THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 262, No. 33, Issue of November 25, pp. 15840-15844, 1987
Printed in U.S.A.
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The Receptor for Antidiabetic Sulfonylureas Controls the Activity of the ATP-modulated K^+ Channel in Insulin-secreting Cells*

(Received for publication, June 12, 1987)

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Sulfonylureas are powerful hypoglycemic drugs that have been used for decades to treat diabetic patients. This paper describes a $^{86}Rb^+$ flux technique that permits one to study easily the properties of ATP-modulated K^+ channels in RINm5F insulinoma cells. Sulfonylureas inhibit this type of K^+ channel under conditions of intracellular ATP depletion. The most potent sulfonylureas (glibenclamide, glipizide, and gliquidone) are acting in the nanomolar range of concentration. Inhibition of the single ATP-modulated K^+ channels by low concentrations of sulfonylureas was also observed using the patch-clamp technique.

The sulfonylurea receptor has been biochemically identified with [^{3}H]glibenclamide. For 10 different sulfonylureas (or sulfonylurea analogs) there was an excellent correlation between efficacy of blockade of ATP-modulated K^+ channels and efficacy of binding to the sulfonylurea receptors using the ^{3}H-ligand.

Insulin secretion from pancreatic β-cells is stimulated by glucose, which evokes a cyclical pattern of electrical activity (1). The slow wave of depolarization that follows glucose application seems to be due to closure of a K^+ channel that is regulated by intracellular ATP (2-7). Sulfonylurea drugs are widely used in the treatment of diabetes mellitus (8). Like glucose, sulfonylureas induce electrical activity in pancreatic islets (1, 9-11), and it has been recently suggested that the two pathways of glucose and sulfonylurea-induced insulin release may converge at the level of the ATP-regulated K^+ conductance (2, 5, 12-21). However, it has also been suggested that sulfonylureas may act on Ca^{2+}-activated K^+ channels (13, 14) and more recently on voltage-sensitive Ca^{2+} channels (22). This paper describes a biochemical technique for measuring the activity of the ATP-sensitive K^+ channel using $^{86}Rb^+$ efflux. It identifies the sulfonylurea receptor in an insulin-secreting cell line with [^{3}H]glibenclamide and demonstrates that this receptor, which binds some of the sulfonylureas with a high affinity (K_d in the nanomolar range or below), is indeed closely associated with blockade of the ATP-regulated K^+ channel.

MATERIALS AND METHODS

Cell Culture—RINm5F cells from an insulin-producing cell line derived from a rat islet cell tumor were grown as described previously (23, 24). Cells were plated at a density of 200,000 cells/well (Falcon 24-well tissue culture plates), or at a density of 3×10^6 cells (Falcon roller bottles, 850-cm^2 style), or in Falcon culture flasks, 75-cm^2-style.

$^{86}Rb^+$ Efflux Experiments—Efflux studies were performed in 24-well culture plates at 37 °C and after overnight equilibration of cells in RPMI 1640 medium supplemented with 10% fetal calf serum, 0.1 μCi/ml ^{86}RbCl, and 0.2 μCi/ml L-[^{3}H]leucine (internal marker of cell recovery). After removing the medium, cells were preincubated, for various times as indicated, in a medium containing 120 mM NaCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM KCl with 20 mM Hepes[1]/NaOH buffer, at pH 7.5, supplemented with 0.1 μCi/ml ^{86}RbCl, 0.24 μg/ml oligomycin, 1 mM 2-deoxy-D-glucose, and ligands as indicated in the figures. $^{86}Rb^+$ efflux studies were initiated by removing the preincubation medium and incubating the cells with 200 μl of the same medium/well without $^{86}Rb^+$, oligomycin, and 2-deoxy-D-glucose. Efflux was stopped as indicated by removing this latter medium and washing the cells three times with 1 ml of 0.1 M $MgCl_2$ at 37 °C. Cells were extracted with 2 × 1 ml of 0.1 N NaOH and counted. Total intracellular concentrations of ATP were measured after extracting the cells with 1% Triton X-100, according to Ref. 25, by using the luciferase-luciferin technique. An intracellular volume of 1 μl/10^6 cells was taken (26) corresponding to 4 μl/mg cell protein.

