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[^{125}I]A-312110, a Novel High-Affinity 1,4-Dihydropyridine ATP-sensitive K^+ Channel Opener: Characterization and Pharmacology of Binding

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ABSTRACT

Although ATP-sensitive K^+ channels continue to be explored for their therapeutic potential, developments in high-affinity radioligands to investigate native and recombinant K_{ATP} channels have been less forthcoming. This study reports the identification and pharmacological characterization of a novel iodinated 1,4-dihydropyridine K_{ATP} channel opener, [^{125}I]A-312110 [(9R)-9-(4-fluoro-3-^{125}iodophenyl)-2,3,5,9-tetrahydro-4H-pyrano[3,4-*b*]thieno[2,3-*e*]pyridin-8(7H)-one-1,1-dioxide]. Binding of [^{125}I]A-312110 to guinea pig cardiac (K_D = 5.8 nM) and urinary bladder (K_D = 4.9 nM) membranes were of high affinity, saturable, and to a single set of binding sites. Displacement of [^{125}I]A-312110 by structurally diverse potassium channel openers (KCOs) indicated a similar rank order of potency in both guinea pig cardiac and bladder membranes (K_i, heart): A-312110 (4.3 nM) > *N*-cyano-*N*′-(1,1-dimethylpropyl)-*N*″-3-pyridylguanidine (P1075) > (−)-*N*-(2-ethoxyphenyl)-*N*′-(1,2,3-trimethylpropyl)-2-nitroethene-1,1-diamine (Bay X 9228) > pinacidil > (−)-cromakalim > *N*-(4-benzoyl phenyl)-3,3,3-trifluro-2-hydroxy-2-methylpropionamine (ZD6169) > 9-(3-cyanophenyl)-3,4,6,7,9,10-hexahydro-1,8-(2H,5H)-acridinedione (ZM244085) ≫ diazoxide (16.7 μM). Displacement by K_{ATP} channel blockers, the sulfonylurea glyburide, and the cyanoguanidine *N*-[1-(3-chlorophenyl)cyclobutyl]-*N*′-cyano-*N*″-3-pyridinyl-guanidine (PNU-99963) were biphasic in the heart but monophasic in bladder with about a 100- to 500-fold difference in K_i values between high- and low-affinity sites. Good correlations were observed between cardiac or bladder-binding affinities of KCOs with functional activation as assessed by their respective potencies to either suppress action potential duration (APD) in Purkinje fibers or to relax electrical field-stimulated bladder contractions. Collectively, these results demonstrate that [^{125}I]A-312110 binds with high affinity and has an improved activity profile compared with other radiolabeled KCOs. [^{125}I]A-312110 is a useful tool for investigation of the molecular and functional properties of the K_{ATP} channel complex and for the identification, in a high throughput manner, of both novel channel blockers and openers that interact with cardiac/smooth muscle-type K_{ATP} channels.

ATP-sensitive potassium (K_{ATP}) channels belong to a family of weak inwardly rectifying K^+ channels that are gated by changes in intracellular ATP/ADP levels thereby coupling cellular metabolism to membrane excitability (Nichols and Lederer, 1991). These channels play critical roles in modulating processes such as insulin secretion, leptin release, synaptic transmission, and excitability of cardiac, vascular, and nonvascular smooth muscle. K_{ATP} channel blockers, such as glyburide and glipizide, are currently used in the management of type II diabetes. Activation of K_{ATP} channels by a variety of structurally diverse chemotypes, including benzopyrans, cyanoguandines, and dihydropyridines, has been increasingly examined for several therapeutic applications. Initially, K_{ATP} channel openers were investigated as

ABBREVIATIONS: K_{ATP}, ATP-sensitive potassium channel; SUR, sulfonylurea receptor; P1075, *N*-cyano-*N*′-(1,1-dimethylpropyl)-*N*″-3-pyridylguanidine; PKF217-744, (3*S*,4*R*)-*N*-[3,4-dihydro-2,2-dimethyl-3-hydroxy-6-(2-methyl-4-pyridinyl)-2H-1-benzopyran-4-yl]-3-[2,6-^{3}H]pyridinecarboxamide; [^{125}I]A-312110, (9*R*)-9-(4-fluoro-3-iodophenyl)-2,3,5,9-tetrahydro-4H-pyrano[3,4-b]thieno[2,3-*e*]pyridin-8(7H)-one 1,1-dioxide; ZD6169, *N*-(4-benzoyl phenyl)-3,3,3-trifluro-2-hydroxy-2-methylpropionamine; ZM244085, 9-(3-cyanophenyl)-3,4,6,7,9,10-hexahydro-1,8-(2H,5H)-acridinedione; Bay X 9228, (−)-*N*-(2-ethoxyphenyl)-*N*′-(1,2,3-trimethylpropyl)-2-nitroethene-1,1-diamine; PNU-99963, *N*-[1-(3-chlorophenyl)cyclobutyl]-*N*′-cyano-*N*″-3-pyridinyl-guanidine; APD, action potential duration; A-278637, (−)-(9*S*)-9-(3-bromo-4-fluorophenyl)-2,3,5,6,7,9-hexahydrothieno[3,2-*b*]quinolin-8(4H)-one 1,1-dioxide; KCO, potassium channel opener; $DiBAC_4(3)$, bis-(1,3-dibutylbarbituric acid)trimethine oxonol.

antihypertensive agents; more recently, however, newer compounds have been studied for their cardioprotective properties and for the treatment of nonvascular smooth muscle disorders such as overactive bladder (Atwal and Grover, 1996; Andersson, 2000; Coghlan et al., 2001).

Considerable effort in the cloning and expression of K_{ATP} channel subunits has resulted in the elucidation of functional and pharmacological properties of diverse channel combinations. K_{ATP} channels are heteromeric complexes composed of four inwardly rectifying K^+ channel subunits belonging to $K_{IR}6.1$ or $K_{IR}6.2$ and four regulatory proteins, the sulfonylurea receptor (SUR) (Aguilar-Bryan and Bryan, 1999; Seino, 1999). The inwardly rectifying K^+ channel subunits are responsible for ion permeation and contain the primary site for ATP inhibition of K_{ATP} channel activity, whereas the sulfonylurea receptors are relatively larger proteins belonging to the ATP-binding cassette superfamily that hosts binding sites for nucleotides, sulfonylureas, and potassium channel openers. Functional analysis of combinations of the various SUR isoforms (SUR1, SUR2A, and SUR2B) with inwardly rectifying K^+ channel subunits ($K_{IR}6.1$ or $K_{IR}6.2$ subunits) has revealed K_{ATP} channels with distinct biophysical and pharmacological properties. SUR2A-$K_{IR}6.2$ is the proposed subunit combination of K_{ATP} channels expressed in the heart (Inagaki et al., 1996; Okuyama et al., 1998). On the other hand, the SUR2B isoform in conjunction with $K_{IR}6.1$ or $K_{IR}6.2$ is thought to constitute the diverse smooth muscle type K_{ATP} channels (Fujita and Kurachi, 1999; Cui et al., 2001). Although molecular analysis has proved to be of great importance toward understanding the nature and diversity of K_{ATP} channels, the precise correlation of biophysical characteristics, nucleotide sensitivity, and pharmacological properties of native K_{ATP} channels with subtypes derived from various recombinant heteromeric combinations continues to be an active area of investigation.

Radioligand binding studies from several laboratories during the past decade has shed light on potassium channel opener, blocker, and nucleotide interactions with sulfonylurea receptors (Uhde et al., 1999; Hambrock et al., 2001). [^{3}H]P1075 has been a widely used radioligand from the cyanoguanidine series in the examination of native channels and, more recently, with channels derived from recombinant SUR2A and SUR2B combinations (Loffler-Walz and Quast, 1998; Schwanstecher et al., 1998; Hambrock et al., 1999). However, the utility of this agent as a tool to study K_{ATP} channels, especially at levels expressed in native tissues, is somewhat limited because of its relatively weak binding affinity, low specific activity, and signal-to-noise ratio. More recently, a benzopyran radioligand, [^{3}H]PKF217-744, has emerged (Manley et al., 2001). Although the pharmacological profile of this novel ligand compares well with studies using [^{3}H]P1075, the affinity of [^{3}H]PKF217-744 is about 20-fold lower than [^{3}H]P1075. Because these known ligands are tritiated with relatively low specific activity, identification of a radioligand with improved properties to enable screening for novel potassium channel openers in a high-throughput fashion and to further investigate the mechanism of action of potassium channel modulators with native and recombinant K_{ATP} channels would be highly desirable. The present study reports on the identification and pharmacological characterization of a novel iodinated 1,4-dihydropyridine K_{ATP} channel opener, [^{125}I]A-312110. The study shows that [^{125}I]A-312110 binds with high affinity to native cardiac and bladder membranes and interacts with both openers and blockers with rank-order potencies that compare well with functional K_{ATP} channel activation.

