proposed that the contractions evoked by field-stimulation may involve muscarinic, purinergic and other mechanisms (Hoyle et al., 1989). The contractions elicited by low frequency field stimulation were only partially sensitive to atropine (EC_{50} = 10.2 ± 3.11 nM, $n = 4$) leaving a residual compo-

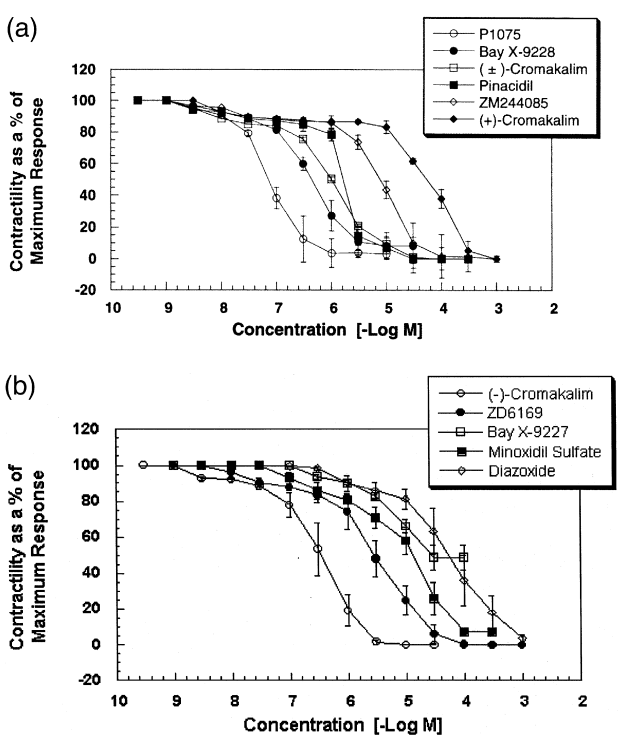

Fig. 1. Concentration–response curves for K^+ channel openers in the field-stimulated pig detrusor. Shown are the effects of P1075, Bay X-9228, (±)-cromakalim, pinacidil, ZM244085 and (+)-cromakalim (panel a) and (−)-cromakalim, ZD6169, Bay X-9227, minoxidil sulfate and diazoxide (panel b). Contractility data are expressed as a percentage of maximum response, mean ± S.E.M. ($n \geq 4$).

Table 2
Effects of K^+ channel openers on field-stimulated and carbachol-stimulated contractile responses in the pig detrusor

	Field-stimulated (0.05 Hz)		Carbachol-stimulated (100 nM)	
	EC_{50} ± S.E.M. (μM)	Inhibition[a]	EC_{50} ± S.E.M. (μM)	Relaxation[b]
P1075	0.057 ± 0.04	100	0.025 ± 0.003	100
(−)-Cromakalim	0.398 ± 0.17	100	0.363 ± 0.05	100
Bay X-9228	0.490 ± 0.10	92	0.063 ± 0.003	100
(±)-Cromakalim	1.5 ± 0.8	100	0.759 ± 0.05	100
Pinacidil	2.0 ± 0.3	100	0.457 ± 0.05	100
ZD6169	3.4 ± 1.8	100	0.692 ± 0.3	97
Bay X-9227	6.3 ± 1.4	52	1.5 ± 0.6	100
ZM244085	9.8 ± 1.9	99	4.1 ± 0.4	100
Minoxidil Sulfate	11.0 ± 3.7	93	25.1 ± 0.8	100
Diazoxide	66.1 ± 15.5	100	19.1 ± 1.0	100
(+)-Cromakalim	79.4 ± 13.6	100	16.2 ± 2.5	100

Shown are the potency and relative efficacy values of the K^+ channel openers examined in the field-stimulated and carbachol-stimulated pig detrusor smooth muscle. Data are expressed as means ± S.E.M. ($n = 4–8$). Inhibition[a], relaxation[b], efficacies expressed as percentage of maximum response relative to P1075.

nent of about 57% of the control response. Application of tetrodotoxin (100 nM) completely inhibited the contractile response and was reversible with rinsing (data not shown). Removal of Ca^{2+} from the assay buffer caused a rapid cessation of the twitch response that returned to control values when Ca^{2+} was re-introduced (data not shown).