[^{3}H]Glibenclamide Binding to Microsomes—RINm5F cells grown in roller bottles and taken at 70% confluency were washed once with an ice-cold 0.3 M sucrose, 40 mM Hepes/NaOH buffer and scraped with the same buffer. Cells were homogenized with five strokes of a Potter-Elvehjem homogenizer and the suspension sonicated for 10 s and centrifuged at 70,000 × *g* for 25 min. The microsome pellet was suspended in 20 mM Hepes/NaOH buffer at pH 7.5. For equilibrium binding assays, microsomes were incubated at 4 °C in a solution containing 20 mM Hepes/NaOH buffer at pH 7.5 with the required concentrations of [^{3}H]glibenclamide. Incubations lasted 60 min and were stopped by rapid filtration through Whatman GF/B filters under reduced pressure. Filters were washed with 100 mM Tris/HCl buffer at pH 7.5 and 4 °C. Nonspecific binding was measured using 1 μM glibenclamide. [^{3}H]Glibenclamide binding was proportional to membrane protein concentrations between 0.2 and 1.2 mg/ml (not shown). Experiments were done in duplicate.

[^{3}H]Glibenclamide Binding to Cells in Suspension—Cells were detached by mechanical means with a medium containing 140 mM *N*-methylglucamine, 10 mM KCl with 20 mM Hepes/NaOH buffer at pH 7.5 at 37 °C. The suspension of dissociated cells was used for binding assay, as described above, using 140 mM *N*-methylglucamine, 1.8 mM Ca^{2+}, 0.8 mM $MgCl_2$, 10 mM KCl in 20 mM Hepes/Tris buffer at pH 7.5 and 4 °C.

Electrophysiological Measurements—Unitary currents carried by ATP-regulated potassium channels were recorded from inside-out membrane patches of RINm5F cells (27). The membrane potential was clamped at −60 mV by a voltage-clamp amplifier (Biologic, France). Calcium currents were recorded in the whole-cell configuration. Cells were voltage-clamped at −80 mV. Both single-channel and whole cell membrane currents were digitized at 0.5-ms intervals by a digital oscilloscope (Nicolet Instrument Corp., Madison, WI) and stored on hard-disc using a computer (Hewlett-Packard Co., Palo Alto, CA) for further analysis. Pipettes were coated with Sylgard resin to reduce electrode capacity and current noise. The composition of both bath and pipette solution used for the single-channel experiments was (in mM): KCl, 150; $MgCl_2$, 2; EGTA, 4; Hepes/KOH, 10; pH 7.2. The composition of both solutions used for the recording of calcium currents were, for extracellular (in mM): CsCl, 100; tetraeth-

* This work was supported by the Centre National de la Recherche Scientifique, the Fondation pour la Recherche Médicale, and the Mutuelle Générale de l'Education Nationale. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)] tetraacetic acid.

ylammonium-Cl, 30; $CaCl_2$, 25; Hepes/CsOH, 10; pH 7.3 and for intracellular (in mM): CsCl, 140; $MgCl_2$, 2; EGTA, 6; Hepes/CsOH, 10; pH 7.2.

Chemicals—The specific radioactivity of [^{3}H]glibenclamide was 26.4 Ci/mmol. Glibenclamide, tolbutamide, and HB699 were from Hoechst-Roussel Pharmaceuticals, Inc., gliquidone was from Boehringer Ingelheim, glibornuride from Roche Laboratories, glisoxepide from Schering-Plough Corp., carbutamide and gliclazide from Laboratoire Servier. Chlorpropamide and glipizide were a gift from Dr. J. Dolais-Kitabgi (Nice). Luciferase-luciferin, stock FLE-50, was from Sigma. Other products were from standard sources.

