0026-895X/95/010155-09$03.00/0

MOLECULAR PHARMACOLOGY, 47:155–163, 1995.

Receptor Binding Characterization in Kidney Membrane of [^{3}H]U-37883, a Novel ATP-Sensitive K^+ Channel Blocker with Diuretic/Natriuretic Properties

KAUSHIK MEISHERI, MICHEL FOSSET, STEPHEN HUMPHREY, and MICHEL LAZDUNSKI

Cardiovascular Pharmacology, Upjohn Laboratories, Kalamazoo, Michigan 49001 (K.M., S.H.), and Institut de Pharmacologie Moleculaire et Cellulaire, 06560 Valbonne, France (M.F., M.L.)

Received September 9, 1994; Accepted November 1, 1994

SUMMARY

U-37883 (4-morpholinecarboximidine-*N*-1-adamantyl-*N*-cyclohexyl), a known blocker of ATP-sensitive K^+ (K_{ATP}) channels, produces natriuresis/diuresis *in vivo* by a direct effect on the kidney. In the present study, the binding characteristics of the U-37883 receptor were investigated using pig kidney cortex microsomes. [^{3}H]U-37883 (0.5–5 nM, 50 Ci/mmol) exhibited specific binding, which was reversible, increased linearly with protein concentration (50–500 μg/ml), and was destroyed after treatment with proteases. Scatchard plots derived from the competition experiments suggested the presence of a single class of low affinity binding sites, with a K_d of 225 nM and a B_{max} of 7.8 pmol/mg of protein. A similar K_d value was derived from complementary studies dealing with association and dissociation kinetics. The binding of [^{3}H]U-37883 was tissue specific, because very little specific binding could be detected in microsomes from rat insulinoma cells (RINm5F) and brain. In contrast, these membranes displayed high affinity specific binding of [^{3}H]glyburide, another K_{ATP} channel blocker. Finally, analogs of U-37883 that were found to be active K_{ATP} channel blockers in isolated rabbit mesenteric artery and active *in vivo* as diuretics/natriuretics were also found to be active in displacing specific binding of [^{3}H]U-37883, whereas the inactive analogs (no vascular K_{ATP} channel-blocking activity and no *in vivo* diuresis/natriuresis) were inactive in this binding assay. We suggest that the U-37883 binding site represents a functional receptor that mediates the K_{ATP} channel antagonism and natriuresis observed with this class of compounds.

In recent years, the role of K^+ channels in general and K_{ATP} channels in particular within the cardiovascular system has come under intense investigation (1, 2). Thus, a significant number of chemically different classes of openers, as well as blockers, of the K_{ATP} channel have been described (2–4). Glyburide (or glibenclamide) remains the most widely used blocker of the K_{ATP} channel in a variety of cell systems for pharmacological and biochemical characterization (5–7). We have described the pharmacological characteristics in vascular smooth muscle of another, structurally different, K_{ATP} channel blocker, U-37883A (8, 9). Like glyburide, U-37883A is an effective blocker of *in vitro* relaxation as well as $^{42}K^+$ efflux induced by K_{ATP} channel openers and is an effective blocker of *in vivo* hypotension produced by various K_{ATP} channel openers (8–10). More recently, we have also provided electrophysiological evidence demonstrating that U-37883A can, again like glyburide, effectively block activation of the endogenous K_{ATP} channel in follicle-enclosed oocytes (11).

Both glyburide and U-37883A have been shown to produce another *in vivo* cardiovascular effect, i.e., natriuresis and diuresis (12, 13). The details of *in vivo* diuresis/natriuresis produced by U-37883 and its alkyl and aryl guanidine analogs have recently been reported (14). Thus, when given intravenously to rats, both glyburide and U-37883A stimulate Na^+ excretion without affecting the glomerular filtration rate (12–14). Such a profile suggests a direct renal tubular effect of these K_{ATP} channel blockers. These effects of glyburide and U-37883A are quite consistent with the postulated role of the renal K_{ATP} channels (15). A small-conductance renal epithelial K_{ATP} channel with a high open probability under physiological conditions has been suggested to play a role in NaCl reabsorption (16, 17). Thus, it has been speculated that the unique, nonkaliuretic, natriuretic properties of glyburide and U-37883A might be due to their ability to block the renal K_{ATP} channel (12, 13).

Significant work has been done characterizing the biochemistry of the glyburide receptor (7, 18). Previously, we demonstrated the presence of a functional receptor for U-37883A in

This research was supported by a grant from the CNRS, France. K.M. was on research sabbatical leave from the Upjohn Co. during the course of this investigation. The membrane binding work was carried out at CNRS in France, whereas the *in vitro* vascular work and the *in vivo* work with rats were carried out at Upjohn Laboratories in the United States.

ABBREVIATIONS: K_{ATP} channel, ATP-sensitive K^+ channel; HCTZ, hydrochlorothiazide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

follicle-enclosed *Xenopus* oocytes (11). We report here the initial characterization of the binding of [^{3}H]U-37883, using porcine kidney membranes. The overall objective is to delineate the biochemical mechanism(s) by which the receptor for U-37883 controls K_{ATP} channel-mediated Na^+ reabsorption in the kidney.

Materials and Methods

Preparation of Pig Renal Cortex Membranes

Pig kidneys were obtained from a regional slaughterhouse and were transported to the laboratory in ice-cold HEPES buffer (20 mM, pH 7.4). The capsule, which is the outer membrane enclosing each kidney, was removed, the kidney halves were separated, and renal cortex was harvested. The tissue was placed in ice-cold (4°) 50 mM HEPES containing 0.3 M sucrose and 1 mM EDTA (10 ml of buffer/1 g of tissue). The tissue was minced with scissors and then homogenized with a Polytron homogenizer (PTA 36–50 rotor) at high speed three times, each time for about 15 sec. The beakers were kept in ice to prevent warming. This was followed by sequential centrifugation at 1000 × *g* (10 min) and 10,000 × *g* (20 min). Each time the pellet was discarded and the supernatant was used for the subsequent step. The final centrifugation was at 60,000 × *g* for 25 min (Beckman ultracentrifuge). The microsomal pellet was resuspended in 20 mM HEPES buffer and used for the binding studies.

Binding Studies

The binding experiments were carried out at room temperature (22–24°). Membranes were incubated in 1 ml of 50 mM HEPES, pH 7.4, at a protein concentration of 500 μg/ml. Initial experiments were done using [^{3}H]U-37883 in the concentration range of 0.5–5 nM, to determine binding as a function of [^{3}H]U-37883 concentration. To determine specific binding, four tubes were assayed simultaneously at each [^{3}H]-U-37883 concentration, as follows: tube A, membranes plus [^{3}H]U-37883; tube B, membranes plus [^{3}H]U-37883 plus unlabeled U-37883A (10 μM); tube C, [^{3}H]U-37883 without membranes; tube D, [^{3}H]U-37883 plus unlabeled U-37883A (10 μM) but without membranes. Tubes C and D were used to determine the propensity of the radiolabel to stick to the filter paper. Thus, at each [^{3}H]U-37883 concentration, the specific binding was calculated as $[(A - B) - (C - D)]$. Incubations lasted for 60 min and were stopped by rapid filtration through Whatman GF/C filters under reduced pressure. Filters were washed three times each with 10 ml of ice-cold buffer, pH 7.4, containing 20 mM Tris and 100 mM NaCl. The radioactivity remaining on the filter was counted using a liquid scintillation counting system. The raw data were converted to yield binding as femtomoles/milligram of protein.