3.2. Effects of K^+ channel openers on field-stimulated (0.05 Hz) contractions of the pig detrusor

A structurally diverse class of compounds including pinacidil [(±)-*N*-cyano-*N′*-4-pyridinyl-*N″*-(1,2,2-trimethylpropyl)-quanidine], (−)-cromakalim [(−)-trans-6-yano-3,4-dihydro-2,2-dimethyl-4-(2-oxo-1-pyrrolidyl)-*H*-1-sbenzopyran-3-ol] and ZD6169 [*N*-(4-benzoylphenyl)-3,3,3-trifluro-2-hydroxy-2-methylpropion amine], inhibited field-stimulated responses in a concentration-dependent manner in the pig detrusor (Fig. 1 a,b). Application of glyburide (10 μM) restored the contractile activity that was reduced by the K^+ channel openers. The rank order of potencies for the K^+ channel openers examined was P1075 > [(−)-cromakalim = Bay X-9228] > [(±)-cromakalim = pinacidil = ZD6169] > [Bay X-9227 = ZM244085 = minoxidil sulfate] > [diazoxide = (+)-cromakalim]. The EC_{50} values and maximum efficacies are summarized in Table 2. Within the set of compounds examined, P1075 was the most potent agent with an EC_{50} value of 57 nM, which was about 35-fold more potent than its structural analog pinacidil. The enantiomers of cromakalim exhibited stereoselectivity as previously demonstrated in both vascular and non-vascular tissues(Zini et al., 1991; Bishop and Doggrell, 1994) with (−)-cromakalim being the most potent (EC_{50} = 398 nM). Similar enantioselectivity was noted with the nitroethylene analogs Bay X-9228 and Bay X-9227 [((−)-*N*-(2-ethoxyphenyl)-*N′*-(1,2,3-trimethylpropyl)-2-nitroethene-1,1-diamine)]. ZD6169 [*N*-(4-benzoylphenyl)-3,3,3-trifluro-2-hydroxy-2-methylpropion amine], an anilide tertiary carbinol inhibited contractile response with an EC_{50} = 3.4 μM whereas ZM244085 [9-(3-cyanophenyl)-3,4,6,7,9,10-hexahydro-

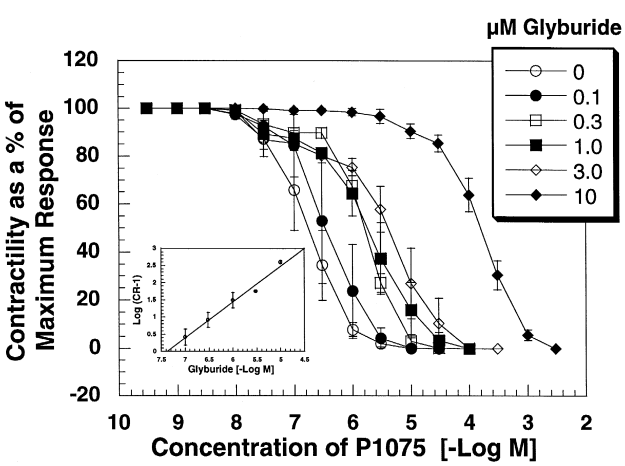

Fig. 2. Concentration-dependent displacement of the P1075 inhibitory–response curve in the field-stimulated pig detrusor by glyburide. Contractility data are expressed as a % of maximum response, ±S.E.M. Inset: Schild analysis of the data (n ≥ 4 for each concentration).