RESULTS AND DISCUSSION

Because the ATP-modulated K^+ channel is known to be permeable to Rb^+ (28), a technique has been devised in which $^{86}Rb^+$ flux experiments have been used to measure the activity of this channel type. Control RINm5F cells have a total internal ATP concentration of 3.8 ± 0.3 mM. ATP depletion was obtained by treating the cells with oligomycin in the presence of 2-deoxy-D-glucose. A decrease of the internal ATP concentration created a component of $^{86}Rb^+$ efflux that is inhibited by 1 μM glibenclamide (Fig. 1*A* and *inset*). Fig. 1*B* shows that the ATP-modulated K^+ transport system was fully active after a 20-min ATP depletion under our conditions. Fig. 1*A* and *inset* show typical direct and semilog plots of $^{86}Rb^+$ efflux kinetics. An intracellular ATP depletion for 20 min from $[ATP]_{in}$ = 3.8 mM to $[ATP]_{in}$ = 0.4 mM increased the rate constant of efflux, k_{efflux}, from 2.2×10^{-4} s^{-1} to 1.9×10^{-3} s^{-1}. This 8.6-fold increase of k_{efflux} was abolished by 1 μM glibenclamide. Introduction of Ca^{2+} into RINm5F cells using the Ca^{2+} ionophore A23187 was without effect on the glibenclamide-sensitive $^{86}Rb^+$ efflux component (not shown), indicating that the drug is without action on Ca^{2+}-activated K^+ channels.

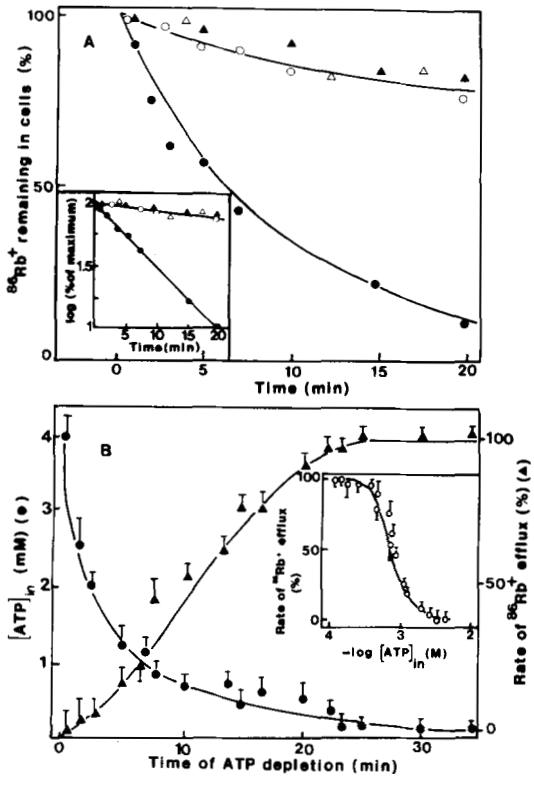

FIG. 1. **Kinetics of $^{86}Rb^+$ efflux from RINm5F cells and effects of ATP depletion on $^{86}Rb^+$ efflux.** *A*, kinetics of $^{86}Rb^+$ efflux without depletion of ATP, in the absence (▲) or presence (△) of 1 μM glibenclamide, and after depletion of ATP for 22 min in the absence (●) or presence (○) of 1 μM glibenclamide. *Inset*, semilog plots of $^{86}Rb^+$ efflux. *B*, time dependence of the rate of glibenclamide-sensitive $^{86}Rb^+$ efflux (▲) and of the $[ATP]_{in}$ concentration (●) following oligomycin and 2-deoxy-D-glucose treatment. Basal $^{86}Rb^+$ efflux before $[ATP]_{in}$ depletion was subtracted. *Inset*, $[ATP]_{in}$ dependence of the rate of glibenclamide-sensitive $^{86}Rb^+$ efflux. The rate of $^{86}Rb^+$ efflux in the presence of 1 μM glibenclamide was subtracted (5–10% of total efflux rate). For *B*, time of $^{86}Rb^+$ efflux for each experimental point was 1 min. Experiments were mean ± S.E. (n = 5) for *B* and *inset*. Other efflux experiments were in duplicate.