Protein Dependence of Specific Binding

For this experiment, [^{3}H]U-37883 was used at a concentration of 2 nM. The protein dependence of the specific binding was determined in the protein concentration range of 50–500 μg of protein/assay tube. At each protein concentration, total binding and binding in the presence of 10 μM unlabeled U-37883A were determined. Several tubes were also run without any membranes. Other assay conditions and the washout procedure remained the same as described above.

U-37883A Competition Curves and Scatchard Plots

Initial experiments suggested that [^{3}H]U-37883 specific binding did not saturate even at 5 nM. Thus, to determine apparent K_d values, competition experiments were carried out. Again, 2 nM [^{3}H]U-37883 and a protein concentration of 500 μg/ml were used. Competition curves using unlabeled U-37883A in the concentration range of 0.001–10 μM were generated. Specific binding was calculated from the data with 10 μM unlabeled U-37883A. These data were also converted to generate Scatchard plots (19, 20). The concentration of radiolabel was determined using a dilution factor, to generate the Scatchard plot.

Effects of Proteases on Specific Binding

To determine whether the specific binding of [^{3}H]U-37883 occurred at a protein, three proteases were used, i.e., trypsin, α-chymotrypsin, and Pronase, each at a final concentration of 1.0 mg/ml. Trypsin solution was prepared in the presence of 10 mM $CaCl_2$ to prevent autodigestion. Kidney cortex membranes were incubated with a given protease and heated at 37° for 1 hr. The incubation mixture was allowed to remain at room temperature for 15 min. In the case of the trypsin treatment group, the membranes were exposed to soluble trypsin inhibitor at a final concentration of 10 mg/ml, to inactivate trypsin. Aliquots of membranes after each protease treatment were assayed for the specific binding of [^{3}H]U-37883, using the experimental conditions described above.

Tissue Distribution of Specific Binding of [^{3}H]U-37883

Tissue distribution was used as another criterion to determine the specific nature of U-37883 binding in the kidney. Thus, [^{3}H]U-37883 binding was compared in membranes from pig kidney cortex, pig brain cortex, and RINm5F cells. The preparation of membranes from brain and RINm5F cells in our laboratory has been described before (18, 21). In each case, the experimental conditions were similar, i.e., 2 nM radiolabel, 500 μg of protein, room temperature, and pH 7.4. Because it is well known that both brain and RINm5F cells contain large amounts of specific binding sites for another K_{ATP} channel blocker, glibenclamide, comparative binding studies in these three membrane preparations were also performed using [^{3}H]glibenclamide (0.2–2.5 nM).

Association and Dissociation Kinetics of [^{3}H]U-37883 Binding

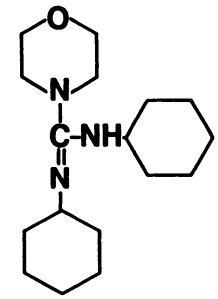
Association kinetics. Because it was not possible to perform saturation analysis of [^{3}H]U-37883 binding, kinetic studies of the binding were carried out to complement the competition experiments. Association kinetics experiments were carried out at 4°. Membranes (500 μg/ml protein) were incubated with about 2 nM [^{3}H]U-37883, and aliquots were taken for filtration every 15 sec starting at time 0. The time course of binding was assayed for 10 min, by which time the binding reached a plateau. Preliminary experiments showed that the binding at 10 min was similar to that at 60 min. Some of the time points were also assayed in the presence of excess unlabeled U-37883A (10 μM), to determine nonspecific binding. The data were plotted as percentage specific binding as a function of incubation time.

Dissociation kinetics. These experiments were also carried out at 4°. The membranes were incubated with about 2 nM [^{3}H]U-37883 for 1 hr, to allow maximum equilibrium binding. At that point, excess unlabeled U-37883A (10 μM) was added and aliquots were taken for filtration every 15 sec starting at time 0. The time course of the loss of binding was assayed for 15 min. Parallel 1-hr incubations, as described above, were also carried out in the presence of excess unlabeled U-37883A (10 μM), to determine nonspecific binding. The data were plotted as decline in percentage specific binding as a function of time. Based on the experimentally derived values for the observed association constant and dissociation constant, the value of K_d was calculated as described by Weiland and Molinoff (19).

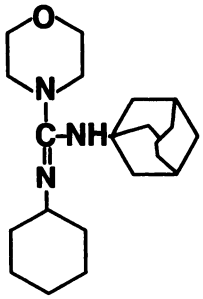
U-37883A competition curve at 4°. Because the kinetic studies were done at 4°, it was considered important to generate a U-37883A competition curve at 4°. The competition experiment was identical to the one described above, with the exception of temperature (room temperature versus 4°).

Functional Significance of [^{3}H]U-37883 Binding

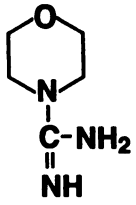
Several chemical analogs of U-37883, i.e., U-18177, U-42069, U-46340, U-50447, and U-52090, were used to determine the functional significance of the observed specific binding. The structures of these compounds as well as U-37883 are shown in Fig. 1. Two types of experiments were carried out with these compounds. In the first set of experiments, the effects of these compounds on [^{3}H]U-37883 binding in pig kidney cortex membranes were determined. The ability of each



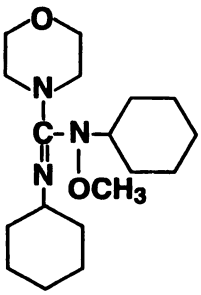
U-18177



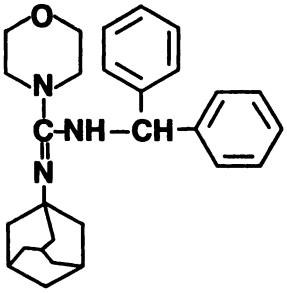
U-37883



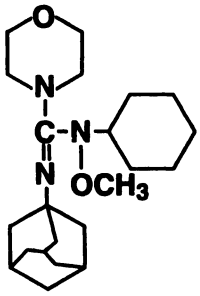
U-42069



U-46340



U-50447



U-52090

Fig. 1. Structures of U-37883 and various analogs used in this study.

compound to inhibit the specific binding of [³H]U-37883 was determined at 0.5, 1, and 5 μM. The experimental conditions were 2 nM [³H]U-37883 and 500 μg of protein/ml, at pH 7.4 and room temperature, with a 1-hr incubation period. In the second set of experiments, the ability of these compounds, at the same concentrations, to act as K_{ATP} channel blockers was determined. Our previous studies established isolated rabbit mesenteric arteries as an *in vitro* preparation for characterizing the K_{ATP} channel-blocking activity of U-37883 and glibenclamide (8–10). Thus, this preparation was used to determine the *in vitro* K_{ATP} channel-blocking activities of each of the aforementioned compounds, relative to U-37883A. The details of this procedure have been provided in our previous publications (8–10). Briefly, isolated tissue bath experiments with rabbit mesenteric artery were carried out. The K_{ATP} channel opener pinacidil (1 μM) maximally relaxes precontracted artery. The ability of a blocker to inhibit this pinacidil-induced relaxation is studied and is indicative of functional K_{ATP} channel blockade (22). The objective was to correlate the ability of each analog to act as a K_{ATP} channel blocker with its ability to compete with [³H]-U-37883 binding.