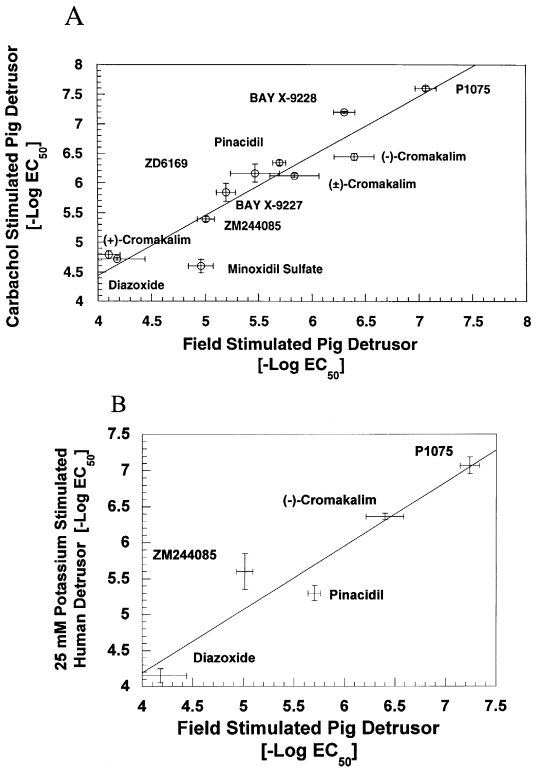

Fig. 3. Correlation of potencies of K^+ channel openers to inhibit field-stimulated and relax carbachol (100 nM) stimulated contractions in the pig detrusor (panel A) and correlation of potencies of K^+ channel openers to inhibit field-stimulated and relaxation of 25 mM KCl stimulated contractions of the human detrusor (panel B). Data are expressed as $-\log EC_{50} \pm$S.E.M. ($n \geq 4$).

1,8-(2*H*,5*H*)-acridinedione], was 3-fold weaker with an EC_{50} of 9.8 μM. Diazoxide [7-chloro-3-methyl-2*H*-1,2,4-benzothiadiazine 1,1-dioxide], was considerably weaker ($EC_{50} = 66.1$ μM).

3.3. Effects of glyburide on K^+ channel opener-mediated inhibition of the field-stimulated twitch in the pig detrusor

In detrusor strips contracted with field-stimulation, glyburide by itself had no effect on the evoked twitch or baseline tension. However, the inhibitory effects of P1075 were reversed by glyburide in a concentration-dependent manner (Fig. 2). Glyburide evoked parallel shifts of the P1075 concentration–response curves with no reduction in the maximum response. Schild analysis of the data yielded a slope of 1.08 ± 0.09 and pA_2 value equal to 7.38 ± 0.43 ($n = 19$; Fig. 2 inset).

3.4. Effects of K^+ channel openers on carbachol-evoked contractions of the pig detrusor

Since activation of the muscarinic M_3 receptor by acetylcholine serves as the physiological stimuli for contraction of the detrusor smooth muscle, we examined the effects of the K^+ channel openers on carbachol-evoked contractions. Carbachol, 10 nM–30 μM, evoked concentration-dependent increases in contractions ($EC_{50} = 977$ nM; maximal contractile response, 15.00 ± 2.29 g; $n = 8$). However, unlike low frequency field-stimulation, the maximal tension produced by carbachol was not sustained and hence cumulative concentration–response curves could not be generated.

The potencies of K^+ channel openers to inhibit field-stimulated responses correlated well with those obtained for the relaxation of tissue strips stimulated with 100 nM carbachol (Table 2). As shown in Fig. 3A, a linear coefficient of regression of 0.93 and a slope of 1.0 was obtained indicating a significant correlation.

It should be noted that in addition to carbachol, other exogenous agents including serotonin, ATP, histamine and depolarization with low KCl (25 mM) evoked contractions similar to carbachol with EC_{50} values and (maximum responses) of 170.0 ± 45.0 nM (10.56 g) $n = 4$; 1.1 ± 0.65 μM (7.43 g) $n = 4$; 6.6 ± 0.80 μM (4.58 g) $n = 8$; and 30.9 ± 3.0 mM (31.62 g) $n = 4$, respectively. Further, when the concentrations of the above agents were adjusted to match the contractions produced by field-stimulation (0.05 Hz), the potency of P1075 to inhibit evoked contractions was comparable (Table 3).

