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Binding of the K^+ Channel Opener [^{3}H]P1075 in Rat Isolated Aorta: Relationship to Functional Effects of Openers and Blockers

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SUMMARY

P1075 [*N*-cyano-*N'*-(1,1-dimethylpropyl)-*N''*-3-pyridylguanidine], an analogue of the K^+ channel opener pinacidil, was shown to be a K^+ channel opener in rat aorta, based on (i) its ability to stimulate $^{86}Rb^+$ efflux, (ii) its ability to relax contractions in response to noradrenaline under normal conditions (5 mM KCl) but not under depolarized conditions (55 mM KCl), and (iii) the sensitivity of these effects to inhibition by the sulfonylurea glibenclamide. In these assays, P1075 was approximately 20 times more potent than cromakalim. Using a tritiated derivative, [^{3}H] P1075, specific binding could not be detected in microsomal preparations from various tissues. However, in rat aortic strips specific binding of [^{3}H]P1075 has been observed and was reduced by lowering the temperature or by decreasing intracellular ATP levels via metabolic inhibition. Specific [^{3}H]P1075 binding was influenced neither by depolarization (55 mM KCl) nor by lowering the pH from 7.4 to 6.0. Binding was inhibited by representatives from all major families of K^+ channel openers, with potencies that correlated well with the potencies obtained in $^{86}Rb^+$ efflux and relaxation studies. However, stimulation of $^{86}Rb^+$ efflux occurred at 40 times higher concentrations than did binding (and vasorelaxation). Of the various inhibitors of the K^+ channel openers tested, only the sulfonylureas inhibited [^{3}H] P1075 binding with the same rank order of potencies as that required for inhibition of P1075-induced $^{86}Rb^+$ efflux, although at higher concentrations. The results show that binding of [^{3}H] P1075 is independent of membrane potential but decreases concomitantly with the intracellular ATP level. The excellent correlation between the potencies of the openers and sulfonylurea blockers in binding assays and functional studies suggests that the 'drug receptor' labeled by [^{3}H]P1075 in rat isolated aorta is of functional relevance. However, the fact that binding of the openers occurred at concentrations considerably lower than those required for K^+ channel opening and that binding of the sulfonylureas was only reflected at concentrations higher than those needed to block the channel requires complex models to link binding and effect, possibly involving two agonist binding sites coupled by negative cooperativity.

Compounds that open plasmalemmal K^+ channels in smooth muscle (KCOs) are under development for use in a wide range of disorders, including ischemic diseases of heart and skeletal muscle, hypertension, and asthma (1–3). At present, several structurally distinct groups of KCOs have been identified (1–3), including the benzopyrans, e.g., cromakalim (4), and the cyanoguanidines, e.g., pinacidil (see Refs. 1–3 for reviews). These compounds produce vasorelaxation associated with an increase in the efflux of $^{42}K^+$ or $^{86}Rb^+$ and membrane hyperpolarization, and these effects are inhibited by the sulfonylurea glibenclamide (reviewed in Refs. 1–3). Consistent with their mechanism of action, the KCOs cannot elicit vasorelaxation when the membrane potential is clamped at depolarized values by elevation of the extracellular potassium concentration above approximately 35 mM (4).

Despite recent progress, the nature of the K^+ channel opened by these compounds remains controversial, and it is not known whether the structurally heterogeneous KCOs bind to the same K^+ channel (1–3). We have recently introduced a tritiated derivative of the pyridylcyanoguanidine P1075 (5), a compound structurally related to pinacidil, as a probe for the binding of KCOs in rat aortic strips (6). P1075 is a potent relaxant of canine cephalic vein precontracted with phenylephrine (pD_2 = 7.6) (5). However, the mechanism of action of this compound has not been studied in detail. In the present study we characterize the pharmacological properties of P1075 in rat aorta. The relationship of the inhibition of [^{3}H]P1075 binding, by both KCOs and their blockers, to their functional effects is examined and the coupling of the [^{3}H]P1075 binding site to

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ABBREVIATIONS: KCO, K^+ channel opener; [^{3}H]QNB, L-[*benzilic*-4,4'-^{3}H]quinuclidinylbenzilate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PSS, physiological salt solution; $[ATP]_i$, intracellular ATP concentration; K_{ATP}, ATP-sensitive K^+ channel; MOPS, 3-morpholinopropanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid.

cell metabolism is described. Some of these data have previously been presented to the German and Japanese Pharmacological Societies (7, 8).

Materials and Methods

Drugs and solutions. Synthesis of [^{3}H]P1075 (specific activity, 86.7 Ci/mmol) will be reported elsewhere.[2] The radiolabel was stored in ethanol at −20°; its purity and stability were periodically assessed by thin layer chromatography and were consistently found to be >95%. ^{86}RbCl and [^{3}H]isradipine ([^{3}H]PN 200-110; specific activity, 85 Ci/mmol) were obtained from Amersham International (Amersham, UK), and [^{3}H]QNB (specific activity, 44 Ci/mmol) was obtained from New England Nuclear (Boston, MA).

Nicorandil was a generous gift from Chugai (Tokyo, Japan), diazoxide from Essex Pharma (München, FRG), and RP 49356 and RP 61674 from Rhône-Poulenc Rorer (Paris, France); the other KCOs used in this study and the blocker tedisamil were synthesized at Sandoz (Basel, Switzerland) according to published procedures. Glibenclamide, oligomycin, and 2-deoxy-D-glucose were purchased from Sigma (Buchs, Switzerland). Glipizide was a gift from Farmitalia (Milano, Italy), AZ-DF 265 from Thomae (Biberach, FRG), glibornuride from Hoffmann-La Roche (Basel, Switzerland), guanethidine and phentolamine from Ciba-Geigy (Basel, Switzerland), and ciclazindol from Pfizer (Sandwich, UK). Substances were dissolved in dimethylsulfoxide and further dilutions were made in twice-distilled water.

Preparation of rat aortic strips. Male Sprague-Dawley rats (200–250 g) were exposed to an atmosphere of carbon dioxide and exsanguinated. The aorta was carefully removed and washed in a cold HEPES-buffered PSS (in mM): NaCl, 139; KCl, 5; $CaCl_2$, 2.5; $MgCl_2$, 1.2; glucose, 11; HEPES, 5; gassed with 95% O_2 5% CO_2 and titrated to pH 7.4 with NaOH at 37°. Adherent fat and some of the adventitia were removed and the aorta was cut into rings. In all experiments tissues were mounted on a metal tissue holder and immersed in PSS.