In Vivo Significance of [³H]U-37883 Binding

To further investigate the pharmacological significance of the [³H]-U-37883 binding site, *in vivo* diuretic effects of the six guanidine compounds described above were also determined in conscious male rats. Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 250 g were acclimated to a diet of Purina Rat Chow and water under 12-hr light/dark conditions. Each compound was examined at three intravenous doses in six separate tests. Six rats were injected at each dose, and four saline-treated control rats were included in each experiment. After injection, two identically treated rats were housed in a stainless steel metabolism cage to permit quantitative urine collection. Two hours later, the urinary bladders of the rats were lightly compressed, the total urine volume was recorded, and an aliquot was retained for Na^+ and K^+ concentration analysis with a NOVA-13 ion-selective electrode analyzer. A computerized analysis of covariance program calculated the means ± standard errors for volume, Na^+ excretion, and K^+ excretion for each guanidine dosage (three cages) and the statistical significance from the pooled vehicle controls (12 cages).

Drugs

The unlabeled drug was U-37883A, which is the hydrochloride salt of U-37883. This hydrochloride salt is freely water soluble. Radiolabeled [³H]U-37883 was prepared at the Upjohn Co., and stock solutions were prepared in methanol. The analogs of U-37883A used in this study were obtained from the Biological Screening Office of the Upjohn Laboratories.

Results

Specificity and protein dependence of [³H]U-37883 binding. The binding of [³H]U-37883 in pig kidney as a function of the concentration of [³H]U-37883 is shown in Fig. 2A. As explained in Materials and Methods, four tubes were assayed in parallel at each concentration of the radioligand. Thus, Fig. 2A, *line A*, shows the total binding observed with kidney microsomes. Fig. 2A, *line B*, shows the binding observed with microsomes in the presence of 10 μM unlabeled U-37883A and represents the nonspecific binding in the presence of microsomes. The amount of binding (i.e., sticking) to the filter paper in the absence of membranes is shown in Fig. 2A, *lines C* and *D*, which represent radioligand alone and with 10 μM U-37883A, respectively. The specific binding of [³H]U-37883 was then determined as $[(A - B) - (C - D)]$. Fig. 2A, *inset*, shows that the specific binding of [³H]U-37883 calculated using this formula increased linearly as a function of [³H]U-37883 concentration within the range of 0.5–5 nM used. Because the binding did not plateau even at 5 nM [³H]U-37883, saturation kinetics studies were not carried any further. Subsequent experiments were carried out using about 2 nM [³H]U-37883.

Fig. 2B shows the protein dependence of the [³H]U-37883 binding. It can be seen that the total binding of [³H]U-37883

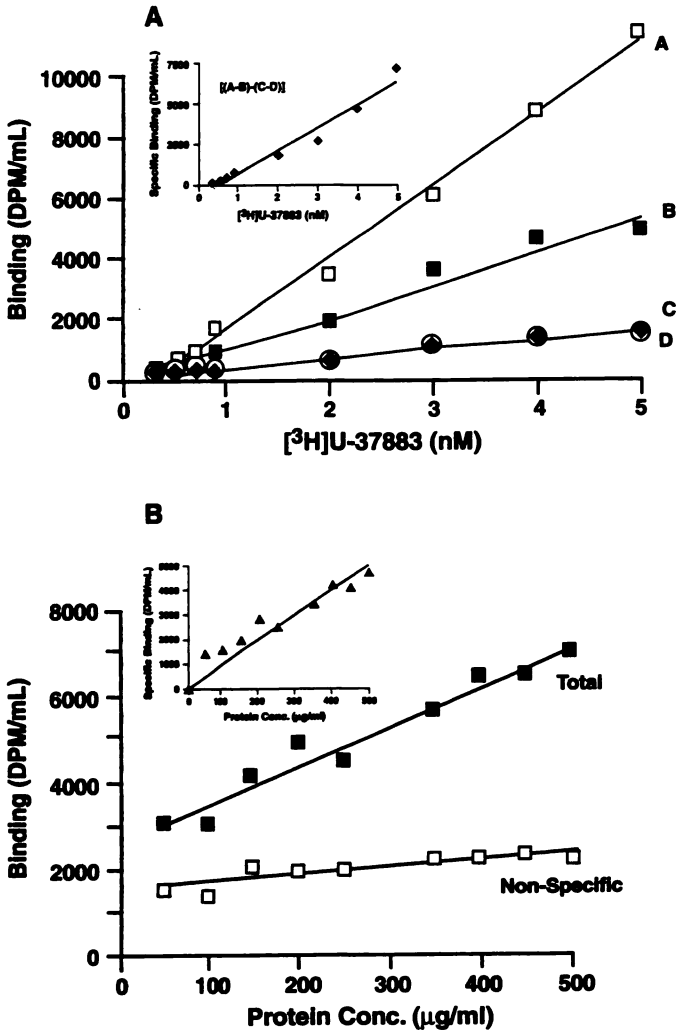


Fig. 2. A, Binding of [^{3}H]U-37883 as a function of ligand concentration. Binding was studied as total binding (*lines A* and *C*) and nonspecific binding (with 10 μM unlabeled U- 37883) (*lines B* and *D*), with membranes (*lines A* and *B*) and without membranes (*lines C* and *D*). *Inset*, specific binding of [^{3}H]U-37883 as a function of ligand concentration, determined as specific binding = [(*A* − *B*) − (*C* − *D*)]. See Materials and Methods for details. B, Binding of [^{3}H]U-37883 as a function of protein concentration. *Inset*, specific binding, which equaled total binding minus nonspecific binding.

increased linearly as a function of protein concentration in the range of 50–500 μg of protein used. In contrast, the nonspecific binding in microsomes in the presence of 10 μM unlabeled U-37883A did not increase when the protein concentration was increased. Because a fixed concentration of [^{3}H]U-37883 (2 nM) was used, the filter-sticking components (i.e., tubes C and D) remained constant and are not shown in Fig. 2B. As shown in Fig. 2B, *inset*, the specific binding increased linearly as a function of protein concentration in the entire range of 50–500 μg of protein used.

U-37883A competition curves. As described above, the initial studies suggested the [^{3}H]U-37883 binding to be of the low affinity type, because it did not saturate. Because of this constraint, experiments were designed to generate a competition curve using unlabeled U-37883A, to obtain apparent K_d values. For these studies, the [^{3}H]U-37883 concentration was 2 nM and the protein concentration was 500 μg/ml. Under these conditions, the specific binding as a percentage of total binding was found to be 60.7 ± 1.8% (four experiments). The absolute amount of specific binding observed was 76.5 ± 4.2 fmol/mg of protein (four experiments). Fig. 3A shows the U-37883A competition curve in the concentration range of 0.001–10 μM. As can be seen in Fig. 3A, no significant displacement of [^{3}H]U-37883 occurred with up to 0.05 μM U-37883A, and the majority of the displacement occurred between 0.05 and 1 μM unlabeled U-37883A. A 50% displacement of the specific binding occurred at about 0.2 μM U-37883A.