The total intracellular ATP concentration dependence of the glibenclamide-sensitive rate of $^{86}Rb^+$ efflux is shown in Fig. 1*B*, *inset*. The channel was blocked at internal ATP concentrations higher than 3 mM and was fully activated below $[ATP]_{in}$ = 0.3 mM ($[ATP]_{in}$ represents the total ATP concentration inside the cell). Half-maximal inhibition by ATP was observed at $K_{0.5}^{ATP}$ = 0.8 ± 0.2 mM. The $[ATP]_{in}$ response was highly cooperative with a Hill coefficient higher than 3. The total intracellular concentration of ATP may have no relationship with the cytoplasmic concentration of ATP.

Fig. 2 and Table I present an analysis of the efficacy of a series of hypoglycemic drugs in inhibiting the ATP-sensitive rate of $^{86}Rb^+$ efflux in RINm5F cells. Half-maximal inhibitions ($K_{0.5}$) of the different hypoglycemic drugs varied widely between 0.06 nM and 40 μM. The rank order of potency is glibenclamide > glipizide > gliquidone > glisoxepide > glibornuride > gliclazide > chlorpropamide and tolbutamide > carbutamide. The non-sulfonylurea part of glibenclamide, HB699, which is also known to be hypoglycemic (29), also

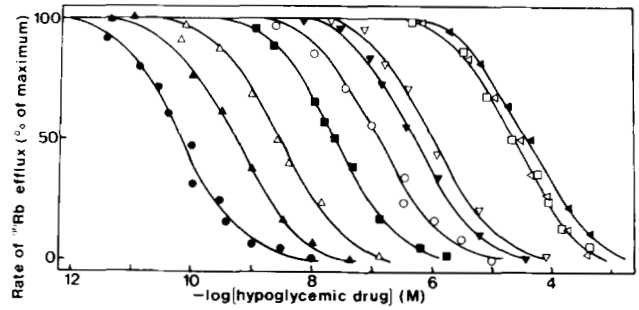

FIG. 2. **Inhibition by various hypoglycemic drugs of ATP and glibenclamide-sensitive rate of $^{86}Rb^+$ efflux from RINm5F cells.** Efflux of $^{86}Rb^+$ was measured as described under "Materials and Methods," in the presence of increasing concentrations of glibenclamide (●), glipizide (▲), gliquidone (△), glisoxepide (■), glibornuride (○), HB699 (▼), gliclazide (▽), chlorpropamide and tolbutamide (□, ◁), and carbutamide (◀). Time of ATP depletion was 20 min, and time of $^{86}Rb^+$ efflux was 1 min. Efflux experiments were in duplicate.

TABLE I

K_d of hypoglycemic drugs for half-inhibition of specific [^{3}H] glibenclamide-binding to RINm5F microsomes in comparison to $K_{0.5}$ for specific half-inhibition of $^{86}Rb^+$ efflux from RINm5F cells and therapeutic doses in man

Hypoglycemic drug	K_d	$K_{0.5}$	Therapeutic doses in man[a]
	nM		*mg/day*
Glibenclamide	0.3	0.06	2.5–15
Glipizide	0.8	0.5	2.5–20
Gliquidone	1	3	15–90
Glisoxepide	11	25	2–16
Glibornuride	90	120	12.5–75
HB699	260	500	
Gliclazide	600	1,000	80–240
Chlorpropamide	9,000	22,000	125–500
Tolbutamide	9,000	22,000	1,000–1,500
Carbutamide	25,000	40,000	1,000

[a] Therapeutic doses are taken either from the French Vidal dictionary or from the German Rote Liste.

blocked the ATP-modulated K^+ channel but was much less potent than glibenclamide (Table I).