Tension studies. Endothelium-intact aortic rings (≈4 mm in length) were mounted for isometric recordings at 37° under an initial tension of 1 g. After a 90-min equilibration period, tissues were exposed to noradrenaline (100 nM) in buffer containing normal KCl (5 mM) or high KCl (55 mM), the latter in the presence of isradipine (100 nM) and reduced NaCl (89 mM). A maintained contraction was allowed to develop. Tissues were then exposed to glibenclamide (100 nM) or solvent and, 20 min later, to increasing concentrations of P1075 or another KCO (or solvent), using a cumulative protocol (with the exception of minoxidil sulfate) (9). Measurements of spontaneous activity of rat portal vein were performed as described (10).

^{86}Rb$^+$ efflux studies. Aortic rings (15–20 mm in length) with intact endothelium were incubated for 120 min in PSS to which had been added ^{86}Rb$^+$ (5 μCi/ml). They were transferred to thermostatic perfusion chambers and perfused with PSS at 2.5 ml/min at 37°. The perfusate was collected at 2-min intervals and counted for radioactivity as previously described in detail (10). The data were expressed as the efflux rate coefficient (k, in 10^{-2} min^{-1}), which represents the radioactivity released per minute expressed as a percentage of the radioactivity remaining in the tissue.

KCO effects were expressed as the difference (Δk) between the peak increase in k and the base-line value before drug application. Inhibition of P1075-stimulated efflux (60 nM, applied for 20 min) was assessed in a double-pulse protocol by comparison of the areas under the curve of the efflux rate coefficient versus time plots in the presence and absence of inhibitor (9).

[^{3}H]P1075 binding assays in membrane preparations. Membranes were prepared from smooth muscle tissue (rat thoracic aorta and guinea pig ileal longitudinal muscle) and also from rat cerebral cortex, lung, and heart. Tissues were homogenized using a Polytron homogenizer (Kinematika, Luzern, Switzerland) and/or a Potter Elverjem homogenizer (Braun, Melsungen, FRG). The homogenates were centrifuged according to published protocols [aorta (11), ileum (12, 13), brain (14), heart (15), and lung (16)]. Aliquots of homogenates, supernatants, or resuspended pellets from the various centrifugation steps were adjusted to protein concentrations of 0.1–2 mg/ml and incubated with the Ca^{2+} antagonist [^{3}H]isradipine (0.1–3 nM), the muscarinic antagonist [^{3}H]QNB (0.1–3 nM), or the KCO [^{3}H]P1075 (0.1–30 nM) for 30–90 min at 37°. Bound and free ligand were separated by rapid filtration over Whatman GF/B or Millipore GSWP 2500 (pore size, 0.22 μm) filters or by centrifugation at 30,000 to 100,000 × g. Pellets were dissolved in 0.5 ml of Lumasolve (Lumac; Facola, Basel, Switzerland), then supplemented with 10 ml of scintillant (Optifluor; Packard, Zürich, Switzerland), and counted for ^{3}H; filters were counted in 10 ml of Optifluor. Nonspecific binding of [^{3}H]isradipine, [^{3}H]QNB, and [^{3}H]P1075 was determined in the presence of isradipine, atropine, and P1075 (1 μM), respectively.

Inhibition of [^{3}H]P1075 binding by unlabeled drugs in rat aortic strips. Aortic rings (2–3 mm in length, 2–4 mg of wet weight) were blotted and cut open, and the endothelium was removed by rubbing with a moistened cotton bud. The tissues were then weighed and, using a randomized design, incubated in quadruplet at 37° for 90 min in PSS containing [^{3}H]P1075 (0.3 nM) and the unlabeled inhibitor of interest. They were then transferred to ice-cold PSS for 1 min, blotted, and assigned to individual vials containing 0.5 ml of Lumasolve. After 2 hr, the samples were supplemented with 0.5 ml of 0.1 M HCl and 16 ml of Optifluor and counted for ^{3}H. Nonspecific binding (≈220 dpm/mg of tissue wet weight) was determined in the presence of unlabeled P1075 (1 μM) and ranged between 35 and 40% of total binding. Concentration-inhibition curves were analyzed by fitting the data to the Hill equation. At the concentration of radiolabel used [0.3 nM, i.e., less than 1/10th of the K_d value obtained for [^{3}H]P1075 binding in saturation experiments (K_d = 6 nM) (6)], the apparent inhibition constant (IC_{50}) of a ligand competing with the radiolabel is approximately equal to the inhibition constant, K_i, of this ligand (17).

Binding assays and determination of tissue ATP levels under varying incubation conditions. Endothelium-denuded strips of rat aorta (≈6–8 mg of wet weight) were preincubated for 60 min under the conditions detailed in the legend to Table 1. Half of the tissues were incubated for an additional period of 60 min, frozen in liquid N_2, and stored at −70° for determination of $[ATP]_i$ as described below. The other half was supplemented with [^{3}H]P1075 (0.3 nM) in the absence and presence of P1075 (1000 nM) and incubated for 60 min under the same conditions, and the bound radioactivity was determined.

For determination of $[ATP]_i$ tissues were prepared, with minor modifications, according to the method of Post and Jones (18). They were thawed in 0.7 ml of $HClO_4$ (5%) on ice and homogenized with a Brinkman Polytron homogenizer. The homogenate was centrifuged at 13,000 × g for 7 min, the supernatant was brought to pH 7.8 with KOH (10 M)/triethanolamine (0.5 M), and the precipitate was removed by centrifugation at 13,000 × g for 20 min. The supernatant was assayed for ATP using a luminescence kit based on the luciferase reaction (bioluminescent somatic cell assay kit; Sigma). The signal was calibrated by spiking the solution with known amounts of exogenous ATP. The extraction of control tissues gave a value of 0.16 ± 0.01 nmol of ATP/mg of tissue wet weight.

For protein determination, tissues were dissolved in 1 M NaOH (250 μl/mg of wet weight) at 95° for 1 hr and then Na_2CO_3 was added to give a 2% solution. Protein was determined according to the method of Lowry *et al.* (19), using bovine serum albumin as the standard, and gave a value of 270 ± 7 μg of protein/mg of tissue wet weight.