The data in Fig. 3A were transformed to generate Scatchard plots (17, 18). For this, the concentration of radiolabel was determined using the dilution factor obtained from the knowledge of the amount of unlabeled U-37883A added. Fig. 3B shows a Scatchard plot from one of the four competition experiments carried out. A computer-generated fit of the data showed that the binding represents a single class of low affinity binding sites. The mean values of K_d and B_{max} derived from four such Scatchard plots are as follows: K_d, 225 ± 21 nM (four

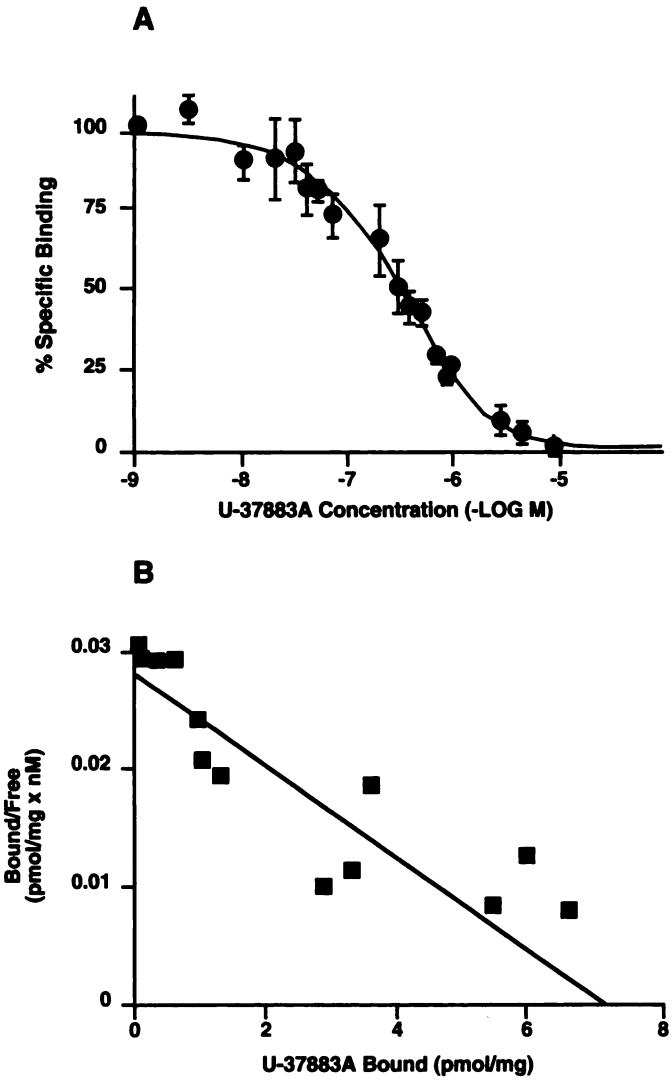


Fig. 3. A, U-37883A competition curve obtained using 2 nM [^{3}H]U-37883 (four experiments). B, Representative Scatchard plot derived from the competition experiment. The mean values of K_d and B_{max} were derived from four such Scatchard plots (K_d = 225 ± 21 nM, four experiments; B_{max} = 7.8 ± 0.4 pmol/mg of protein, four experiments).

experiments); B_{max}, 7.8 ± 0.4 pmol/mg of protein (four experiments).

Effects of proteases on [³H]U-37883 binding. To determine whether the [³H]U-37883 binding site is a protein, the susceptibility of the specific binding to various proteases was determined. Data showing the effects of trypsin, α-chymotrypsin, and Pronase pretreatment (all at 1 mg/ml concentration) on the specific binding of [³H]U-37883 in pig kidney are shown in Fig. 4. Each of the three proteases used produced a dramatic loss of the specific binding of [³H]U-37883.

Tissue specificity of [³H]U-37883 binding. Fig. 5 shows the comparative data for the specific binding of [³H]U-37883 and [³H]glyburide in membranes from pig kidney, pig brain, and RINm5F cells. The data for [³H]U-37883 binding are shown in Fig. 5A. Similar incubation conditions were used for all three membrane preparations, i.e., 2 nM [³H]U-37883 and 500 μg of protein, at room temperature, at pH 7.4. In contrast to the kidney, very little specific binding of U-37883 could be detected in membranes from brain or RINm5F cells. Fig. 5B shows the data for [³H]glyburide (0.2–0.4 nM), which displayed very large amounts of specific binding in both brain and RINm5F cells. In contrast, at 0.5 nM [³H]glyburide very little specific binding could be detected in kidney membranes (Fig. 5B, *Kidney2*). When the concentration of [³H]glyburide was increased to 2.5 nM, significant specific binding could be found in the kidney membranes (Fig. 5B, *Kidney1*). Thus, both U-37883 and glyburide displayed low affinity binding in the kidney. U-37883, in contrast to glyburide, did not display significant specific binding in brain or RINm5F cell membranes.

Association and dissociation kinetics of [³H]U-37883 binding in kidney. To complement the competition studies, the kinetics of association as well as dissociation were also investigated. Fig. 6A shows the association time course. These data were converted to generate Fig. 6A, *inset*, as described in detail by Weiland and Molinoff (19), showing the natural logarithm of fractional specific binding at a given time point as a function of time. The slope of this linear transformation gives the observed association rate (k_{obs}), which was calculated to be 16.4 sec^{-1}. The association rate constant (k_1) was calculated using the following equation, as described in Ref. 19: $k_{obs} = k_1[(L)T \times (R)T/(LR)_e]$, where $(L)T$ is the ligand concentration used (1.8 nM), $(R)T$ is the maximum binding capacity (B_{max} = 5.4 pmol/mg of protein, as determined from the Scatchard plot of the competition experiment performed at 4°), and $(LR)_e$ is the receptor-ligand concentration (i.e., equilibrium binding in this experiment equals 41 fmol/mg of protein). From these, the derived value of $k_1 = 7.0 \times 10^7$ M^{-1} sec^{-1} was obtained.

The dissociation time course of [³H]U-37883 binding after equilibrium is shown in Fig. 6B. A linear transformation of these data is presented in Fig. 6B, *inset*, which shows the natural logarithm of fractional equilibrium specific binding at a given time point as a function of time. The slope of this line provides the value of the dissociation rate constant, k_{-1} = 14.5 sec^{-1}.

The apparent K_d was derived from these data using the equation $K_d = k_{-1}/k_1$ (19) and was calculated to be 206 nM. Thus, this value of K_d derived from the kinetic experiments is in good agreement with the K_d value (225 nM) derived from the competition experiments.

Because the kinetic experiments were carried out at 4°, one experiment was also done to study the U-37883A competition curve at 4°. The U-37883A competition curve at 4° (data not shown) was very similar to that obtained at room temperature.

Functional significance of [³H]U-37883 binding. Several guanidine analogs of U-37883A, i.e., U-18117, U-42069, U-46340, U-50447, and U-52090 (see Fig. 1 for structures), were used to investigate the functional significance of the [³H]U-37883 binding. Fig. 7 shows the relationship between the ability of various analogs to inhibit specific binding of [³H]U-37883 in