Many other non-sulfonylurea molecules have been assayed which were without effect on the glibenclamide and ATP-sensitive K^+ channel. These molecules were Na^+ channel blockers (30) such as tetrodotoxin (1 μM), Ca^{2+} channel blockers (31, 32) such as (+)-PN 200-110 and (−)-desmethoxyverapamil (1 μM), blockers of a variety of K^+ channels and of Ca^{2+}-activated K^+ channels (30, 33–35) such as tetraethylammonium (20 mM), and 3,4-aminopyridine, apamin, toxin I from the snake *Dendroaspis polylepis polylepis*, the mast cell degranulating peptide from bee venom (34), and phencyclidine (36, 37) at 1 μM. Drugs such as metformine, fenfluramine, tetracaine, atropine, γ-aminobutyric acid, alprenolol, prazosin, librium, and chlorpromazine were also without effect on the ATP-modulated $^{86}Rb^+$ efflux when they were used at 1 μM.

The activity of the ATP-regulated K^+ channel was also studied at the single-channel level using the patch-clamp technique (Fig. 3). K^+ channel activity could be recorded on excised patches in the presence of a very low concentration of ATP on the cytoplasmic face (ATP = 1 μM) as described previously (3). When the ATP concentration on the cytoplasmic side of the patch was raised to 2.5 mM, the K^+ channel activity disappeared. Another way to suppress the ATP-regulated K^+ channel activity was to leave the ATP concentration at 1 μM and to apply 20 nM glibenclamide, a concentration of sulfonylurea that, from the $^{86}Rb^+$ efflux results of Fig. 2, is indeed expected to block the activity of the channel. Whereas a concentration of 25 nM glisoxepide, a less active sulfonylurea according to ^{86}Rb efflux data presented in Fig. 2, failed to block the ATP-regulated K^+ channel (not shown), a concentration of 500 nM (*i.e.* 20 times higher than the $K_{0.5}$ value found in Fig. 2 (see Table I)) completely blocked the channel. Therefore, there is an excellent agreement between ^{86}Rb efflux data and single-channel recording from excised patches of the plasma membrane.

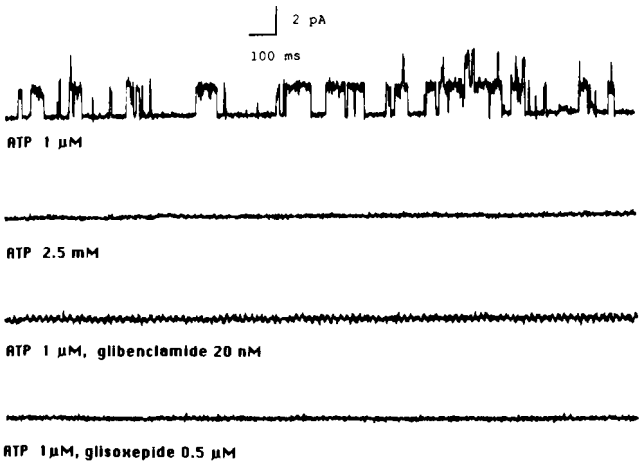

FIG. 3. **Single-channel currents recorded from inside-out patches.** An upward deflection in the traces corresponds to an inward current. The membrane potential was −60 mV and the currents were filtered at 500 Hz. ATP-regulated K^+ channels that opened at low concentrations of ATP (1 μM, *upper trace*) could be blocked by either 2.5 mM ATP (*second trace*) or sulfonylureas (20 nM glibenclamide and 500 nM glisoxepide, *lower traces*).