Materials and Methods

Materials. A-312110 was synthesized as described by Carroll et al. (2001). 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. [^{125}I]A-312110 routinely had a radiochemical purity of >99% as determined by high-performance liquid chromatography. (−)-Cromakalim, P1075, ZD6169, ZM244085, Bay X 9228, and PNU-99963 were synthesized in house or obtained from the Abbott compound library. Other compounds including glyburide and pinacidil were purchased from RBI/Sigma (Natick, MA). Unlabeled isradipine was obtained from Dr. D. J. Triggle (SUNY, Buffalo, NY). Compounds were prepared as 10 mM stock solutions in 100% dimethyl sulfoxide and diluted before use. All other chemicals and inorganic salts were purchased from Sigma Chemical Co. (St. Louis, MO).

Membrane Preparation. Cardiac membranes were prepared by quickly removing the ventricles from male guinea pigs (300–400 g; Charles River Laboratories, Wilmington, MA) and placing them 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) containing protease inhibitors (Complete tablets; Roche Applied Science, Indianapolis, IN). Tissues were separated from any adherent connective tissue, chopped into small pieces, and then homogenized using a Tekmar Polytron homogenizer at 13,500 rpm at an interval of 2 × 30 s in 15 volume/gram weight. Homogenates were filtered through double layers of prewetted cheesecloth and centrifuged at 500*g* for 10 min at 4°C. The supernatant was transferred into a second centrifuge tube kept on ice. Ice-cold buffer was added to the remaining pellet, and the procedure was repeated. The second supernatant was collected, combined with the first, and both supernatants were centrifuged at 45,000*g* for 45 min at 4°C. The supernatant was discarded and the membrane pellet was resuspended in assay buffer.

Preparation of bladder membranes entailed removing urinary bladders from male guinea pigs (300–400 g; Charles River Laboratories) and placing them in ice-cold buffer (20 mM HEPES and 0.25M sucrose, pH 7.4) containing protease inhibitors (Complete tablets; Roche Applied Science). Bladders were cut into small pieces and then homogenized using a Tekmar Polytron homogenizer at 13,500 rpm at an interval of 2 × 30 s in 15 vol./g wt. The homogenate was centrifuged at 500*g* for 10 min at 4°C. The supernatant was transferred into a second centrifuge tube kept on ice. Ice-cold buffer was added to the remaining pellet, and the procedure was repeated. The second supernatant was collected, combined with the first, and both supernatants were centrifuged at 40,000*g* for 30 min at 4°C. The supernatant was discarded and the membrane pellet was 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) for radioligand binding studies. Protein concentrations of cardiac and bladder membranes were determined using bovine serum albumin as a standard (Lowry et al., 1951).

Radioligand Binding. Before use, the desired amount of [^{125}I]A-312110 was dried under a gentle stream of nitrogen gas to remove ethanol and reconstituted in assay buffer. Membranes (300 μg/tube) 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 of creatine phosphokinase, and 1 mM Na_2ATP) at 37°C for 90 min. These conditions have been previously employed wherein the levels of ATP are consistently maintained because free ADP is converted back to ATP by creatine phosphate and creatine is regenerated to creatine phosphate by the enzyme creatine phosphokinase (Gopalakrishnan et al., 1991; Loffler-Walz and Quast, 1998). To

determine the optimal ATP dependence for maximal specific binding, the assay mixture was incubated with varying concentrations of Na_2ATP (0.01–3 mM). For saturation experiments, membranes were incubated with increasing concentrations of radioligand (0.25–30 nM). In competition experiments, membranes were incubated with varying concentrations of test compounds. Specific radioligand binding was defined by subtracting the nonspecific binding defined by the inclusion of 10 μM unlabeled A-312110 from total binding. Binding was terminated by rapid vacuum filtration through GF/B filters (PerkinElmer Wallac, Boston, MA). Filters were 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% (PerkinElmer Wallac).

To assess the sulfonylurea selectivity of A-312110, [^{3}H]glyburide binding to rat brain membranes was employed as described previously (Gopalakrishnan et al., 1991). Briefly, crude rat brain membranes were homogenized, centrifuged at 45,000*g* for 30 min and the pellet resuspended in ice-cold 50 mM Tris HCL, pH 7.2, containing protease inhibitors (Complete tablets; Roche Applied Science). [^{3}H]Glyburide binding assay was carried out in a final volume of 250 μl with [^{3}H]glyburide (1 nM; PerkinElmer Life Sciences, Boston, MA) and rat brain membranes (100 μg/tube) in the presence of varying concentrations of unlabeled glyburide or A-312110 for 60 min at room temperature. Specific binding was defined by the addition of 10 μM glyburide. Incubations were terminated by rapid vacuum filtration over GF/B filters (PerkinElmer Wallac). Filters were washed three times with ice-cold 50 mM Tris HCl, pH 7.2, and radioactivity bound to the filters was assessed by liquid scintillation spectroscopy at an efficiency of 45% (LS 5000 TD; Beckman Coulter, Fullerton, CA).

Membrane Potential Assays. Changes in membrane potential responses in guinea pig bladder smooth muscle cells were assessed using the bis-oxonol dye $DiBAC_4(3)$ (Molecular Probes, Eugene, OR) in the fluorometric imaging plate reader (Molecular Devices, Sunnyvale, CA) as detailed previously (Gopalakrishnan et al., 1999). Briefly, smooth muscle cells were isolated by enzymatic dissociation from urinary bladders removed from anesthetized male guinea pigs (Charles River Laboratories). The bladder sections were incubated in 5 ml of Dulbecco's phosphate-buffered saline solution (Invitrogen, Carlsbad, CA) containing 1 mg/ml collagenase (type VIII; Sigma) and 0.2 mg/ml pronase (Calbiochem, La Jolla, CA) at 37°C for 30 min. The mixture was spun at 1300*g* and the cell pellet was resuspended in 5 ml of growth media (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 units/ml streptomycin, and 0.25 mg/ml amphotericin B) and plated in clear-bottomed black 96-well plates (ViewPlate-96; PerkinElmer Life Sciences) for fluorescence studies at a density of 20,000 cells/well. Cells were maintained in a cell incubator with 90% air/10% CO_2 for 5 to 7 days. Assays were carried out in buffer (20 mM HEPES, 120 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM glucose, pH 7.4, adjusted with NaOH) 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.

Whole-Cell Patch-Clamp Recording. Whole-cell currents from guinea pig bladder smooth muscle cells were measured using the conventional tight-seal, whole-cell clamp method as described previously (Shieh et al., 2001). Guinea pig urinary bladders were transferred directly into preoxygenated physiological saline solution (137 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 0.42 mM KH_2PO_4, 4.17 mM $NaHCO_3$, 10 mM HEPES, 10 mM glucose, pH 7.4, adjusted with NaOH). Pieces of bladder smooth muscle were incubated with 1 mg/ml collagenase and single smooth muscle cells were obtained by trituration using a fire-polished, large-bore Pasteur pipette. Measurements were carried out within 48 h after cell dissociation, during which period the cells appeared elongated and contractile. The intracellular pipette solution contained 107 mM KCl, 1.2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM EGTA, 5 mM HEPES, and 0.1 mM ATP, pH 7.2, adjusted with KOH; total K^+, 140 mM). The bath solution contained 60 mM KCl, 100 mM NaCl, 2.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 5 mM HEPES, pH 7.4, adjusted with NaOH). The microelectrodes filled with pipette solution had a resistance of 2 to 5 MΩ. After a tight seal was formed, the membrane was ruptured and the capacitance transient integrated on-line to estimate cell capacitance. Uncompensated series resistance was typically 3 to 10 MΩ. The whole-cell currents were amplified using Axopatch-200B amplifier (Axon Instruments, Union City, CA) and low-pass filtered at 5 kHz (−3 dB, 4-pole Bessel filter) before digitization by Digidata 1200B at a sampling rate of 10 kHz (Axon Instruments).