Data fitting and model calculations. Parameters of concentration-response curves were estimated by performing unweighted least squares fits of the data to the Hill equation. Errors in these parameters

[2] P. W. Manley, U. Quest, H. Andres, and K. M. Bray. Synthesis of and radioligand binding studies with a tritiated pinacidil analogue: Receptor interactions of structurally different classes of potassium channel openers and blockers. Manuscript in preparation.

were calculated by propagation of errors using linear approximations and neglecting covariances (20).

Results

Effects of P1075 on $^{86}Rb^+$ efflux and tension in rat aorta. P1075 stimulated $^{86}Rb^+$ efflux from rat aorta. Thus, at 0.1 μM, P1075 induced an increase in $^{86}Rb^+$ efflux similar to that induced by cromakalim (1 μM), when areas under the curve or peak increases (Δk) were compared (Fig. 1A). At the higher concentration of P1075 used (3 μM), the marked increase in k was transient and decayed rapidly during the 20-min application period. In Fig. 1A, *inset*, the concentration-effect curves of P1075 and cromakalim for $^{86}Rb^+$ efflux are compared, giving midpoints of 0.16 and 2.7 μM, respectively. The $^{86}Rb^+$ efflux stimulated by P1075 (60 nM) was reduced to ≈20% of control by the sulfonylurea glibenclamide (300 nM) (Fig. 1B).

In rat aortic rings, preincubation with P1075 (0.3–1000 nM) produced a concentration-dependent inhibition of noradrenaline (100 nM)-induced tone (Fig. 2), with a pD_2 value of 8.52 ± 0.03. In the presence of glibenclamide (100 nM), the concentration-relaxation curve for P1075 was shifted 10-fold to the right (pD_2 = 7.56 ± 0.01), with no effect on maximum relaxation. In depolarizing medium (55 mM KCl, in the presence of the dihydropyridine calcium antagonist isradipine to block KCl-induced tension), P1075 relaxed noradrenaline-induced contractions only at very high concentrations (≥10 μM), i.e., at concentrations 10,000-fold higher than those effective under nondepolarizing conditions (Fig. 2).

Activity of [^{3}H]P1075 in rat portal vein. The biological activity of the radiolabel was tested in rat portal vein. Superfusion of the tissues with [^{3}H]P1075 (10 nM) completely abolished myogenic activity. This effect was observed as a gradual reduction of the contraction amplitude to ≈50% of the predrug level, accompanied by a reduction in frequency to 0, over 30 min (four experiments, data not shown). Similar results were obtained with the unlabeled substance (data not shown), indicating that radiolabeling did not affect the biological activity of the compound.

Lack of specific [^{3}H]P1075 binding in membrane preparations. Rat aortae were homogenized under conditions known to minimize proteolysis (Ca^{2+}-free buffer, in the presence of 1 mM EGTA and phenylmethylsulfonyl fluoride, bestatin, and pepstatin at 1 μM each) and/or to preserve the phosphorylation state of the preparation with the presence of 50 mM NaF and 0.1 mM MgATP. The crude homogenate and the pellets and supernatants resulting from centrifugations at 1000, 10,000, and 100,000 × g (11) were assayed for [^{3}H]P1075 binding (0.1–30 nM) but showed only nonspecific binding. In contrast, specific binding of [^{3}H]QNB and of [^{3}H]isradipine (the latter in the presence of Ca^{2+}) was readily detected in most microsomal preparations. Variation of the ionic composition of the buffer (in particular, the concentrations of Ca^{2+}, K^+, and Mg^{2+}), variation of the buffering ion (Tris, HEPES, MOPS, or inorganic phosphate), addition of ATP, lysis of the microsomes by repeated hypotonic shock, variation of the incubation conditions (time, temperature, and pH), and separation of bound from free ligand by two different procedures (rapid filtration and centrifugation) were tried without success; binding of [^{3}H]P1075 was always the same regardless of the presence or absence of unlabeled P1075 (1 μM). Nonspecific binding was linearly related to protein and radiolabel concentration. The search for specific binding of [^{3}H]P1075 in crude homogenates and microsomal fractions prepared from guinea pig ileal longitudinal muscle or rat cerebral cortex, lung, or heart was similarly unsuccessful, whereas specific binding of [^{3}H]isradipine and [^{3}H]QNB in these preparations was saturable and of high affinity, in agreement with published data (data not shown).

[^{3}H]P1075 binding in rat aortic strips and dependence on temperature, pH, membrane potential, and the metabolic state of the tissue. In rat aortic strips, specific binding of [^{3}H]P1075 was observed that was saturable and of nanomolar affinity (6, 8). In the present study, the influence of different incubation conditions on specific binding of [^{3}H]P1075 (0.3 nM) was determined together with the changes in $[ATP]_i$. The results are listed in Table 1.

Decreasing temperature led to a reduction in binding that was very marked below 20°. At 1°, after 2 and 4 hr of incubation binding was reduced to 15% and ATP levels to ≈50% (see Table 1). A moderate decrease in pH (to 6.0) and depolarization of the tissue (55 mM KCl) did not appreciably affect binding.

Several protocols were used to inhibit cell metabolism (Table 1). Hypoxia and inhibition of glycolysis (replacement of 11 mM glucose by 1 mM deoxy-D-glucose) each reduced binding and $[ATP]_i$ by approximately 50%; their combination reduced both parameters by >90%. A buffer designed to mimic the conditions of mild ischemia (elevated KCl, reduced pH, glucose, and O_2, and the presence of lactate) (21) and metabolic inhibition by CN^- (2 mM) (22) in the absence of glucose were moderately effective in reducing binding and/or $[ATP]_i$. Inhibition of mitochondrial function by oligomycin (in the presence of 2-deoxy-D-glucose) (23) reduced binding and $[ATP]_i$ concentration dependently to very low levels, as did the alkylating agent iodoacetic acid (1 mM, (22)), which was more effective than *N*-ethylmaleimide. These results suggest that $[ATP]_i$ is a major determinant of [^{3}H]P1075 binding.