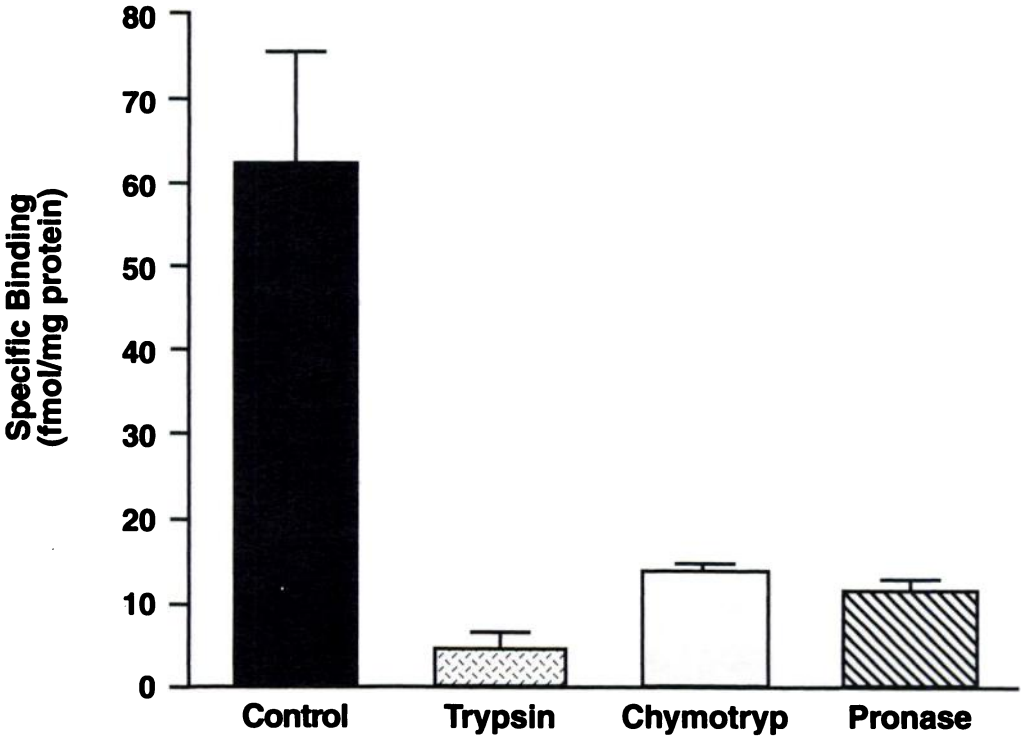


Fig. 4. Effects of proteases on the specific binding of [³H]U-37883. Trypsin, α-chymotrypsin (*Chymotryp*), and Pronase were all used at a final concentration of 1 mg/ml.

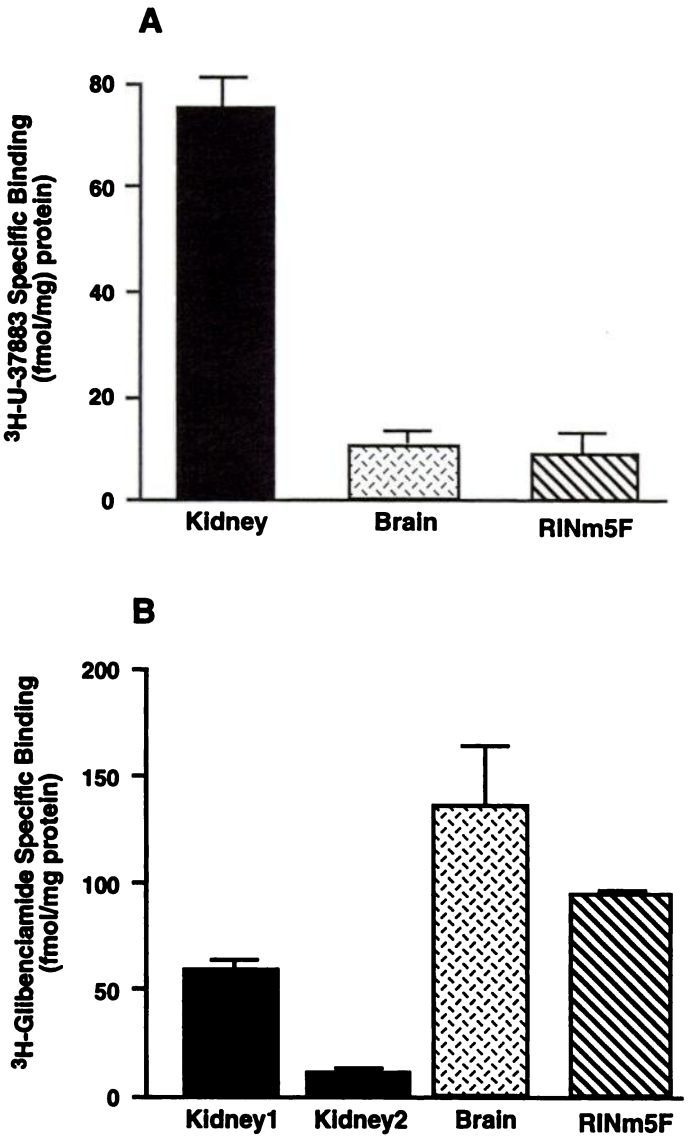


Fig. 5. Comparison of specific binding of [^{3}H]U-37883 (A) and [^{3}H]-glyburide (B) in pig kidney cortex, pig brain cortex, and RINm5F cell membranes. The [^{3}H]U-37883 concentration was about 2 nM. The [^{3}H]-glyburide concentration for brain and RINm5F cells was about 0.5 nM and for kidney was 0.5 nM (*Kidney2*) or 2.5 nM (*Kidney1*). See Materials and Methods for details.

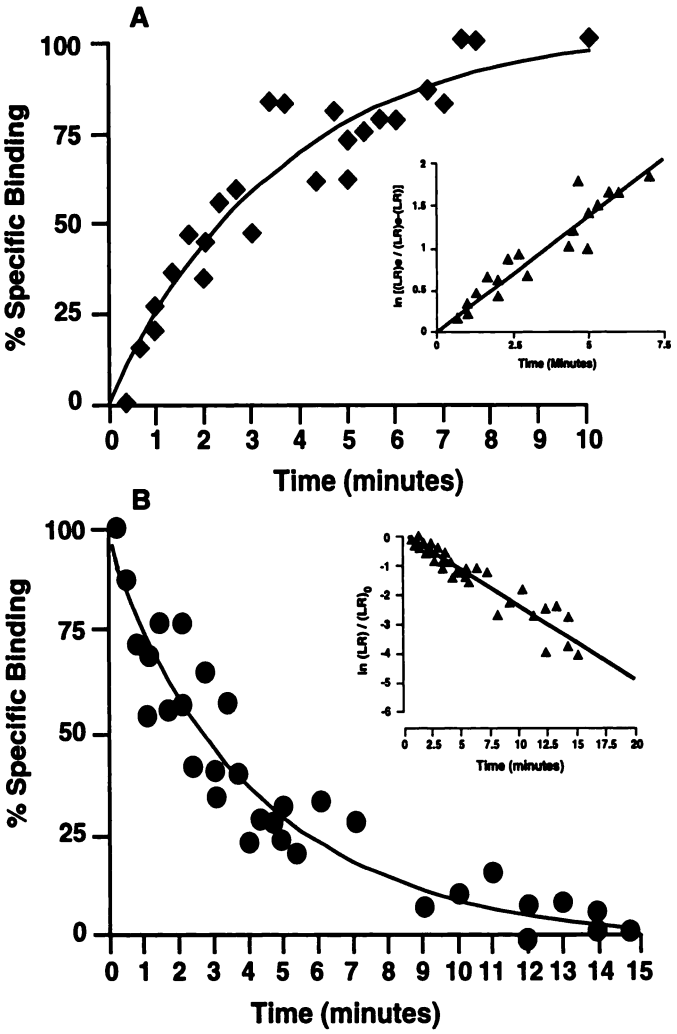


Fig. 6. A, Time course of association of the specific binding of [^{3}H]U-37883. *Inset*, logarithmic transformation of the data, as described in the text. The slope gives the observed association rate (k_{obs}), which was calculated to be 16.4 sec^{-1}. B, Time course of dissociation of the specific binding of [^{3}H]U- 37883. *Inset*, logarithmic transformation of the data, as described in the text. The slope gives the dissociation rate constant (k_{-1}), which was determined to be 14.5 sec^{-1}. The apparent K_d for U-37883 calculated from these kinetic data was 206 nM. See text for details.

kidney membranes and their ability to act as a K_{ATP} channel blocker in isolated rabbit mesenteric artery. Linear regression analysis showed a correlation coefficient of 0.926. Thus, the U-37883 analogs that were good K_{ATP} channel blockers also recognized the [^{3}H]U-37883 receptor site.