3.5. Analysis of ATP-sensitive K^+ channels in the human detrusor

In a limited set of experiments, K^+ channel openers were tested for their effects in relaxing human detrusor strips pre-contracted with KCl (25 mM). The rank order of potency (EC_{50}) was P1075 (85.1 ± 28.1 nM) > (−)-cromakalim (437.0 ± 47.3 nM) > [ZM244085 (2.5 ± 2.0 μM) = pinacidil (5.0 ± 1.4 μM)] > diazoxide (70.8 ± 17.6 μM). Comparison of K^+ channel opener potencies obtained from the human detrusor to those generated from the field-stimulated pig detrusor yielded a coefficient of linear regression of 0.95 and a slope of 0.88 (Fig. 3B). Further comparison of the results from human detrusor with that of the 25 mM KCl stimulated pig detrusor, yielded a similar coefficient of linear regression, 0.97 and a slope of 0.95.

To investigate the molecular components of ATP-sensitive K^+ channels in the pig detrusor and to compare them

Table 3
Comparison of the potencies of P1075 in the pig detrusor stimulated with various agonists

Agonist	Concentration	Response ± S.E.M. (g)	Potency EC_{50} ± S.E.M. (nM)
Serotonin	3.0 μM	2.10 ± 0.10	18.2 ± 5.3
Carbachol	0.1 μM	2.98 ± 0.60	18.2 ± 5.4
ATP	3.0 μM	5.71 ± 0.39	22.4 ± 9.7
Histamine	3.0 μM	2.69 ± 0.30	12.6 ± 3.3
KCl	25 mM	4.63 ± 0.74	38.0 ± 12.7

The concentration of agonists was chosen so as to generate tension responses to a level equi-effective to that generated by field stimulation (0.05 Hz). The efficacy of P1075 was 100% in all cases. Data are expressed as maximum response in grams of contractility ± S.E.M. and potency, EC_{50}, ± S.E.M., $n \geq 4$.

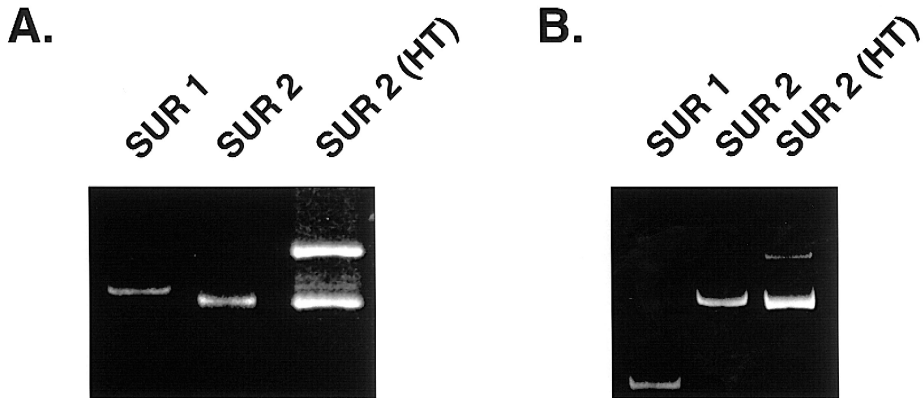

Fig. 4. Analysis of ATP-sensitive K^+ channel subunits using total RNA isolated from pig (panel A) and human (panel B) detrusor. Products of the expected size (see Table 1) were amplified for SUR1 and SUR2B from both species. No message was detected for SUR2A although a fragment of the correct size corresponding to SUR2A was amplified from heart (HT) in a positive control (top band, SUR2A; bottom band, SUR2B). Negative controls lacking reverse transcriptase in the cDNA synthesis step did not reveal any detectable band.