Inhibition of [^{3}H]P1075 binding by KCOs. KCOs belonging to different structural families inhibited binding of [^{3}H]P1075 in rat aortic strips (see Table 2 for the parameters of the concentration-response curves, i.e., the midpoint, pK_i, the extent of inhibition, A_{max}, and the Hill slope, n_H). Inhibition was stereoselective, as seen from the eudismic ratios of ≈20 for the enantiomers of pinacidil and >100 for the enantiomers of cromakalim (BRL 38227 and BRL 38226) as well as for the enantiomeric pair SDZ PCO 400 and SDZ PCO 399. A_{max} was approximately 65% of total binding and the Hill coefficients were close to unity.

Table 2 also shows the parameters for the concentration-dependent stimulation of $^{86}Rb^+$ efflux from rat aortic rings or rat portal vein. Both preparations gave similar results, with the exception of the flux amplitude (A_{max}), which was approximately twice as large in aorta as in portal vein. The pK values obtained in the flux assays correlated very well with the pK_i values from the binding assay (r = 0.97, slope = 1.0 ± 0.1, ordinate intercept = −1.6 ± 0.6; Fig. 3); however, inhibition of binding occurred at about 40 times lower concentrations than did stimulation of $^{86}Rb^+$ efflux.

Pronounced differences between the KCOs were found when the amplitudes and Hill coefficients of their concentration-relaxation curves were considered (Table 2). However, the potencies of the KCOs derived from the binding assay compared very well with their vasorelaxant potencies (Table 2). Linear correlation analysis of the double-logarithmic plot of pD_2 (va-

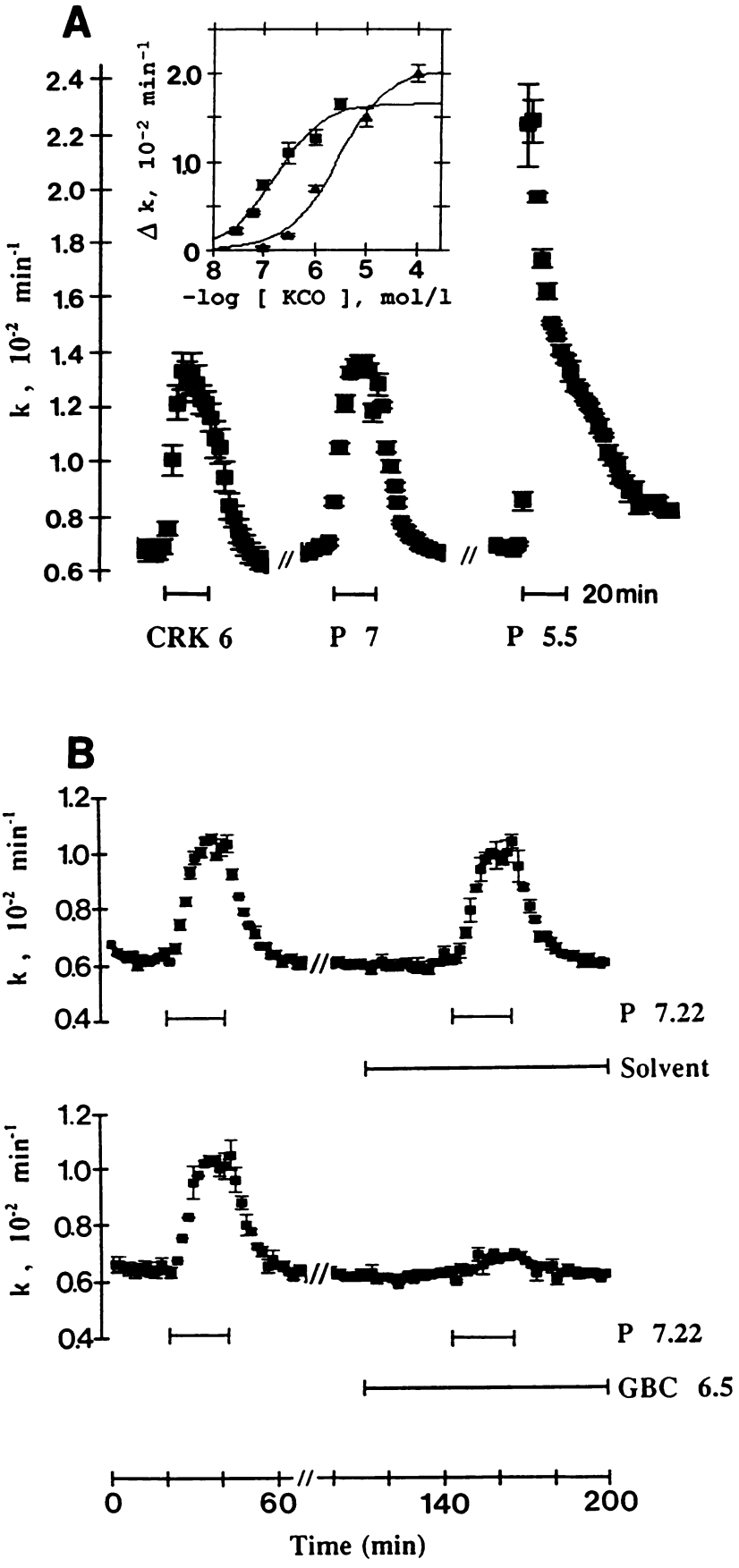

Fig. 1. Stimulation of $^{86}Rb^+$ efflux by P1075 (*P*) or cromakalim (*CRK*) in rat aorta. A, Increases in the rate of constant of $^{86}Rb^+$ efflux (*k*) induced by 20-min superfusions of cromakalim (1 μM) and P1075 (0.1 and 3 μM); means ± standard errors from four to six experiments. *Inset*, concentration dependence of the effect on tracer efflux. ■, P1075; ▲, cromakalim. The fit of the data to the Hill equation gave the following parameters (P1075/cromakalim): midpoint (μM), 0.16 ± 0.06/2.73 ± 0.1; maximum effect (10^{-2} min^{-1}), 1.66 ± 0.21/2.05 ± 0.18; Hill coefficient, 0.94 ± 0.27/0.88 ± 0.19; four to six experiments. B, Inhibition of P1075-induced $^{86}Rb^+$ efflux by glibenclamide (*GBC*). *Upper trace* (control), P1075 (60 nM) was superfused for 20 min at the times indicated. *Lower trace*, glibenclamide (300 nM) was superfused 20 min before the second application of P1075 until the end of the experiment and inhibited the response to P1075 by 78 ± 5% (three experiments). *Numbers near the bars*, ligand concentration, in −log (M).