***In vivo* significance of [^{3}H]U-37883 binding.** Table 1 summarizes the diuretic/natriuretic effects of the aforementioned guanidine compounds in fasted male rats. The mean 2-hr urinary excretion values for the control rats were 1.8 ml of urine, 0.08 mEq of Na^+, and 0.05 mEq of K^+. U-37883A caused dose-dependent increases in urine volume and Na^+ excretion. Similarly, all other guanidine compounds except U-42069 produced significant dose-dependent diuresis and natriuresis in rats. U-42069 was completely inactive at all intravenous doses used.

To assist in evaluating these guanidine compounds, the standard diuretics HCTZ and furosemide were also examined over similar intravenous dose ranges. In this test system, maximal diuresis was achieved with 5.0 mg/kg HCTZ (1.18 ± 0.10 mEq of Na^+ and 0.20 ± 0.01 mEq of K^+), and nearly maximal diuresis was obtained with 10.0 mg/kg furosemide (2.12 ± 0.31 mEq of Na^+ and 0.35 ± 0.02 mEq of K^+). Compared with these standards, four of the five active guanidine compounds were considerably more effective than HCTZ in terms of facilitating Na^+ excretion, and U-37883 and U-52090 approached the high natriuresis of furosemide but with slightly less K^+ loss. Using these data, it is possible to rank all compounds by their natriuretic efficacy, calculated as the net increase in Na^+ excretion above control/cumulative intravenous dose and reflecting the areas under the respective dose-response curves. Expressed in this manner, the rank order is furosemide (236 μEq of Na^+ increase/mg/kg) > U-50447 (202 μEq of Na^+ increase/mg/kg) > U-37883 (194 μEq of Na^+ increase/mg/kg) ≈ U-52090 (193 μEq of Na^+ increase/mg/kg) > U-46340 (176 μEq of Na^+ increase/mg/kg) > U-18177 (119 μEq of Na^+ increase/mg/kg)

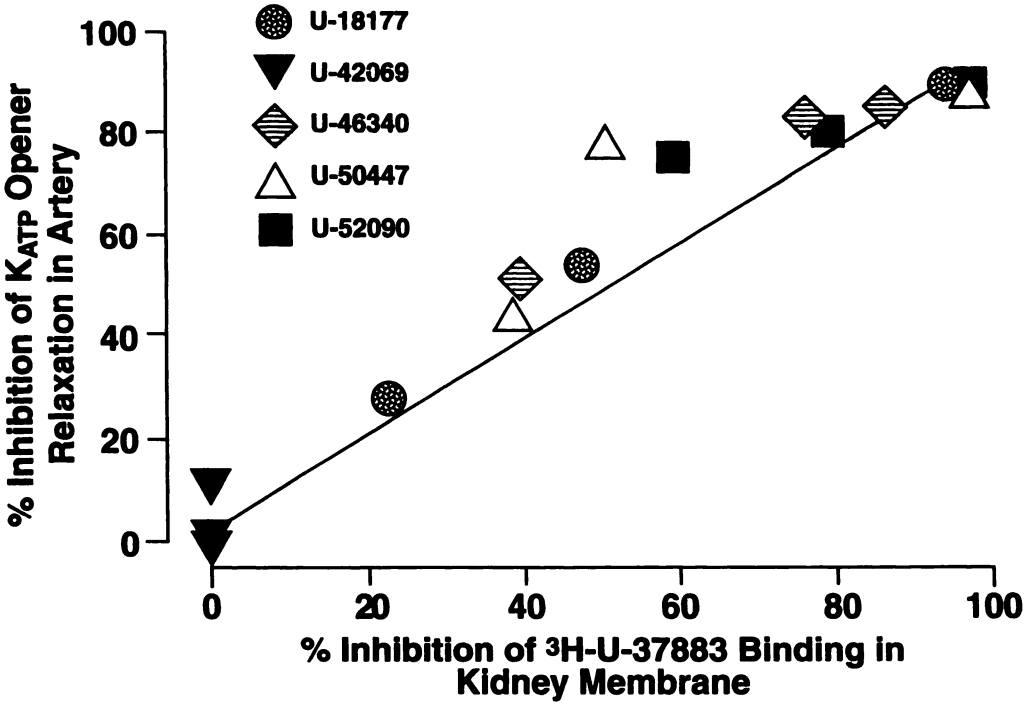


Fig. 7. Correlation between the effects of U-37883 analogs on [³H] U-37883 binding in pig kidney membranes (*x*-axis) and their effects as K_{ATP} channel blockers in isolated rabbit mesenteric artery (*y*-axis), as described in the text. Linear regression analysis showed a correlation coefficient of 0.926.

TABLE 1
Diuretic effects of selected guanidine diuretics in fasted male rats

Compound	Intravenous dose	2-hr post-treatment urinary excretion		
		Volume	Na^+	K^+
	mg/kg	*ml*	*mEq*	*mEq*
Vehicle	0	1.8 ± 0.3	0.08 ± 0.02	0.05 ± 0.01
U-37883	1.5	3.6 ± 0.6*	0.40 ± 0.02*	0.09 ± 0.01
	5.0	14.3 ± 1.0*	1.79 ± 0.14*	0.30 ± 0.03*
	15.0	15.7 ± 0.2*	2.23 ± 0.05*	0.32 ± 0.02*
U-18177	1.5	5.4 ± 0.3*	0.52 ± 0.07*	0.15 ± 0.01*
	5.0	8.0 ± 1.5*	0.89 ± 0.21*	0.22 ± 0.04*
	15.0	9.4 ± 1.2*	1.39 ± 0.14*	0.26 ± 0.05*
U-42069	1.5	2.2 ± 0.4	0.11 ± 0.03	0.08 ± 0.02
	5.0	2.3 ± 0.1	0.10 ± 0.01	0.10 ± 0.01
	15.0	2.4 ± 0.3	0.15 ± 0.03	0.11 ± 0.02
U-50447	1.5	5.1 ± 0.8*	0.50 ± 0.10*	0.20 ± 0.03*
	5.0	11.3 ± 0.4*	1.35 ± 0.08*	0.29 ± 0.03*
	10.0	12.9 ± 0.3*	1.73 ± 0.05*	0.31 ± 0.01*
U-46340	1.5	8.0 ± 0.3*	0.85 ± 0.04*	0.20 ± 0.01*
	5.0	11.2 ± 1.0*	1.33 ± 0.20*	0.27 ± 0.01*
	15.0	14.6 ± 1.0*	1.86 ± 0.06*	0.27 ± 0.03*
U-52090	1.5	8.6 ± 0.6*	0.95 ± 0.11*	0.17 ± 0.02*
	5.0	10.7 ± 0.6*	1.49 ± 0.00*	0.24 ± 0.01*
	15.0	13.3 ± 0.7*	1.96 ± 0.05*	0.27 ± 0.03*

* Significantly different ($p < 0.05$), compared with the vehicle control.

= HCTZ (119 μEq of Na^+ increase/mg/kg) ≫ U-42069 (5 μEq of Na^+ increase/mg/kg).

In summary, the guanidine compounds that competed well with [³H]U-37883 binding in kidney membranes demonstrated *in vivo* efficacy as diuretics/natriuretics and were also effective as *in vitro* K_{ATP} channel blockers. In contrast, the guanidine compound that was inactive in the binding assay failed to demonstrate *in vivo* diuresis or *in vitro* K_{ATP} channel blockade.