Bladder Strip Relaxation. Urinary bladders were removed and immediately placed in Krebs-Ringer bicarbonate solution (120 mM NaCl, 20 mM $NaHCO_3$, 11 mM dextrose, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.5 mM $MgSO_4$, 1.2 mM KH_2PO_4, equilibrated with 5% CO_2/95% O_2, pH 7.4, at 37°C). 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 to a Grass FT03 transducer at a basal preload of 1.0 g. 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 for field stimulation (0.05 Hz, 0.5 ms at 20 V). Tissues were allowed to equilibrate for at least 60 min before addition of test compounds. Contractions were evoked by electrical field-stimulation using a frequency of 0.05 Hz, 0.5 ms at 20 V. Cumulative concentration response curves were generated for each tissue before the addition of glyburide (10 μM) at the conclusion of the concentration response curve to assess reversibility of K_{ATP} channel-mediated effects.

Purkinje Fiber Activity. The functional effects of KCOs on cardiac K_{ATP} channels were evaluated by assessing potential changes in action potential duration (APD) of canine cardiac Purkinje fibers (Gintant et al., 2001). Free-running fibers were excised, placed in a warmed (37°C) superfusion chamber, and stimulated (0.5 Hz, 2× threshold, biphasic wave form, 1–2 ms in duration) using platinum electrodes located in the chamber floor. Fibers were superfused with Tyrode's solution (131 mM NaCl, 18 mM $NaHCO_3$, 1.8 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 5.5 mM dextrose, 4 mM KCl, 2 mM $CaCl_2$, pH 7.2, at room temperature) and aerated with 95% O_2/5% CO_2. Electrical activity was monitored using 3 M KCl-filled microelectrodes (10–30 mΩ) connected to high impedance electrometers (IE-210; Warner Instruments, Hamden, CT) and recorded digitally (Digidata 1200; Axon Instruments). In most cases, impalements were maintained throughout the experiment. Fibers were considered suitable for study if the following criteria were satisfied: 1) the membrane potential just before the action potential upstroke was negative to −80 mV, 2) the APD range was between 300 and 500 s (spanning ~1.2 S.D. from the mean value of 405 ms), and 3) the normal automatic rate did not exceed the 2-s stimulation cycle length. The APD was defined as the interval between the upstroke and the time at which repolarization was within 10 mV of the maximum diastolic potential. The APD values were measured from the average of three consecutive action potentials.

Data Analysis. The concentration-dependence of maximal steady-state changes in fluorescence, changes in tension responses, and the displacement of specific binding was fitted by nonlinear regression analysis (Prism; GraphPad Software, San Diego, CA) to obtain EC_{50} or IC_{50} values as appropriate. The ligand affinity (equilibrium dissociation constant or K_D) and maximal receptor density (B_{max}) were derived from nonlinear regression analysis of the saturation binding isotherm using GraphPad Prism. The K_i values were calculated from the IC_{50} values (Cheng and Prusoff, 1973). Data are expressed as means ± S.E.M.

Results

Characterization of A-312110 as a K_{ATP} Channel Opener. A range of structurally diverse 1,4-dihydropyridine

analogs with iodo-substitutions in the aromatic ring were synthesized (Carroll et al., 2001) and evaluated for potential K_{ATP} channel opening activity initially in guinea pig urinary bladder smooth muscle cells using a $DiBAC_4(3)$-based membrane potential assay (Fig. 1A). Unlabeled A-312110 evoked concentration-dependent decreases in membrane potential responses with an EC_{50} value of 2.32 nM (log EC_{50} = 8.68 ± 0.06; slope = 1.63 ± 0.11; n = 16; Fig. 1B). This effect was reversed by subsequent addition of glyburide (5 μM).

The effect of A-312110 on K_{ATP} channel currents was directly assessed by whole-cell patch-clamp studies in dissociated guinea pig bladder smooth muscle cells. Application of A-312110 (10 μM) activated an inward current with an amplitude of 56.5 ± 18.6 pA (n = 4) when cells were voltage-clamped at a potential of −80 mV in bath solution containing 140 mM extracellular and 60 mM intracellular K^+. Upon coaddition of 5 μM glyburide with A-312110, the currents reversed to baseline values (Fig. 2A).

Functional effects of A-312110 were also evaluated by assessing electrical field-stimulated contractions of isolated guinea pig detrusor smooth muscle. A stable twitch response in the detrusor strips was produced using low-frequency stimulation (0.1 Hz, 0.5 ms at 20 V) with a mean tension of 3.76 ± 0.14 g (n = 3). Upon addition of A-312110, the low frequency contractions were abolished in a concentration-dependent manner (Fig. 2B). Addition of 10 μM glyburide reversed the effects of A-312110. The IC_{50} value for guinea pig bladder relaxation by A-312110 was 18.5 nM (−log IC_{50} = 7.75 ± 0.09; slope = 1.6 ± 0.11; n = 3; Fig. 2C). Collectively, these studies demonstrate that A-312110 is an opener of K_{ATP} channels in bladder smooth muscle tissues.

Selectivity of A-312110. A-312110, although structurally related to 1,4-dihydropyridine calcium channel antagonists, did not displace the binding of [^{3}H]isradipine to L-type channel sites in rat brain tissues (23 ± 0.6% inhibition at 10 μM). To further assess potential interactions with L-type calcium channels, A-312110 was also evaluated in a concentration-dependent manner (ranging from 0.1 nM to 30 μM) for effects on relaxation of depolarization (80 mM K^+) evoked contractility of aortic strips, conditions in which L-type calcium channel antagonists are highly effective. A-312110 did not inhibit 80 mM K^+ stimulated rat thoracic aorta strips up to 30 μM, under conditions in which the 1.4-dihydropyridine-calcium channel antagonist, nifedipine, effectively relaxed aortic strips (−log IC_{50} = 7.0). Similar lack of interaction with L-type calcium channel has previously been reported with another dihydropyridine K_{ATP} channel opener, A-278637 (Gopalakrishnan et al., 2002).

In a radioligand binding screen that contained representatives of G-protein-coupled receptors as well as many ligand- and voltage-gated ion channel binding sites (receptor binding and enzyme profile; CEREP, Inc., Redmond, WA), A-312110 did not displace binding to more than 70 receptor/ion channel sites (<30% at 10 μM). In particular, A-312110 did not show significant displacement of [^{3}H]glyburide, a high-affinity radioligand for SUR1-containing K_{ATP} channels. Additional [^{3}H]glyburide studies confirmed that A-312110 did not displace the high-affinity SUR1 binding to rat brain membranes, a preparation in which high-affinity binding interactions correlate well with those derived from recombinant SUR1 channels (Gopalakrishnan et al., 2000). Under similar conditions, unlabeled gluburide displaced binding with a K_i value of 0.28 ± 0.09 nM (n_H = 0.72 ± 0.15; n = 3; Fig. 3A) in agreement with previous observations. Accordingly, A-312110 was chosen for labeling with ^{125}I and subsequent radioligand binding studies. Initial experiments using [^{125}I]A-312110 showed high specific binding to both bladder and cardiac membranes and experiments were carried out using both these preparations.

ATP-Dependence of [^{125}I] A-312110 Binding. [^{125}I]A-312110 binding was found to be dependent on the concentration of ATP. The dependence of specific binding as a function of ATP concentration in the presence of an ATP regeneration system (creatine phosphokinase and creatine phosphate) is shown in Fig. 3B. Specific binding increased with increasing concentrations of ATP with maximal binding observed at 0.3 and 1 mM ATP. The EC_{50} value of MgATP to support [^{125}I]A-312110 binding was found to be 105.6 ± 12.9 μM (n = 5). At higher ATP concentrations, 3 and 10 mM, specific binding began to decrease, which could be caused by possible inhibition by ADP, the primary product of ATP hydrolysis that may occur at higher concentrations (Gopalakrishnan et al., 1991).