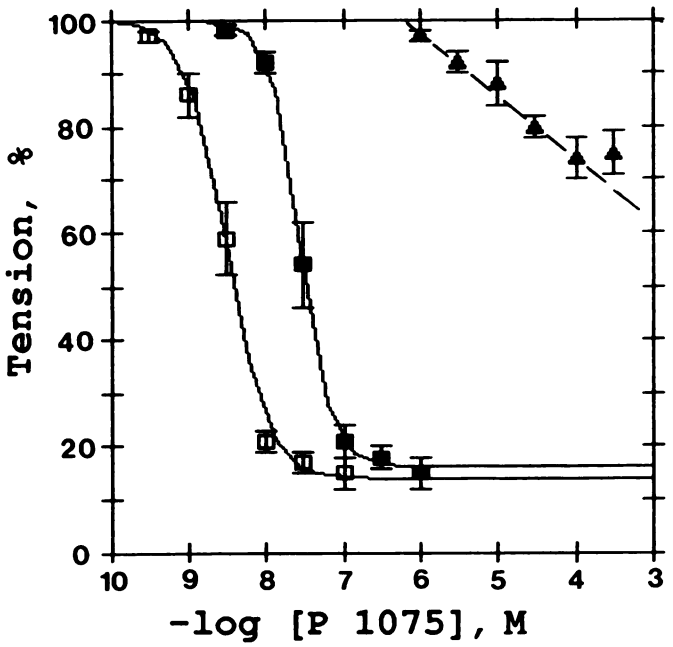

Fig. 2. Vasorelaxant effects of P1075 in noradrenaline (100 nM)-precontracted aorta. □, Control conditions; ■, in the presence of glibenclamide (0.1 μM); ▲, depolarizing conditions (55 mM KCl) and in the presence of isradipine (0.1 μM, to block KCl-induced tension). Hill analysis of the data in normal salt solution (5 mM KCl) yielded the following parameter values (without glibenclamide/with glibenclamide): midpoint (nM), 3.0 ± 0.2/27 ± 1; maximum relaxation (% inhibition of initial tension), 86 ± 2/84 ± 1; Hill coefficient, 1.67 ± 0.19/2.17 ± 0.15; six to 12 experiments.

TABLE 1

Changes in [^{3}H]P1075 binding and tissue ATP levels with changes in incubation conditions and after metabolic inhibition

Specific binding is defined as the difference in [^{3}H]P1075 (0.3 nM) binding in rat aortic strips in the absence and presence of unlabeled P1075 (1 μM); the control value was 1.6 ± 0.1 fmol/mg of tissue wet weight (120 experiments). [ATP]$_i$ was determined as described in Materials and Methods; the control value was 0.16 ± 0.01 nmol of ATP/mg of tissue wet weight, corresponding to 0.59 ± 0.04 nmol of ATP/mg of protein (three or four experiments).

Conditions[a]	Specific binding	[ATP]$_i$
	% of control	% of control
22°	82 ± 8	ND[b]
10° (2 or 4 hr)	46 ± 13	87 ± 13
1° (2 or 4 hr)	15 ± 6	53 ± 17
pH 6	105 ± 7	ND
55 mM KCl	83 ± 7	ND
N_2 + G	44 ± 7	50 ± 12
O_2 + D	61 ± 9	59 ± 10
N_2 + D	8	3 ± 0.05
Kwan buffer	72 ± 9	ND
CN^- (2 mM) − G	60 ± 1	47 ± 13
Oligomycin (0.24 μg/ml) + D	52 ± 9	19 ± 9
Oligomycin (1 μg/ml) + D	15 ± 5	8 ± 5
Iodoacetate (1 mM)	≈0	2 ± 1
N-Ethylmaleimide (20 μM)	32 ± 6	9 ± 1

[a] Conditions: N_2, bubbling with N_2 (95%)/CO_2 (5%); O_2, bubbling with O_2 (95%)/CO_2 (5%); D, 2-deoxy-D-glucose (1 mM) and no glucose; G, glucose (11 mM); Kwan buffer (mM), KCl (7.6), glucose (4), lactate (1), N_2, pH 6.8 (21); total exposure time, 2 hr unless stated otherwise.
[b] ND, not determined.

sorelaxation) versus pK_i (binding) gave a correlation coefficient of $r = 0.94$ and the fitted line was close to the line of identity (slope = 1.07 ± 0.26, ordinate intercept = −0.2 ± 1.3; data not shown).

K$^+$ channel blockers. Table 3 compares K$^+$ channel blockers in their ability to inhibit P1075-induced ^{86}Rb$^+$ efflux and [^{3}H]P1075 binding in rat aortic strips. Glibenclamide and glipizide ('second-generation' structurally extended sulfonylureas), glibornuride ('first-generation' short sulfonylurea), and the sulfonylurea-related compound AZ-DF 265 (for chemical structure, see Ref. 24) inhibited binding with the same rank order of potencies as that with which they inhibited P1075-stimulated ^{86}Rb$^+$ efflux. Linear correlation analysis of the pK values for inhibition of flux versus the pK_i values from the binding assays gave a correlation coefficient of $r = 0.99$; the slope of the fitted line was 1.09 ± 0.12 and the ordinate intercept was approximately 0 (data not shown). Inhibition of binding required 2–4 times higher concentrations than did inhibition of ^{86}Rb$^+$ efflux (Table 3).

Other structurally diverse inhibitors such as tedisamil (25), ciclazindol (26), phentolamine (27), and guanethidine (28) were quite potent as inhibitors of P1075-stimulated ^{86}Rb$^+$ efflux, although very weak as inhibitors of [^{3}H]P1075 binding (Table 3). Note also the low Hill coefficient values for tedisamil, ciclazindol, and guanethidine in the binding assay.

Discussion

The major findings of the present study are that (i) the cyanoguanidine P1075 is a potent and specific KCO in rat aorta, (ii) specific binding of [^{3}H]P1075 in rat aortic strips is diminished concomitantly with reductions in [ATP]$_i$, and (iii) binding of the KCOs occurs at concentrations 40 times lower than those required for K$^+$ channel opening, whereas binding of the sulfonylurea blockers occurs at higher concentrations than those required for K$^+$ channel blockade.