with those from the human detrusor, we carried out RT-PCR studies. RNA was isolated from Landrace pig (three different bladders) and human detrusor tissue (two different bladders) reverse transcribed to generate cDNA prior to amplification by PCR. This yielded products of the predicted sizes for SUR1 (355 bp) and SUR2B (312 bp) in the pig and SUR1 (134 bp) and SUR2B (312 bp) in the human (Fig. 4). Analysis of the inward rectifier K^+ channel subunits revealed the presence of Kir 6.2 (316 bp) in the pig and both Kir 6.2 (636 bp) and Kir 6.1 (737 bp) in the human. Each of the products was sequenced to confirm its identity. No message corresponding to SUR2A was detected in both species while positive controls for these subunits were found in heart for both of these species.

4. Discussion

4.1. Summary

Our present studies demonstrate that the profile of K^+ channel openers to inhibit field-stimulated contractions in the pig detrusor compares well with those evoked by the muscarinic receptor agonist, carbachol and furthermore, to relaxation of the pre-contracted human detrusor. In support of the latter, RT-PCR analysis demonstrated mRNA for the drug binding sulfonylurea receptors, SUR2B and SUR1 in both pig and human detrusor.

4.2. Pharmacology of ATP-sensitive K^+ channels in the field-stimulated pig detrusor

Low frequency field-stimulation provides a reproducible twitch response that allowed for the construction of repetitive, cumulative inhibition curves. Analogs belonging to various structural classes including benzopyrans, cyanoguanidines and nitroethylenes all inhibited contractile responses of the pig detrusor in a concentration-dependent manner with a rank order potency profile reported similar to those previously in the guinea pig (Gopalakrishnan et al., 1999). ZD6169, a tertiary carbinol analog with demonstrated in vivo uroselectivity (Howe et al., 1995) was equipotent to (±)-cromakalim and pinacidil. Glyburide was effective in inhibiting or reversing the effects of all K^+ channel openers examined suggesting that the site of action was the ATP-sensitive K^+ channel. The potency of glyburide to inhibit the responses of P1075 was independent of the inhibitor concentration used suggesting functional competitive antagonism (parallel shifts, no reduction in maximum response and slopes of unity) as previously demonstrated in vasculature and other tissues (Eltze, 1989; Zini et al., 1991). Since interactions of glyburide and P1075 with the sulfonylurea receptors are possibly allosteric in nature, this may reflect equal but opposite contributions of the antagonist and activator effects. (Bray and Quast, 1992). It should be noted that glyburide had no effect on the baseline tension or the maximum amplitude of the twitch of the detrusor smooth muscle.

4.3. Carbachol-evoked responses in the pig detrusor: modulation by K^+ channel openers

Application of carbachol produced a contractile response that differed in kinetics, onset and decay compared to the rapid twitch response evoked by field-stimulation. However, a good correlation of potencies for K^+ channel openers in relaxing/inhibiting contractions evoked by the two stimuli was noted. The potencies of K^+ channel openers to relax carbachol-evoked contractions were consistently half-log unit higher than those observed for the inhibition of field-stimulated contractions. This may be attributed to the fact that field stimulation releases transmitters other than just those causing muscarinic receptor activation such as ATP, necessitating the need for higher concentrations of the K^+ channel openers.