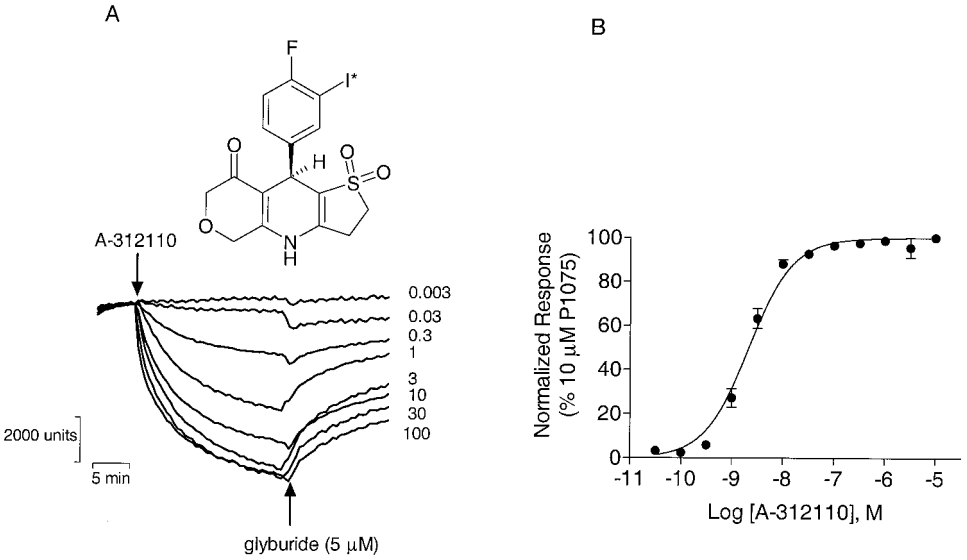

Fig. 1. Effect of A-312110 on membrane potential responses in guinea pig bladder smooth muscle cells assessed by the bis-oxonol dye, $DiBAC_4(3)$. A, representative trace showing typical changes in fluorescence responses upon addition (indicated by down arrow) of varying concentrations of A-312110 (0.003, 0.03, 0.3, 1, 3, 10, 30, and 100 nM) and reversal (indicated by up arrow) by the addition of glyburide (5 μM). Inset, structure of A-312110. * indicates the site of iodination. B, concentration response curve for fluorescence changes evoked by A-312110 (EC_{50} = 2.32 ± 0.3 nM). Responses are normalized to the response evoked by the prototypical cyanoguanidine KCO P1075 (10 μM). Depicted are means ± S.E.M. of 16 separate determinations.

Therefore, all subsequent experiments were carried out in the presence of 1 mM MgATP in the assay buffer.

A critical component of [125I]A-312110 binding is the ATP regeneration system that functions to maintain a consistent ATP concentration. Competition studies in the presence and absence of the ATP regeneration system revealed that A-312110 displaced specific binding with a K_i value of 4.29 ± 0.07 nM (n_H = 0.99 ± 0.14; n = 3) in the presence of the ATP regeneration system. However, no specific binding was detected in the presence of just ATP alone (Fig. 3C). Under these conditions, specific binding defined by unlabeled A-312110 was maximal at 37°C and increased linearly with increasing protein concentrations (up to 300 μg per reaction).

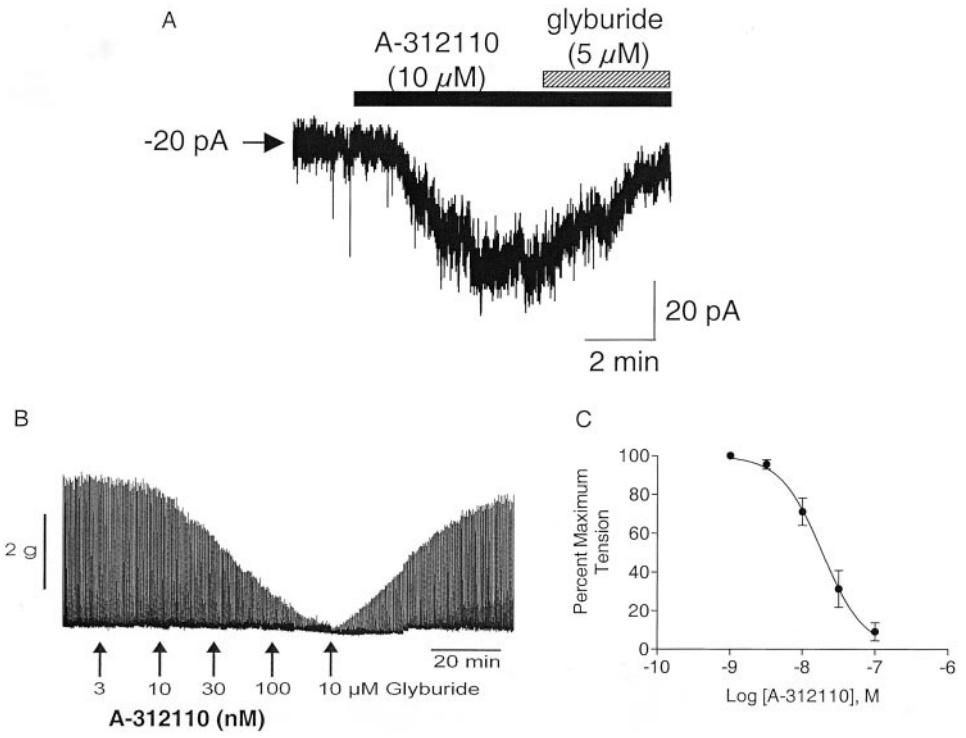

Fig. 2. Effect of A-312110 on K_{ATP} channel currents and bladder strip relaxation in the guinea pig. A, application of A-312110 (10 μM) evoked an increase in inward whole-cell current in guinea pig bladder cells (■) that was reversed upon addition of 5 μM glyburide (▨). Cells were voltage-clamped at a potential of −80 mV, and changes in membrane currents were measured under conditions of 140 mM extracellular and 60 mM intracellular [K^+]. B, representative experiment showing suppression of guinea pig bladder strip contractions after addition of increasing concentrations of A-312110 (3, 10, 30, and 100 μM). Glyburide (10 μM) reversed the effects of A-312110. C, concentration response curve of bladder smooth muscle relaxation evoked by A-312110 (IC_{50} = 18.5 ± 3.8 nM). Depicted are means ± S.E.M. of three separate determinations.

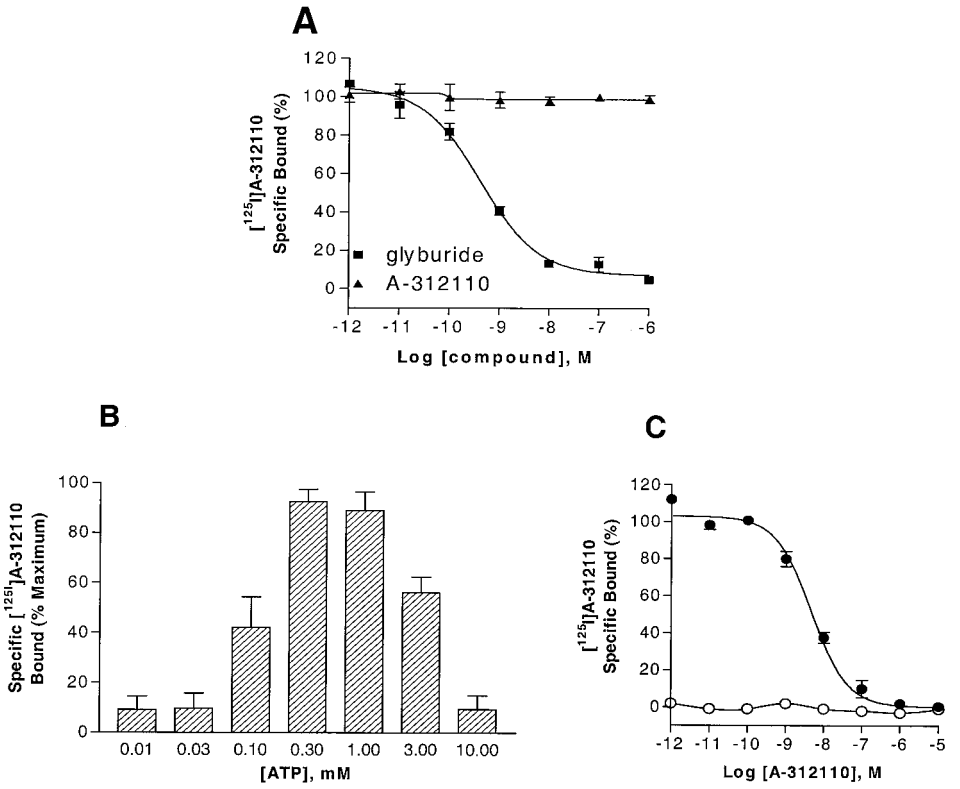

Fig. 3. Selectivity and ATP-dependence of binding. A, displacement of high-affinity [3H]glyburide. SUR1 binding to rat brain membranes. Shown are displacement profiles of unlabeled glyburide and A-312110. Each data point represents means ± S.E.M. of three separate determinations, each performed in duplicate. The K_i and n_H values are summarized under *Results*. B, concentration-response relationship of ATP dependence of specific [125I]A-312110 binding. Cardiac microsomal membranes were incubated with varying concentrations of ATP before determination of specific binding. The *y*-axis, expressed as a percentage of specific binding, is normalized to the values obtained in the presence of 1 mM ATP. The concentration of [125I]A-312110 was 1 nM. C, displacement of specific binding by unlabeled A-312110 in the presence (●) and absence (○) of the ATP regeneration system. Data shown is representative of three independent determinations.