Discussion

This report represents our first attempt at a detailed characterization of the receptor binding of [³H]U-37883 in pig kidney membranes. We demonstrate that the binding is specific, reversible, sensitive to proteases, and tissue selective and appears to be pharmacologically and functionally relevant. The analyses using competition data as well as kinetic data revealed that the U-37883 receptor represents a single class of low affinity binding sites, with a K_d of 225 nM and a B_{max} of about 7 pmol/mg of protein. These characteristics are discussed below.

U-37883, a guanidine compound, represents a new chemical class of K_{ATP} blockers, in comparison with glyburide, a sulfonylurea. However, U-37883 possesses a distinct pharmacological profile, compared with glyburide. Whereas the primary *in vivo* effect of glyburide is insulin secretion from pancreas, the

primary *in vivo* tissue targets of U-37883 appear to be kidney and vascular smooth muscle (5, 8, 13, 14). In fact, the main *in vivo* pharmacological activity of U-37883 is to produce natriuresis/diuresis (13). The details of the *in vivo* natriuretic and diuretic activities of U-37883 and its alkyl and aryl guanidine analogs have been recently described (14). Whereas glyburide works at nanomolar concentrations in β cells (21), U-37883 works at submicromolar/micromolar concentrations in vascular smooth muscle and kidney (8, 13). In contrast to glyburide, which at much higher concentrations does produce effects on vascular smooth muscle and kidney, U-37883 appears to be without effect on the pancreatic β cells (10, 13). Based on these types of *in vitro* and *in vivo* functional data, our hypothesis has been that kidney and smooth muscle probably possess low affinity receptor sites for U-37883 that mediate the selective pharmacological effects via K_{ATP} channel blockade. Using membranes from one target tissue, i.e., kidney, we provide direct experimental support for the presence of low affinity receptor sites for U-37883. This binding is specific, reversible, and recognized by structural analogs of U-37883. Unlike high affinity binding sites, the low affinity binding sites are experimentally difficult to characterize. Thus, several different criteria were used to further verify the specific binding of U-37883. The specific binding was found to increase linearly with the protein concentration used. Also, the specific binding was susceptible to proteases. Trypsin, α-chymotrypsin, and Pronase all dramatically reduced specific binding. Thus, the U-37883 receptor site appears to be a protein.

Another important criterion used was tissue specificity of the binding. Under similar incubation conditions, we found no significant specific binding of U-37883 in tissues such as brain and RINm5F cells. We have previously shown that, unlike glyburide, U-37883 does not block K_{ATP} channels in the RINm5F cells (11, 21). Another study has shown that, in contrast to glyburide, U-37883 does not alter plasma glucose levels in rats (13). In the *in vivo* studies, U-37883 was not known to exert any specific effects in the brain (13). Thus, the lack of specific binding of U-37883 in tissues where there are no pharmacological effects of U-37883 provides strong support for the specificity of the binding observed in the kidney, which is the primary pharmacological target tissue for U-37883.

The lack of U-37883 specific binding in brain and RINm5F cells is apparently not an artifact, because by using these same membrane preparations we could demonstrate high levels of specific binding of [^{3}H]glyburide, another K_{ATP} channel blocker. These two tissues are known to contain high affinity receptors for glyburide-type sulfonylureas (5, 18, 21). It is interesting to note that we could detect low affinity binding of [^{3}H]glyburide in kidney membranes but not high affinity binding. Preliminary competition curve experiments demonstrated that the apparent K_d for glyburide was about 1 μM.[1] Glyburide has been shown to cause a similar type of natriuresis/diuresis as does U-37883 (12, 13). Thus, it may be speculated that glyburide and U-37883 both utilize their respective low affinity receptors to produce K_{ATP} channel-mediated diuresis/natriuresis. Several laboratories have implicated the presence of low affinity binding sites for glyburide (23–25), but their function has not been understood. The present study suggests a potential role of low affinity binding sites in the actions of glyburide in the kidney. Furthermore, because K_{ATP} channel blockade by glyburide in the vasculature is also of low affinity (K_i of roughly 100 nM), the low affinity binding sites could also play a role in the actions of glyburide in the vasculature. The issue of interaction between the binding sites in the kidney for [^{3}H]U-37883 and [^{3}H]glyburide described here might be complex and needs to investigated in more detail. First, characterization of the [^{3}H]glyburide binding site in the kidney, similar to that described here for U-37883, is necessary. Our preliminary experiments show that glyburide does not compete very effectively with renal [^{3}H]U-37883 binding (data not shown). This is consistent with our previous study showing that glyburide did not displace [^{3}H]U-37883 from its binding site in membranes from follicle-enclosed *Xenopus* oocytes (11). However, in vascular smooth muscle we previously reported a functional synergyism between glyburide and U-37883 as K_{ATP} channel blockers (26). It appears that a larger quantitative biochemical database needs to be developed to understand the interactive effects of these two K_{ATP} channel blockers on the relevant target tissues.

Another valuable criterion used in this study was the use of several chemical analogs of U-37883. As shown in Fig. 7, there is an excellent correlation between the ability of a guanidine compound to act as a K_{ATP} channel blocker and its ability to recognize the [^{3}H]U-37883 receptor. All compounds except one were active in both assays to varying degrees. One compound, U-42069, was inactive in both assays even up to a 100 μM concentration (data not shown). Additionally, the *in vivo* data presented here also show that receptor binding predicts the diuretic effects of these compounds. Thus, the abilities of guanidine analogs to compete with [^{3}H]U-37883 binding, produce *in vitro* K_{ATP} channel blockade, and produce *in vivo* diuresis/natriuresis correlate well. Although the *in vitro* K_{ATP} channel-blocking activity was determined using vascular smooth muscle, this correlation is probably meaningful. As pointed out earlier, kidney and vascular smooth muscle are the two primary tissue targets of U-37883. The discovery of U-37883 as a K_{ATP} channel blocker was derived from studies on vascular smooth muscle (8, 9). Using *Xenopus* oocytes, which experimentally mimic the vascular smooth muscle K_{ATP} channel model, we have provided electrophysiological evidence that U-37883 acts as a K_{ATP} channel blocker and shows the presence of a specific binding site (11). Furthermore, U-52090 was active electrophysiologically and competed with [^{3}H]U-37883 binding, whereas U-42069 was without effect in both assays in the aforementioned study (11). Finally, direct electrophysiological evidence, using the patch-clamp technique, demonstrating the ability of U-37883A to block the renal K_{ATP} channel has recently been presented.[2] Thus, all data available collectively suggest that the U-37883 receptor site modulates the renal tubular K_{ATP} channel-blocking activity.

The mechanism by which the U-37883 receptor site modulates K_{ATP} channel activity remains to be established. The cloning of a renal ATP-regulated K^+ channel has been recently described (27). It would be of interest to know whether U-37883A can directly block the ROM-K-induced K^+ current when expressed in *Xenopus* oocyte or a mammalian cell system. Alternatively, the U-37883A receptor site may represent an

[1] K. D. Meisheri, unpublished observations.

[2] T. Wang, W. H. Wang, G. Klein-Robbenhaar, and G. Giebisch. Effects of a novel K_{ATP} channel blocker on renal tubule function and K^+ channel activity. Submitted for publication.

accessory protein for the K_{ATP} channel complex. This would be analogous to the proposed mechanism for the K_{ATP} channel blockade produced by glyburide-type sulfonylureas, because the cloned cardiac K_{ATP} channel, unlike the native channel, was found not to be inhibited by glyburide (28).