Neuronally mediated contractions of the pig bladder has been extensively reviewed and compared to that observed in the human (Sibley, 1984). The partial inhibition by atropine of the field-stimulated twitch responses provides

evidence that the released acetylcholine significantly contributes to the functional response. Our studies also show a significant non-adrenergic, non-cholinergic (NANC) component (57% residual) after atropine treatment consistent with earlier observations demonstrating that this NANC component could be attributed to the release of ATP (Sibley, 1984; Burnstock et al., 1972, 1978; Hoyle et al., 1989; Hashitani and Suzuki, 1995). For example, Sibley (1984) using the pig bladder demonstrated that electrically evoked contractions might be attributed to the co-release of ATP and acetylcholine. The NANC component observed in our study (57%) is higher than reported by Sibley (22%). This may be attributed to the lower frequency (0.05 Hz) used in our study that may release a higher proportion of ATP than the higher frequency (1.0 Hz) employed by Sibley (1984). The ability of tetrodotoxin to completely eliminate the evoked twitch responses in our study supports a role for released neurotransmitters in mediating the contractile response. It has been suggested that the normal human bladder has little or no atropine resistant component, whereas under diseased conditions, a significant amount of the contraction (50%) becomes atropine-resistant (Sjögren et al., 1982). Therefore, contractions elicited by field stimulation in the pig detrusor may be more similar to those seen in the diseased human bladder.

Although higher frequencies of field stimulation do not maintain a steady state of contractility, the potency and efficacy of P1075 was similar over a wide range of frequencies (0.05–4.0 Hz). Exogenous agents such as serotonin, carbachol, ATP, histamine and KCl also evoked contractions of the detrusor smooth muscle. However, none of these agents could be used for the generation of repetitive concentration–responses in a cumulative fashion since the stimulated responses tended to wane. It should be noted that when the concentrations of the above agents were adjusted to match the contractions produced by the field-stimulation (0.05 Hz), the potency of P1075 to inhibit evoked contractions were comparable. This suggests that the effect of K^+ channel openers on bladder relaxation is independent of the pathway involved in triggering contractile responses.

4.4. Analysis of ATP-sensitive K^+ channels in the human detrusor

KCl (25 mM) was used to produce a contractile response. In our initial studies, low frequency stimulation of the human detrusor strips did not evoke a reliable response. Sibley (1984) suggested that in the human detrusor smooth muscle, a single electrical impulse was incapable of releasing enough transmitters from the intramural nerve endings to evoke a response.

Our current findings extend previous observations that the inhibitory effect of K^+ channel openers such as cromakalim are mediated by ATP-sensitive K^+ channels in the human detrusor (De Moura et al., 1993). The relative potencies of the limited number of K^+ channel openers evaluated in the human detrusor including P1075, (−)-cromakalim, pinacidil, ZM244085 and diazoxide showed a good correlation with the data obtained from the pig detrusor suggesting similarities in ATP-sensitive K^+ channels expressed in these tissues. This is supported by RT-PCR data demonstrating a presence of the drug binding sulfonylurea receptors, SUR2B and SUR1 in both the pig and human detrusor.

Since no sequence information was previously known for pig ATP-sensitive K^+ channel subunits, the data obtained from sequencing each of the fragments that were amplified in these reactions yielded novel information. Comparison of this data obtained from pig bladder with that from human tissue showed that in the regions amplified, the homology is 87% for Kir 6.2, 99% for SUR1 and 94% for SUR2B. A single difference noted between the pig and human bladder was the presence of message for Kir 6.1 in human preparations, which was not detected in the pig detrusor. Although the implications of this molecular difference remains to be addressed, it may be reasoned that the activities of K^+ channel openers described in our study demonstrates an interaction with the binding SUR subunit, which were found to be similar in both species. Our study also provides preliminary evidence that channels may exist as a hetero-oligomer composed of SUR2B, SUR1, Kir6.2 (and/or Kir6.1) although additional studies at the protein level using subunit-specific antibodies are warranted to elucidate the molecular composition of native ATP-sensitive K^+ channels.

In conclusion, our study provides evidence that the pharmacological and molecular properties of ATP-sensitive K^+ channels present in pig and human detrusor smooth muscle are similar, a notion that had previously been proposed (Crowe and Burnstock, 1989; Chapple and Smith, 1994; Sibley, 1985). The field-stimulated pig detrusor that has been well characterized in the present study provides a rapid in vitro model for identifying K^+ channel openers with potential for alleviating the symptoms of an overactive bladder.

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