Saturation Binding of [^{125}I]A-312110. [^{125}I]A-312110 bound in a saturable manner to preparations of guinea pig heart and urinary bladder membranes (Fig. 4, A and B, respectively). Specific binding to cardiac membranes represented about 80% of total binding at the K_D value and 60% at the highest ligand concentration tested. Specific binding was shown to be independent of structurally diverse unlabeled KCOs such as A-312110, P1075, or (−)-cromakalim, which were used to define the specific binding of the [^{125}I]A-312110 assay. Nonlinear regression analysis of the saturation binding data from guinea pig heart indicated that specific binding was to a single set of saturable binding sites with a K_D value of 5.83 ± 0.77 nM and a maximum binding capacity of 107.7 ± 12.7 fmol/mg protein (n = 3; Fig. 4C). Similarly, saturation analysis of guinea pig bladder membranes also yielded a single set of binding sites with a K_D value of 4.9 ± 0.54 nM and a B_{max} value of 68 ± 11.2 fmol/mg protein (n = 3; Fig. 4D).

Kinetics of [^{125}I]A-312110 Binding. A representative time course of association of specific binding to cardiac membranes is shown in Fig. 5A. Analysis of the data revealed monophasic association kinetics that reached a plateau at about 90 min, with a calculated association rate constant k_1 = 0.025 ± 0.01 nM^{-1} min^{-1} (n = 3). The time course of dissociation was assessed by addition of an excess of unlabeled A-312110 (10 μM) at equilibrium (Fig. 5B). Analysis of specific data revealed a monophasic dissociation kinetics with a rate constant, k_{-1}, of 0.046 ± 0.01 min^{-1} (n = 3). The calculated ratio of k_{-1}/k_1, a measure of the apparent dissociation constant (2.40 ± 0.64 nM), is comparable with the K_D determined from saturation analysis.

Pharmacological Profile of [^{125}I]A-312110 Binding in Heart. In addition to unlabeled A-312110, compounds previously described to activate K_{ATP} channels also displaced specific binding in cardiac membranes (Fig. 6, A and B). These compounds included a related dihydropyridine analog (ZM244085), cyanoguanidines (pinacidil and P1075), a benzopyran [(−)-cromakalim], a tertiary carbinol (ZD6169), a nitroethene (Bay X 9228), and the benzothiadiazine (diazoxide). The rank order of potency (K_i) value for displacement of inhibition by these compounds is as follows: A-312110 (4.29 nM) > P1075 > Bay X 9228 > pinacidil > (−)-cromakalim ~ ZD6169 > ZM244085 ≫ diazoxide (16.7 μM; Table 1). The pseudo Hill coefficients of the displacement curves were close to unity, except for Bay X 9228, which had a slope less than unity (n_H = 0.54 ± 0.13).

Specific [^{125}I]A-312110 binding was also displaced by two K_{ATP} channel blockers, the sulfonylurea glyburide and the cyanoguanidine analog PNU-99963 (Fig. 6C). When data points were fitted to a single site, the concentration response curves were shallow, with Hill slopes significantly less than 1 indicating negative cooperativity. Nonlinear regression analysis revealed that the displacement by glyburide had a better fit to a two-site model with K_i values of 2.23 nM (K_{i_H}) and 223.20 nM (K_{i_L}) compared with a one-site model (K_i = 64.36 ± 13.7 nM; n = 3). The relative populations of high- and low-affinity sites determined from the two-site analysis were 37 and 63%, respectively. Similarly, the displacement of [^{125}I]A-312110 by PNU-99963 corresponded to a two-site model with K_{i_H} and K_{i_L} values of 0.10 and 48.62 nM, respectively, rather than a one-site model (K_i = 8.19 ± 2.6 nM; n = 3). Again, the population of high- and low-affinity sites, 39 and 61%, respectively, were comparable with the populations defined by glyburide displacement.

Pharmacological Profile of [^{125}I]A-312110 Binding in Bladder. Unlabeled A-312110 displaced specific binding

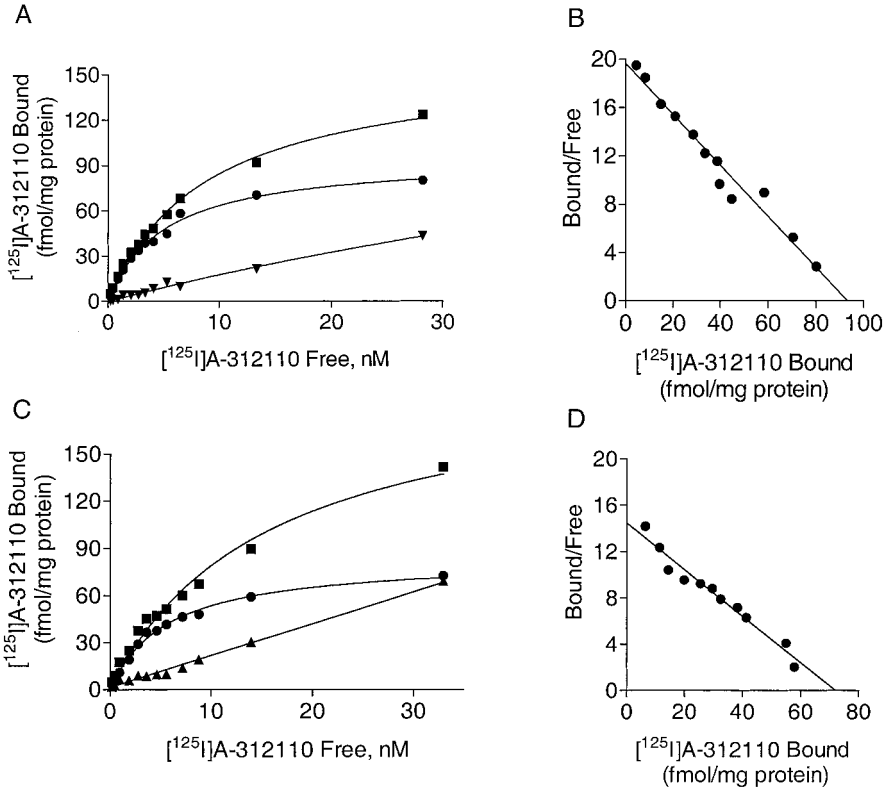

Fig. 4. Saturation binding of [^{125}I]A-312110 to guinea pig cardiac and bladder membranes. A, representative plots of increases in total (■), specific (●), and nonspecific (▲) binding with increasing radioligand concentrations in cardiac (A) and urinary bladder (C) membranes. Specific binding was defined by the inclusion of 10 μM unlabeled A-312110. Scatchard analysis of the specific binding data for guinea pig heart (C) and bladder (D) indicate mean K_D and B_{max} values of 5.83 ± 0.77 nM and 107.7 ± 12.7 fmol/mg of protein and 4.9 ± 0.54 nM and 86 ± 11.2 fmol/mg of protein, respectively.

with a K_i value of 4.9 ± 0.80 nM (n_H = 0.86 ± 0.1) in guinea pig urinary bladder which is identical to the K_D value of [125I]A-312110 derived from saturation analysis (K_D = 4.9 nM). Displacement studies were carried out using the same set of KCOs tested in guinea pig cardiac membranes (Fig. 7, A and B). The rank-order potency of these compounds is as follows: A-312110 (4.9 nM) > P1075 ≈ Bay X 9228 > pinacidil > (−)-cromakalim > ZD6169 > ZM244085 > diazoxide (15.7 μM; Table 1). In all cases, the displacement curves exhibited a pseudo Hill coefficient near unity, except for ZD6169, which had a slope less than unity (n_H = 0.56 ± 0.05). With the exception of A-312110, the K_i values of other KCOs showed an overall trend toward higher (2–5 fold) binding affinities for bladder compared with cardiac membranes.