K$^+$ channel-opening properties of P1075. Three pharmacological properties of P1075 characterize this compound as a potent and specific KCO. Firstly, P1075 stimulated ^{86}Rb$^+$ efflux from rat aortic rings and was approximately 10 times more potent than cromakalim. Secondly, the concentration-vasorelaxation curve for P1075 was shifted 10,000-fold to the right upon depolarization of the tissue by elevated KCl (55 mM). In principle, the vasorelaxant effect of a compound acting solely via K$^+$ channel opening should be abolished by depolarization (4); the large shift of the P1075 curve, therefore, indicates that this compound is a particularly 'pure' KCO. This is in contrast to the small (10-fold) shift found with the structurally related (−)-*R*-enantiomer of pinacidil, indicating that the latter compound has additional vasorelaxant mechanisms of action (29). Thirdly, the vasorelaxant and ^{86}Rb$^+$ efflux-stimulating effects of P1075 were inhibited by glibenclamide, the prototypical sulfonylurea inhibitor of the KCOs (24). From the 9-fold shift of the P1075 relaxation curve induced by glibenclamide (100 nM), an inhibition constant (K_i) of 12.5 nM can be estimated for glibenclamide (adopting a competitive formalism based on the fact that binding of glibenclamide and [^{3}H]P1075 is mutually exclusive (6)). Quantitative evaluation of the inhibition by glibenclamide of P1075-induced ^{86}Rb$^+$ efflux at different P1075 concentrations gives a similar inhibition constant (≈20 nM).[3] Taken together, these results show that P1075 is a potent and specific KCO, which is an important prerequisite for its development as a potential radioligand.

Lack of specific [^{3}H]P1075 binding in membranes. A bioassay in rat portal vein showed that [^{3}H]P1075 was equipotent to P1075 as a vasorelaxant, suggesting that radiolabeling

[3] U. Quast, unpublished observations.

TABLE 2

KCO binding properties, stimulation of $^{86}Rb^+$ efflux and vasorelaxation in rat aorta

Binding of KCOs was determined by inhibition of [^{3}H]P1075 (0.3 nM) binding to rat aortic strips. The parameters of the inhibition-concentration curves were derived by nonlinear least squares fits to the Hill equation [pK_i = −log [midpoint (M)]; A_{max} = maximum inhibition of total binding (%); n_H = Hill slope]. Stimulation of $^{86}Rb^+$ efflux was determined in rat aortic strips (α) or rat portal vein (β) and the data were analyzed as above. The midpoint is approximately the same in the two preparations; A_{max} in portal vein is about 50% of that in aorta. The vasorelaxant properties of the KCOs were assessed in rat aortic rings precontracted with noradrenaline (0.1 μM) [pD_2 = −log[midpoint (M)]; A_{max} = maximum relaxation, in percentage of total tension]. Six to 12 experiments were performed throughout.

Substance[a]	Structural class[b]	Binding			$^{86}Rb^+$ efflux				Relaxation		
		pK_i	A_{max}	n_H	pK	A_{max}	n_H		pD_2	A_{max}	n_H
		(% of total binding)			(10^{-2} min^{-1})				(% of tension)		
P1075	CG	8.54 ± 0.03	65 ± 5	0.90 ± 0.05	6.80 ± 0.16	1.7 ± 0.2	0.94 ± 0.3	(α)	8.52 ± 0.03	86 ± 1	1.69 ± 0.20
RP 61674	TA	8.25 ± 0.04	64 ± 5	0.93 ± 0.07	7.25 ± 0.21	1.6 ± 0.2	0.74 ± 0.2	(α)	9.40 ± 0.01	76 ± 1	1.97 ± 0.08
(−)-Pinacidil	CG	7.66 ± 0.06	65 ± 5	0.90 ± 0.11	5.57 ± 0.03	0.6 ± 0.1	1.40 ± 0.1	(β)	7.38 ± 0.03	93 ± 2	1.65 ± 0.16
Minoxidil sulfate	O	7.45 ± 0.10	57 ± 7	0.92 ± 0.16	6.13 ± 0.12	0.2 ± 0.02	1.30 ± 0.5	(α)	7.35 ± 0.10	88 ± 7	1.06 ± 0.24
BRL 38227	BP	7.33 ± 0.06	57 ± 8	1.02 ± 0.11	5.80 ± 0.08	2.0 ± 0.3	1.00 ± 0.1	(α)	7.43 ± 0.06	78 ± 2	1.15 ± 0.02
SDZ PCO 400	BP	7.21 ± 0.06	65 ± 5	0.98 ± 0.05	5.90 ± 0.1	0.8 ± 0.2	1.00 ± 0.1	(β)	7.42 ± 0.02[c]	65 ± 1[c]	1.82 ± 0.26[c]
RP 49356	TA	6.75 ± 0.05	65 ± 5	0.84 ± 0.07	5.10 ± 0.1	0.9 ± 0.2	1.20 ± 0.1	(β)	6.77 ± 0.03	84 ± 2	2.00 ± 0.2
(+)-Pinacidil	CG	6.27 ± 0.16	65 ± 5	0.92 ± 0.10	4.30	≈0.4	1.00 ± 0.3	(β)	6.49 ± 0.05	100 ± 3	0.98 ± 0.08
SDZ 114-712	CG	5.86 ± 0.05	65 ± 5	1.15 ± 0.13	≈4	≈0.5	≈1	(β)	5.54 ± 0.01	78 ± 1	1.73 ± 0.04
Nicorandil	O	5.31 ± 0.06	65 ± 5	0.86 ± 0.11	3.90 ± 0.1	0.8 ± 0.2	3.90 ± 2.4	(β)	6.21 ± 0.04	100	1.35 ± 0.09
BRL 38226	BP	5.02 ± 0.09	65 ± 5	0.80 ± 0.12	<4	>0.5	?[d]	(β)	5.23 ± 0.06	56 ± 2	1.37 ± 0.20
SDZ PCO 399	BP	5.06 ± 0.03	65 ± 5	0.83 ± 0.04	<4	>0.5	?	(β)	4.80 ± 0.03	69 ± 2	1.27 ± 0.09
Diazoxide	O	4.66 ± 0.03	65 ± 5	1.49 ± 0.12	≈3.3	≈0.7	1.10 ± 0.1	(β)	5.00 ± 0.02	91 ± 2	1.90 ± 0.2
SDZ 214-391	CG	4.44 ± 0.05	65 ± 5	1.00 ± 0.10	<4	?	?	(β)	4.84 ± 0.07	94 ± 5	1.22 ± 0.22

[a] For structural formulae, see Refs. 1–3; SDZ PCO 399, (+)-3*R*,4*S*-enantiomer of SDZ PCO 400; SDZ 114-712, *N*-3-pyridinyl-*N*′-(1,1-dimethylethyl)urea; SDZ 214-391, (*E*)-*N*-4-pyridinyl-*N*′-(2,2-dimethylpropyl)-2-nitro-1,1-ethenediamine.