Acknowledgments

The authors would like to acknowledge the analytical support provided by Michael P. Smith of the Upjohn Co.

References

1. Takano, M., and A. Noma. The ATP-sensitive K^+ channel. *Prog. Neurobiol.* **41**:21–30 (1993).
2. Edwards, G., and A. H. Weston. The pharmacology of ATP-sensitive potassium channels. *Annu. Rev. Pharmacol. Toxicol.* **33**:597–637 (1993).
3. Robertson, D. W., and M. Steinberg. Potassium channel modulators: scientific applications and therapeutic promise. *J. Med. Chem.* **33**:1529–1541 (1990).
4. Wilde, A. M., and J. J. Michiel. Electrophysiological effects of ATP-sensitive potassium channel modulation: implications for arrhythmogenesis. *Cardiovasc. Res.* **28**:16–24 (1994).
5. De Weille, J. R., M. Fosset, C. Mourre, H. Schmid-Antomarchi, H. Bernardi, and M. Lazdunski. Pharmacology and regulation of ATP-sensitive K^+ channels. *Pfluegers Arch.* **414**:S80–S87 (1989).
6. Aschcroft, F. M. Adenosine-triphosphate-sensitive potassium channels. *Annu. Rev. Neurosci.* **11**:97–118 (1988).
7. Ashcroft, S. J. H., and F. M. Ashcroft. The sulfonylurea receptor. *Biochim. Biophys. Acta* **1175**:45–59 (1992).
8. Meisheri, K. D., S. J. Humphery, S. A. Khan, L. A. Cipkus-Dubray, M. P. Smith, and A. W. Jones. 4-Morpholinecarboximidine-*N*-1-adamantyl-*N'*-cyclohexylhydrochloride (U-37883A): pharmacological characterization of a novel antagonist of vascular ATP-sensitive K^+ channel openers. *J. Pharmacol. Exp. Ther.* **266**:655–665 (1993).
9. Ohrnberger, C. E., S. A. Khan, and K. D. Meisheri. Synergistic effects of glyburide and U-37883A, two structurally different vascular ATP-sensitive potassium channel antagonists. *J. Pharmacol. Exp. Ther.* **267**:25–30 (1993).
10. Meisheri, K. D., S. A. Khan, and J. L. Martin. Vascular pharmacology of ATP-sensitive K^+ channels: interactions between glyburide and K^+ channel openers. *J. Vasc. Res.* **30**:2–12 (1993).
11. Guillemare, E., E. Honore, J. De Weille, M. Fosset, M. Lazdunski, and K. Meisheri. Functional receptors in *Xenopus* oocytes for U-37883A, a novel ATP-sensitive K^+ channel blocker: comparison with rat insulinoma cells. *Mol. Pharmacol.* **46**:139–145 (1994).
12. Clark, M. A., S. J. Humphrey, M. P. Smith, and J. H. Ludens. Unique natriuretic properties of the ATP-sensitive K^+ channel blocker glyburide in conscious rats. *J. Pharmacol. Exp. Ther.* **265**:933–937 (1993).
13. Ludens, J. H., M. A. Clark, M. P. Smith, and S. J. Humphrey. Natriuretic effects of chemically distinct ATP-sensitive K^+ channel blockers in conscious rats. *FASEB J.* **8**:A835 (1994).
14. Perricone, S. C., S. J. Humphrey, L. L. Skaletzky, B. E. Graham, R. A. Zandt, and G. R. Zins. Synthesis and diuretic activity of alkyl and aryl guanidine analogs of *N,N'*-dicyclohexyl-4-morpholinecarboxamidine in rats and dogs. *J. Med. Chem.* **37**:3693–3700 (1994).
15. Wang, W., H. Sackin, and G. Giebisch. Renal potassium channels and their regulation. *Annu. Rev. Physiol.* **54**:81–96 (1992).
16. Wang, W., A. Schwab, and G. Giebisch. Regulation of small-conductance potassium channel in apical membrane of rat cortical collecting tubule. *Am. J. Physiol.* **259**:F494–F502 (1990).
17. Wang, W. H., S. Whites, J. Giebel, and G. Giebisch. A potassium channel in the apical membrane of rabbit thick ascending limb of Henle's loop. *Am. J. Physiol.* **258**:F244–F253 (1990).
18. Bernardi, H., M. Fosset, and M. Lazdunski. Characterization, purification, and affinity labeling of the brain [³H]glibenclamide-binding protein, a putative neuronal ATP-regulated K^+ channel. *Proc. Natl. Acad. Sci. USA* **85**:9816–9820 (1988).
19. Weiland, G. A., and P. B. Molinoff. Quantitative analysis of drug-receptor interactions. I. Determination of kinetic and equilibrium properties. *Life Sci.* **29**:313–330 (1981).
20. Molinoff, P. B., B. B. Wolfe, and G. A. Weiland. Quantitative analysis of drug-receptor interactions. II. Determination of the properties of receptor subtypes. *Life Sci.* **29**:427–443 (1981).
21. Schmid-Antomarchi, H., J. R. de Weille, M. Fosset, and M. Lazdunski. The receptor for antidiabetic sulfonylureas controls the activity of the ATP-modulated K^+ channels in insulin secreting cells. *J. Biol. Chem.* **262**:15840–15844 (1987).
22. Meisheri, K. D., M. A. Swirtz, S. S. Purohit, L. A. Cipkus-Dubray, S. A. Khan, and J. J. Oleynek. Characterization of K^+ channel-dependent as well as -independent components of pinacidil-induced vasodilation. *J. Pharmacol. Exp. Ther.* **256**:492–499 (1991).
23. Niki, I., R. P. Kelly, S. J. H. Ashcroft, and F. M. Aschcroft. ATP-sensitive K^+ channels in HIT T15 β-cells studied by patch-clamp methods, ^{86}Rb efflux, and glibenclamide binding. *Pfluegers Arch.* **415**:47–55 (1989).
24. Zini, S., Y. Ben-Ari, and M. L. J. Ashford. Characterization of sulfonylurea receptors and the action of potassium channel openers on cholinergic neurotransmission in guinea pig isolated small intestine. *J. Pharmacol. Exp. Ther.* **259**:566–573 (1991).
25. Gopalakrishnan, M., D. E. Johnson, R. A. Janis, and D. J. Triggle. Characterization of binding of the ATP-sensitive potassium channel ligand [³H]-glyburide to neuronal and muscle preparations. *J. Pharmacol. Exp. Ther.* **257**:1162–1171 (1991).
26. Ohrnberger, C., S. A. Khan, and K. D. Meisheri. Synergistic effects of glyburide and U-37883A, two structurally different vascular ATP-sensitive potassium channel antagonists. *J. Pharmacol. Exp. Ther.* **266**:25–30 (1993).
27. Ho, K., C. G. Nichols, W. J. Lederer, J. Lytton, P. M. Vassilev, M. V. Kanazirska, and S. C. Hebert. Cloning and expression of an inwardly rectifying ATP-regulated potassium channel. *Nature (Lond.)* **362**:31–38 (1993).
28. Ashford, M. L. J., C. T. Bond, T. A. Blair, and J. P. Adelman. Cloning and funtional expression of a rat heart K_{ATP} channel. *Nature (Lond.)* **370**:456–459 (1994).

Send reprint requests to: Kaushik D. Meisheri, Cardiovascular Pharmacology, 7243-209-315, Upjohn Laboratories, Kalamazoo, MI 49001.