In addition to KCOs, two K_{ATP} channel blockers, the cyanoguanidine PNU-99963 and the sulfonylurea glyburide, also

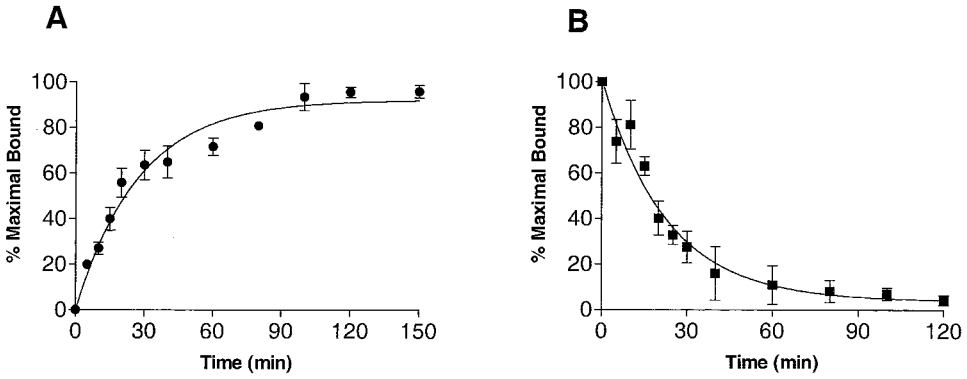

Fig. 5. Kinetics of [125I]A-312110 binding. Shown are time courses of association (A) and dissociation (B) of binding to cardiac membranes. Each data point expresses a percentage of maximal specific [125I]A-312110 bound and represents the mean ± S.E.M of duplicate determinations from three different experiments. The mean values for K_{on} and K_{off} are 0.025 ± 0.01 nM^{-1} min^{-1} and 0.046 ± 0.01 min^{-1}, respectively.

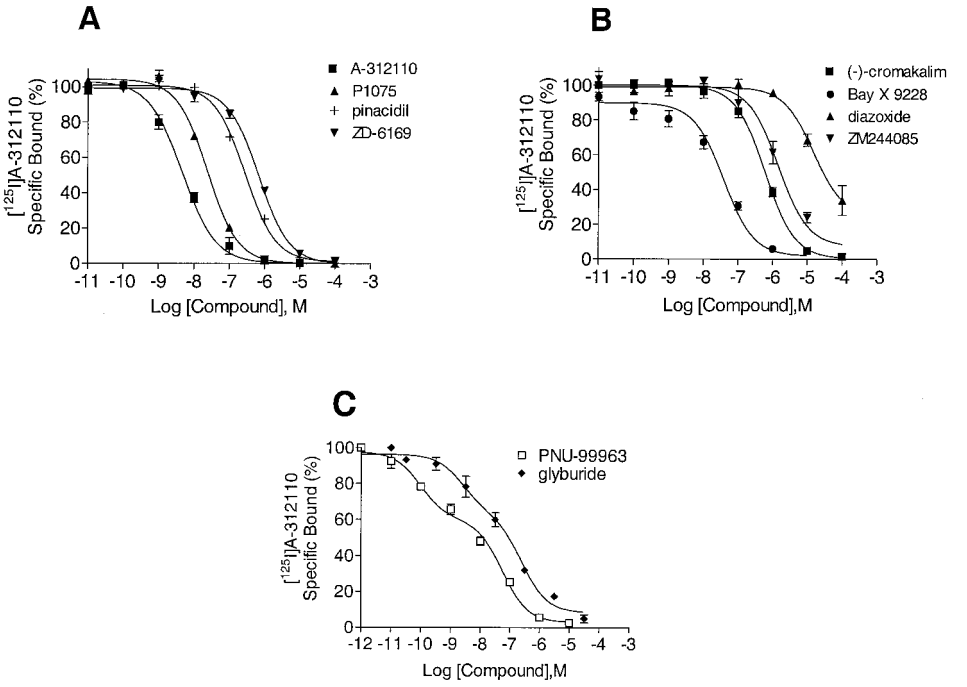

Fig. 6. Displacement profile of [125I]A-312110 binding by KCOs and sulfonylurea analogs in cardiac membranes. Shown are displacement curves of structurally diverse KCOs (A and B). Each data point represents means ± S.E.M. of three to five separate determinations, each performed in duplicate. The K_i and n_H values are summarized in Table 1. C, inhibition of [125I]A-312110 binding by K_{ATP} channel blockers, glyburide, and PNU-99963. Each data point expresses mean ± S.E.M. of three separate determinations each performed in duplicate. The data were best fit to a two-site model than to a single-site model. The K_i values and population of high- and low-affinity sites defined by the displacement curves are summarized in *Results*. Assays were carried out using 1.0 nM radioligand.

TABLE 1

Inhibition of [125I]A-312110 binding by K_{ATP} channel openers

Values represent mean ± S.E.M. error of three to five separate determinations in duplicate. Binding assays were performed with [125I]A-312110 (1 nM) and varying concentrations of compounds as described under *Materials and Methods*.

Compound	Guinea Pig Heart		Guinea Pig Bladder	
	K_i	n_H	K_i	n_H
	nM		*nM*	
A-312110	4.29 ± 0.07	0.99 ± 0.14	4.9 ± 0.80	0.86 ± 0.10
P1075	20.61 ± 1.02	1.04 ± 0.06	10.5 ± 4.02	0.92 ± 0.07
Bay X 9228	41.34 ± 5.87	0.54 ± 0.13	16.0 ± 6.67	0.65 ± 0.17
pinacidil	251.0 ± 7.60	0.91 ± 0.01	104 ± 11.7	0.73 ± 0.17
(−)-Cromakalim	542.20 ± 49.96	0.96 ± 0.09	157.0 ± 63.6	0.71 ± 0.03
ZD6169	629.80 ± 94.35	0.88 ± 0.06	451 ± 138	0.56 ± 0.05
zm244085	2,774 ± 1075	0.93 ± 0.35	671 ± 156	0.94 ± 0.11
diazoxide	16,710 ± 215	1.90 ± 0.70	15,675 ± 5,388	0.68 ± 0.03

displaced high-affinity [^{125}I]A-312110 binding in a monophasic manner, with K_i values of 37.3 ± 10.4 nM (n_H = 1.06 ± 0.06) and 4.5 ± 1.78 μM (n_H = 0.86 ± 0.08) respectively, unlike the biphasic displacement observed in the cardiac membranes (Fig. 7C).

Comparison of [^{125}I]A-312110 Binding versus Functional Responses. The binding affinities of KCOs were compared with their functional profile in heart and bladder smooth muscle as measured by their effects on APD of isolated canine Purkinje fibers, a model for drug-induced arrhythmogenesis (Gintant et al., 2001) and electrically stimulated bladder strip relaxation (Gopalakrishnan et al., 1999), respectively. The compounds evaluated in Purkinje fibers, A-312110, P1075, ZD6169, pinacidil, and diazoxide, all evoked concentration-dependent shortening of APD. For example, A-312110 decreased Purkinje fiber repolarization in a concentration-dependent manner as reflected in the shortening of APD by 11.36 ± 2.18% and 72.6 ± 3.2% at 10 and 100 nM, respectively. The reduction of APD with KCOs was associated with negative inotropic effects and a reversible reduction in the amplitude of diastolic depolarization. The potencies (−log EC_{50} ± S.E.M.) of KCOs for suppressing APD are as follows: A-312110 (7.63 ± 0.19) ≈ P1075 (7.72 ± 0.009) > ZD6169 (6.26 ± 0.23) > pinacidil (5.62 ± 0.05) > diazoxide (4.12 ± 0.08). As shown in Fig. 8A, the rank order of potencies for these KCOs to evoke a 50% shortening of APD correlated well (r^2 = 0.93) with the K_i values obtained from [^{125}I]A-312110 binding. In the bladder, [^{125}I]A-312110 binding affinities of KCOs were found to be 2- to 4-fold greater compared with their potencies for relaxation of electrical field-stimulated guinea pig bladder strips. However, as shown in Fig. 8B, a comparison of the −log K_i values of KCOs with their relaxation potencies indicated a good correlation (r^2 = 0.83).