[b] BP, benzopyran; CG, pyridinylcyanoguanidine or related structure (pyridinylurea or pyridinylnitroethenediamine); TA, pyridinyl-thioacetamide; O, other.

[c] High affinity component only.

[d] ?, not determined because saturation of concentration-response curve could not be reached.

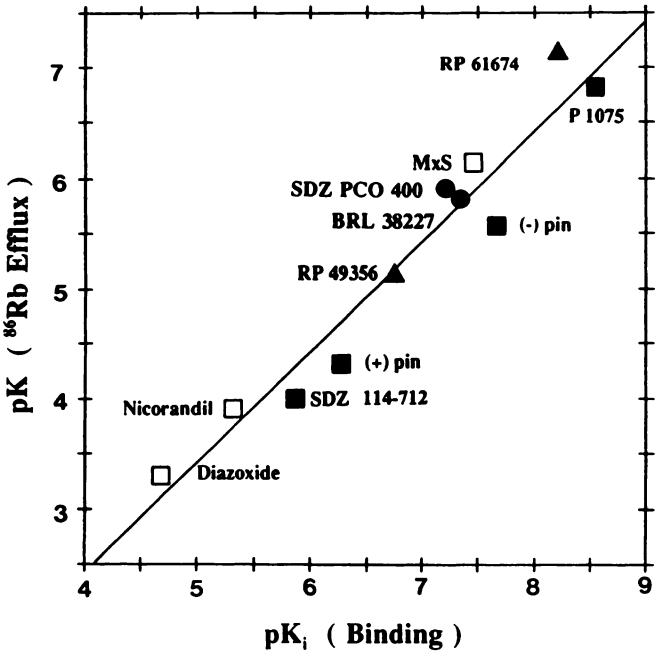

Fig. 3. Correlation of the potencies of KCOs in inducing $^{86}Rb^+$ efflux (pK) with the pK_i values determined in the binding assay. The fitted *line* (r = 0.97) has a slope of 1.0 ± 0.1 and an ordinate intercept of −1.6 ± 0.6. Different symbols were chosen for the different structural classes of KCOs; *pin*, pinacidil; *MxS*, minoxidil sulfate.

had not changed the biological activity of the compound. Extensive investigations were then undertaken in an attempt to detect specific binding of [^{3}H]P1075 in crude homogenates and membrane preparations from rat aorta, because specific binding of [^{3}H]P1075 had been found in intact rings of this tissue (7, 8). Despite the adopting of conditions that minimize proteolysis and/or preserve the phosphorylation state of the preparation after homogenization, these attempts were unsuccessful, as were assays in membrane preparations from other tissues in which effects of the KCOs and/or specific binding of [^{3}H] glibenclamide have been reported, e.g., guinea pig ileum longitudinal muscle (including myenteric neurons), brain, and lung (for reviews see Refs. 3, 30).

The failure to detect specific [^{3}H]P1075 binding in rat aortic microsomes, in contrast to the positive results in intact tissue rings, may be due any of the following reasons: (i) denaturation of the drug receptor by the membrane preparation procedure, (ii) loss of a cytosolic factor (other than ATP) necessary for binding, or (iii) the cytosolic nature of the drug receptor (which would then be an entity different from the channel). Efforts to distinguish between these possibilities are ongoing.

Binding of [^{3}H]P1075 in rings of rat aorta under varying incubation conditions and metabolic inhibition:Correlation with [ATP]$_i$. Analogous to earlier reports on specific binding of [^{3}H]isradipine in vascular strips (31), we have identified specific binding of [^{3}H]P1075 in intact strips of rat aorta (K_d = 6 ± 1 nM; B_{max} = 21 ± 3 fmol/mg of tissue wet weight) (6, 8). The present study shows that specific binding was little affected by decreasing the temperature from 37° to 22°, indicating that binding is accompanied by only a small change in enthalpy, i.e., it is essentially entropy driven. This is in contrast to the thermodynamic properties of [^{3}H]glibenclamide binding in rat brain membranes (13). At temperatures below 10°, binding was almost completely reduced. However, this cannot be attributed to a direct effect of temperature alone, because [ATP]$_i$ was also significantly decreased (Table 1), an effect that also leads to a reduction in binding (see below). Depolarization of the tissue by elevated KCl (55 mM) had no effect on [^{3}H]P1075 binding, indicating that binding of the KCO is not voltage dependent. This is in agreement with the observation that the cromakalim-induced K^+ current in rabbit portal vein cells shows no pronounced voltage dependence (32).

Because the KCOs preferentially show vasodilation in is-

TABLE 3

Inhibition of [^{3}H]P1075 binding and of P1075-stimulated $^{86}Rb^+$ efflux by KCO blockers

Binding of KCO blockers was determined by inhibition of [^{3}H]P1075 (0.3 nM) binding in rat aortic strips. Parameters were derived from the fit of the inhibition-concentration curves to the Hill equation [pK_i = −log[midpoint (M)]; n_H = Hill slope]. All blockers inhibited total binding by maximally 65 ± 5%. Inhibition of KCO-stimulated $^{86}Rb^+$ efflux was measured with P1075 (60 nM) as the stimulus; all blockers inhibited stimulated efflux by 100% at saturation. Three or four experiments were performed throughout.