Discussion

ATP-sensitive potassium channel openers have recently emerged from the 1,4-dihydropyridine structural class, which has given rise to selective receptor-ion channel molecules, the most noteworthy of which are the clinically available L-type calcium channel antagonists (Triggle, 1994). ZM244085 and A-278637, compounds belonging to the category of ATP-sensitive K^+ channel openers, have been shown to relax urinary bladder smooth muscle strips by activating ATP-sensitive potassium current (Li et al., 1996; Brune et al., 2002; Gopalakrishnan et al., 2002). Systematic chemical modification of the dihydropyridine core has led to the identification of A-312110 (Fig. 1). A-312110 selectively activates K_{ATP} channels with a higher potency than that of the prototypical cyanoguanidine analog, P1075. This, together with the opportunity to label this dihydropyridine analog with a higher specific activity by [^{125}I] relative to [^{3}H], led to the identification of [^{125}I]A-312110.

In the present investigation, several lines of evidence derived from whole-cell patch clamp, fluorescence-based membrane potential measures, and relaxation studies clearly demonstrate the K_{ATP} channel activator properties of A-312110. Direct interaction with native K_{ATP} currents was confirmed by whole-cell current measurements in bladder smooth muscle cells, where A-312110 was efficacious in activating glyburide-sensitive currents in a manner similar to other KCOs such as P1075 and ZD6169 (Petkov et al., 2001; Shieh et al., 2001). Compared with other known 1,4-dihydropyridine analogs, including A-278637 (EC_{50} = 102 nM) and ZM244085 (EC_{50} = 6.1 μM; Gopalakrishnan et al., 1999; 2002), A-312110 is more potent in evoking membrane hyperpolarization responses in smooth muscle cells and relaxing bladder strips in guinea pig.

[^{3}H]P1075 and [^{3}H]Bay X 9228 are two earlier known KCO radioligands (Bray and Quast, 1992); initial studies have

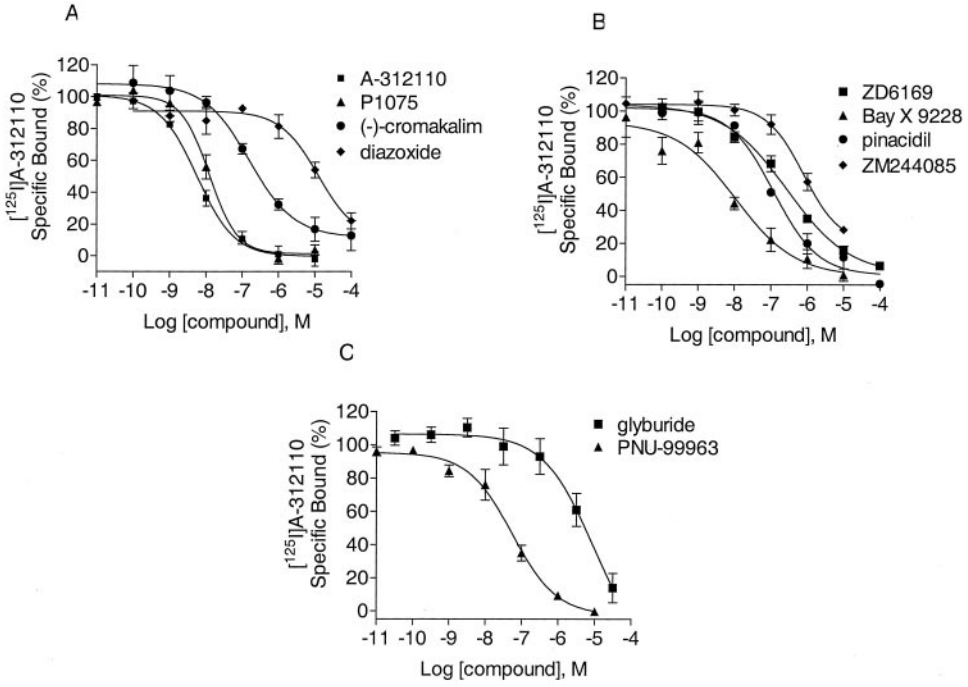

Fig. 7. Displacement profile of [^{125}I]A-312110 binding by KCOs and sulfonylurea analogs in bladder membranes. Shown are displacement curves of structurally diverse KCOs (A and B). Each data point represents means ± S.E.M. of three to five separate determinations each performed in duplicate. C, inhibition of [^{125}I]A-312110 binding by glyburide and PNU-99963. Each data point from the displacement curves represents means ± S.E.M. of three separate determinations, each performed in duplicate. Assays were carried out using 1.0 nM radioligand. The mean K_i and n_H values are summarized in Table 1.

demonstrated binding to intact cells or tissue preparations. Subsequently, it was revealed that the binding of [3H]P1075 to native (Dickinson et al., 1997) or recombinant (Hambrock et al., 1998; Schwanstecher et al., 1998) channels could be measured only when hydrolyzable nucleotides and Mg^{2+} were present. The observed lack of high-affinity binding of [125I]A-312110 in the absence of an ATP-regeneration system (Fig. 3) is consistent with the notion that KCO binding requires ATP. The EC_{50} value of MgATP to support [125I]A-312110 binding (106 μM) was comparable with previous reports of [3H]P1075 binding in cardiac membranes (100 μM; Loffler-Walz and Quast, 1998). More recently, nucleotide dependence of binding has also been demonstrated with a tritiated benzopyran analog [3H]PKF217-744 (Manley et al., 2001). Collectively, these studies indicate that specific binding of openers is critically dependent on the presence of nucleotide (ATP), irrespective of their structural class. It has been suggested that nucleotide binding and hydrolysis by the nucleotide binding folds could serve to modulate interactions of KCOs within the second transmembrane domain of SUR, an essential determinant for KCO sensitivity (D'hahan et al., 1999).

Specific [125I]A-312110 binding to cardiac and bladder membranes, in the presence of an ATP-regeneration system, was found to be saturable and exhibited pharmacological specificity in its interaction with diverse K_{ATP} openers and inhibitors. The K_i value of unlabeled A-312110 derived from displacement analysis (4.3 nM) was comparable with values derived from saturation (5.8 nM) and kinetic studies (2.4 nM) of the radioligand. Because A-312110 structurally resembles other 1,4-dihydropyridines that interact with L-type voltage-sensitive calcium channels, isradipine, a selective L-type calcium channel antagonist, was examined and found not to displace specific [125I]A-312110 binding. Furthermore, A-312110 did not relax depolarization-evoked (80 mM K^+) rat aortic strips, a model of contractility in which a prototypical L-type calcium channel antagonist, nifedipine, was quite effective. In addition, the lack of binding to a variety of receptor and ion channels, including the high-affinity SUR1 binding site, demonstrate that the interactions of [125I]A-312110 are specific to the cardiac/smooth muscle K_{ATP} channel complex.

Structurally divergent KCOs displaced [125I]A-312110 binding to cardiac membranes with Hill slopes near unity, which indicates an interaction with a single set of binding sites that does not show cooperative behavior. The rank-order potencies of KCOs for displacing high-affinity binding seem to be comparable among the present study and those previously reported with [3H]P1075 in rat cardiac membranes. A similar rank order of potency was observed in the bladder where, besides A-312110, specific binding was displaced, albeit less potently, by KCOs including pinacidil, P1075, (−)-cromakalim, Bay X 9228, ZD6169, ZM244085, and diazoxide, with Hill slopes near unity (Table 1). It is now known that the sulfonylurea receptor accommodates binding sites for the KCOs, sulfonylureas, and nucleotides and that there is a positive allosteric interaction between nucleotide and opener binding that enables SUR to bind openers with high affinity (Hambrock et al., 1998; Schwanstecher et al., 1998; Ashcroft and Gribble, 2000). Equilibrium binding experiments with [125I]A-312110 and the displacement studies are consistent with the existence of a single class of binding sites for the KCOs examined. Although studies with recombinant subunits will provide a definitive conclusion, it is likely that dihydropyridine KCOs such as A-312110 may interact with sites akin to those described for benzopyrans and cyanoguanidines within the transmembrane helix 17 (for example, Thr^{1253} and Leu^{1249} of the SUR2A subunit) (Moreau et al., 2000).

In contrast to the profile observed with KCOs, displacement by known K_{ATP} channel blockers such as glyburide and PNU-99963 were biphasic with a high-affinity component corresponding to about 60% of the inhibition curve in both instances. For glyburide, the difference in affinities between the high- and low-affinity sites were approximately 100-fold, which is similar to previous reports with [3H]P1075 in cardiac membranes (Loffler-Walz and Quast, 1998). Although biphasic displacement curves of [3H]P1075 binding sites have been shown in cardiac membranes, monophasic profiles were noted in vascular smooth muscle (rat aorta and A10 cells) (Quast, 1993; Russ et al., 1997). In the present study, similar results were noted with [125I]A-312110 binding in bladder smooth muscle membranes. Unlike the biphasic profile observed in cardiac membranes, the displacement of [125I]A-

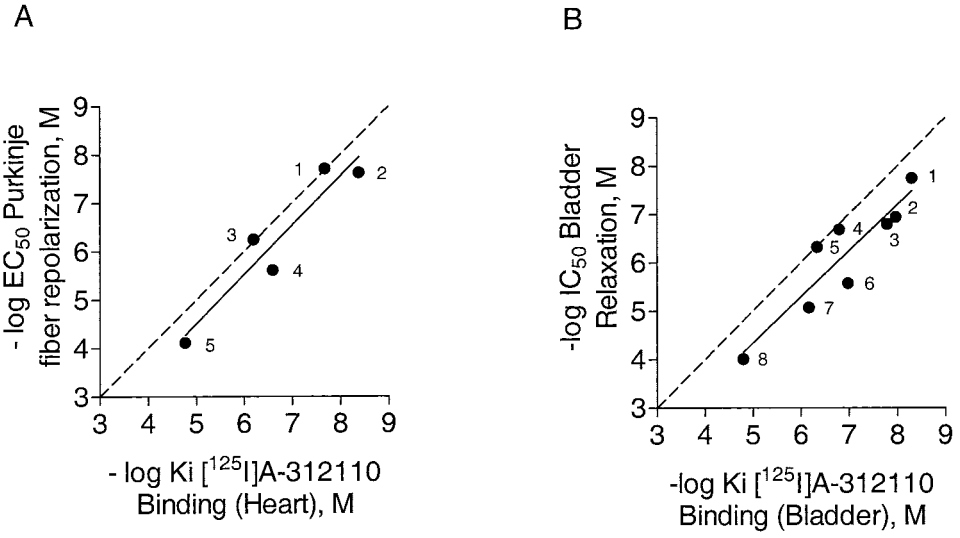

Fig. 8. Correlation of affinities of KCOs to displace [125I]A-312110 binding in guinea pig cardiac and bladder membranes (−log K_i) with their potencies (−log IC_{50}) to suppress action potential duration (APD) in Purkinje fibers (A) and with inhibition of contractile responses (−log IC_{50}) of electrically stimulated guinea pig bladder smooth muscle strips (B), respectively. The numbers shown in A correspond to the following compounds: 1, A-312110; 2, P1075; 3, ZD6169; 4, pinacidil; and 5, diazoxide; those indicated in B correspond to the following compounds: 1, A-312110; 2, P1075; 3, Bay X 9228; 4, (−)-cromakalim; 5, ZD6169; 6, pinacidil; 7, ZM244085; and 8, diazoxide. The −log IC_{50} values of KCOs (except A-312110, this study) for relaxation of field-stimulated tissue strips are taken from Gopalakrishnan et al. (1999). The solid line represents linear regression through data points (A, r^2 = 0.93; B, r^2 = 0.83); the dashed lines correspond to 1:1 correlation.

312110 by K_{ATP} channel blockers PNU-99963 and glyburide was monophasic in bladder. The affinity for inhibition of [^{125}I]A-312110 binding to bladder membranes by glyburide (K_i = 3.7 μM) is comparable with that reported for SUR2B-expressing K_{ATP} channels using either [^{3}H]P1075 (K_i = 2.8 μM; Hambrock et al., 1999) or [^{3}H]PKF217-744 (K_i = 4.78 μM; Manley et al., 2001). The precise nature of these cardiac high- and low-affinity antagonist sites remains to be elucidated, although it is likely that they may emerge from interactions with multiple SUR subtypes or discrete combinations of SURs with K_{IR}6.1 or 6.2 subunits. For example, analysis of cardiac tissue samples have shown the presence of both SUR2A (derived from myocytes) and SUR2B mRNA (derived from endothelial cells) (Isomoto et al., 1996; Schnitzler et al., 2000), whereas smooth muscle tissues, including bladder, have shown the presence of SUR2B, and not SUR2A, consistent with the proposed composition of smooth muscle K_{ATP} channels in heteromeric studies. Alternatively, the affinity differences may be attributable to a population of binding sites that may not be coupled to the K_{IR} subunit. Studies using recombinant SUR2B alone have shown that glyburide inhibition of [^{3}H]P1075 binding is monophasic, with substantially lower affinities (K_i = 2.8 μM; Hambrock et al., 1999). In contrast, the affinities derived for SUR2B coexpressed with K_{IR}6.1 are about 100-fold higher (30 nM; Russ et al., 1999) and are comparable with the potency of glyburide to inhibit K_{IR}6.1-SUR2B or K_{IR}6.2-SUR2B containing channels (40 nM; Dorschner et al., 1999; Russ et al., 1999).

Radioligand binding studies with recombinant SUR2A and SUR2B-containing K_{ATP} channels have been previously performed with [^{3}H]P1075 and [^{3}H]PKF217-744 (Hambrock et al., 1999; Manley et al., 2001). From kinetics studies using [^{3}H]P1075, SUR2B expressing-K_{ATP} channels have a calculated K_D of 2.2 nM, whereas the binding affinity for the SUR2A subtype (K_D = 19 nM) is about 9-fold lower (Hambrock et al., 1999). Similar studies with the benzopyran radioligand [^{3}H]PKF217-744 have shown an overall lower binding affinity for the SUR2 subtypes compared with [^{3}H]P1075, and a modest 3-fold lower affinity for SUR2A (K_D = 40 nM) compared with SUR2B (K_D = 12 nM; Manley et al., 2001). The K_D value of [^{125}I]A-312110 obtained from Scatchard analysis (K_D = 4.9 nM) was found to be comparable with the value reported in cardiac membranes (K_D = 5.8 nM), although the B_{max} value in bladder (68 fmol/mg protein) was somewhat lower compared with that of cardiac membranes (108 fmol/mg protein). Similarly, the K_i values from displacement studies of unlabeled A-312110 in cardiac (4.3 nM) and bladder (4.9 nM) were also quite comparable. Thus, under the present experimental conditions and using crude microsomal membrane preparations, it is unlikely that [^{125}I]A-312110 can discriminate between these two SUR2 splice variants, SUR2A and SUR2B, that constitute the distinct K_{ATP} channels in both these tissues. Studies with recombinant subunits would be required to conclusively determine SUR2B/SUR2A selectivity.

The rank-order potency of KCOs to displace [^{125}I]A-312110 binding to bladder membranes was found to show good 1:1 correlation (r^2 = 0.83) with the potencies for relaxation of electrically stimulated guinea pig bladder strips, an established model for assessing K_{ATP} channel function in the bladder (Buckner et al., 2002). Similarly, despite comparing across species (guinea pig versus canine) and tissues (Purkinje fibers versus ventricle), a good correlation (r^2 = 0.93) was also observed between binding affinities of KCOs in the heart with their potencies to suppress action potential duration. These observations collectively suggest that interactions with binding sites of [^{125}I]A-312110 reflect activation of plasmalemmal K_{ATP} channels. Notably, the crude cardiac membrane preparations could contain heterogeneous contributions from different cell types including myocytes, endothelial cells, fibroblasts and smooth muscle cells. Therefore, a strict comparison with specific cell types or membrane localization cannot be determined at present.

In summary, this study has characterized the K_{ATP} channel properties of a novel dihydropyridine analog, A-312110, by whole-cell patch clamp, fluorescence-based membrane potential, and tissue relaxation measures. [^{125}I]A-312110 binding was found to be of high affinity in both cardiac and bladder membranes, with a pharmacological profile consistent with K_{ATP} channel activation in these tissues. [^{125}I]A-312110 offers many advantages of an ^{125}I-labeled ligand, such as higher specific activity (2000 Ci/mol) compared with [^{3}H]P1075 (~ 110 Ci/mmol), and typically exhibited specific binding in the range of 75 to 90% compared with [^{3}H]P1075, in which specific binding is about 50% or lower under similar conditions. These studies demonstrate that [^{125}I]A-312110 is a useful tool for investigation of the molecular and functional properties of K_{ATP} channel complex and to enable further identification, in a high throughput manner, of novel channel blockers and openers that interact with cardiac/smooth muscle type channels.

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