Substance	Structural class[a]	Binding		Inhibition of stimulated $^{86}Rb^+$ efflux	
		pK_i	n_H	pK	n_H
Glibenclamide	S	6.36 ± 0.04	1.12 ± 0.09	7.01 ± 0.05	1.20 ± 0.16
(±)-AZ-DF 265	S*	5.95 ± 0.03	1.02 ± 0.06	6.30 ± 0.05	1.30 ± 0.20
Glipizide	S	5.20 ± 0.08	1.00 ± 0.17	5.82 ± 0.06	1.25 ± 0.26
Glibornuride	S	4.43 ± 0.10	0.76 ± 0.10	4.77 ± 0.04	1.66 ± 0.24
Tedisamil	Sp	3.74 ± 0.24	0.44 ± 0.12	7.66 ± 0.04	1.10 ± 0.10
Ciclazindol	Is	3.96 ± 0.14	0.52 ± 0.10	6.84 ± 0.06	0.76 ± 0.06[b]
Phentolamine	Im	3.59 ± 0.03	1.28 ± 0.13	6.12 ± 0.04	1.20 ± 0.13
Guanethidine	G	<3.5	<0.5	5.82 ± 0.03	0.88 ± 0.06

[a] S, sulfonylurea; S*, sulfonylurea-related; Sp, sparteine derivative; Is, isoindole; Im, imidazoline; G, guanidine derivative.
[b] Inhibition of cromakalim (1 μM)-induced $^{86}Rb^+$ efflux.

chemic tissue (1, 3) and because their target in various tissues may be K_{ATP} (33, 23), [^{3}H]P1075 binding was investigated under conditions where $[ATP]_i$ was decreased. Whatever the method used to lower $[ATP]_i$, binding was concomitantly reduced. Additional experiments suggest that this reduction in binding is primarily due to a loss of binding sites.[3] However, because specific binding is only observed in intact tissue one cannot assert that a direct interaction of ATP with, and/or phosphorylation of, the KCO receptor (channel?) is required to allow agonist binding. It is intriguing, however, that K_{ATP} is modulated by phosphorylation and direct nucleotide binding (for reviews see Refs. 30 and 34), and it remains to be seen whether similar modulatory mechanisms also apply to the KCO binding site described here. In view of the known sensitivity of the nucleotide binding site(s) of K_{ATP} to sulfhydryl-alkylating agents (35, 36), the effects of *N*-ethylmaleimide and of iodoacetic acid on [^{3}H]P1075 binding were investigated. However, both agents decreased both binding and $[ATP]_i$; the observed reduction in binding cannot, therefore, be attributed solely to alkylation of the drug receptor.

Inhibition of [^{3}H]P1075 binding by other KCOs and implications for the mechanisms of K^+ channel opening and vasorelaxation. The excellent correlation observed for representatives from different structural classes of KCOs between the potencies to inhibit binding of [^{3}H]P1075 and either to stimulate $^{86}Rb^+$ efflux or to induce vasorelaxation strongly suggests that the binding site labeled by [^{3}H]P1075 in rat aorta mediates the effects of the different KCOs in this tissue. On the other hand, there is a large shift (about 40-fold) between the midpoints of the binding curves and the $^{86}Rb^+$ efflux curves (with binding being to the left; see Table 2 and Fig. 3). This shift is surprising because KCO-stimulated $^{86}Rb^+$ efflux qualitatively reflects $^{42}K^+$ efflux (9, 37) and thus the fraction of K^+ channels opened by the KCO. A relatively simple model, compatible with these data, assumes that the channel has two (identical) agonist binding sites coupled by negative cooperativity and that binding of the second agonist molecule (at 40 times higher concentrations than the first) opens the channel. Similarly, a model assuming two independent intrinsically different agonist sites would also fit the data. Similar schemes describe the opening of the nicotinic acetylcholine receptor channel by agonists (38). An alternative explanation is that high concentrations of the KCOs may open more than one type of channel; evidence for such nonselectivity has been obtained with cromakalim in rat aorta (9, 37). According to the good correlation between binding and $^{86}Rb^+$ efflux, the structurally different KCOs must then all show a similar nonselectivity at high concentrations. An indirect (ternary complex) activation model, in which the opener binds to a mediator, which then activates the effector (channel), predicts that the agonist binding curve coincides with, or lies to the right of, the effect ($^{86}Rb^+$ efflux) curve (39). Therefore, such a model, without the incorporation of additional features, e.g., negative cooperativity or heterogeneity of agonist sites, cannot explain the data.

In this context, note again the paradox that, if K^+ channel opening is the cause of vasorelaxation, the KCO-induced $^{86}Rb^+$ efflux (which qualitatively reflects K^+ channel opening) is shifted to 40-fold higher concentrations (see also Ref. 3). Clearly, the link between the KCO-induced K^+ current, hyperpolarization, and smooth muscle relaxation is highly nonlinear and the magnitude of K^+ current required for a certain degree of relaxation is generally unknown. In addition, previous work using the K^+ channel blocker tedisamil, which inhibits cromakalim-induced $^{42}K^+$ efflux more potently than it does vasorelaxation (25, 40), suggests that this KCO possesses vasorelaxant mechanisms in addition to those involving the opening of plasmalemmal K^+ channels. These mechanisms are sensitive to inhibition by glibenclamide but not tedisamil (25, 40).

Inhibition of [^{3}H]P1075 binding by blockers. Of the various inhibitors of the KCOs tested, only the sulfonylureas were recognized by the [^{3}H]P1075-labeled site in a meaningful manner. This suggests that these compounds interfere with the ligand-gated opening of the channel, whereas the mode of channel block by the other inhibitors may be different. Additional evidence (6) indicates that the binding sites for glibenclamide and P1075 are distinct but coupled by negative allosterism.

The sulfonylureas were 2–4 times more potent in inhibiting P1075-induced $^{86}Rb^+$ efflux than [^{3}H]P1075 binding. Correcting for the fact that the flux assay was performed at a much higher P1075 concentration than the binding assay (60 versus 0.3 nM), this apparent difference in potency approximates to 10–20-fold. However, this discrepancy may be explained within the framework of the negative cooperativity model of channel opening discussed above; under the conditions of the binding assay, the sulfonylureas would interact with the monoligated channel-agonist complex (RA), whereas in the flux assay they interact with the open channel form, i.e., the biligated complex

RA_2. The results therefore would suggest that the affinity of the sulfonylureas is higher for the biligated form RA_2 than for RA. Further investigation of this and of the other intriguing observations described in this study requires the biochemical characterization of the drug receptor or electrophysiological experiments at the single-channel level.

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