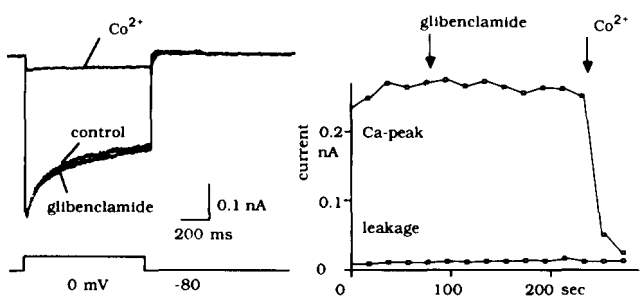

FIG. 4. **Voltage-dependent calcium currents.** These currents elicited by stepping the membrane potential from a holding potential of −80 mV to 0 mV, were blocked by approximately 5 mM cobalt chloride but were not affected by 50 nM glibenclamide (*left panel*). The calcium peak current (measured at 0 mV) and leakage current (measured at −80 mV by applying 10-mV hyperpolarizations) remained stable after adding glibenclamide to the bath solution (*right panel*).

Because sulfonylureas increase the intracellular Ca^{2+} concentration of an insulin-secreting cell line, it was recently proposed that they could be active on voltage-dependent Ca^{2+} channels (22). Ca^{2+} channel activity was recorded in RINm5F cells using the whole-cell patch-clamp technique (Fig. 4). It was blocked by Co^{2+} (5 mM) as expected for a Ca^{2+} channel (38), but it was completely unaffected by glibenclamide (50

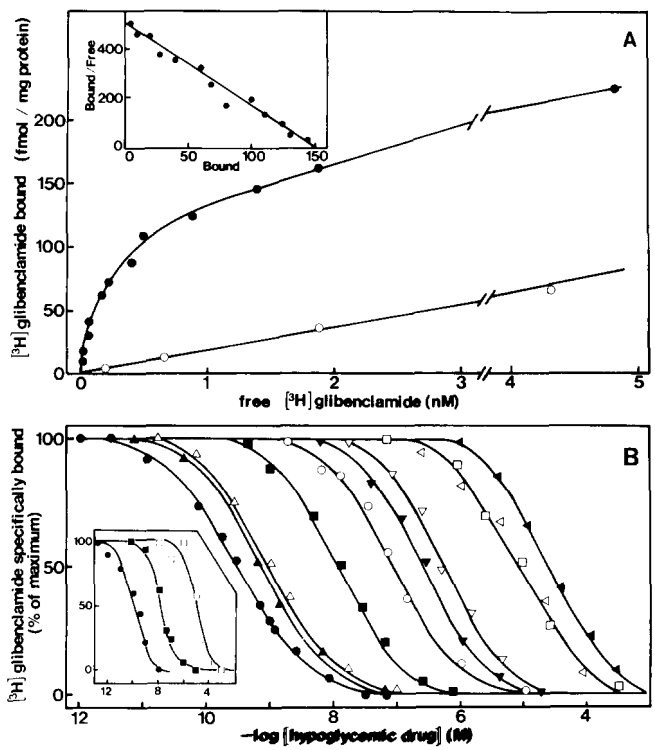

FIG. 5. **Equilibrium binding of [^{3}H]glibenclamide to microsome membranes or to suspension from RINm5F cells and inhibition of binding by various hypoglycemic drugs.** *A*, equilibrium binding was measured in 20 ml of buffer using increasing concentrations of [^{3}H]glibenclamide and 0.3 mg/ml microsome proteins (pH 7.5 and 4 °C). *Main panel*, binding of [^{3}H]glibenclamide to microsomes in the absence (●) or presence (○) of 1 μM glibenclamide. *Inset*, Scatchard plot for the specific [^{3}H]glibenclamide-binding component. Bound is in fmol/mg protein. *B*, inhibition by hypoglycemic drugs of [^{3}H]glibenclamide binding to RINm5F cell microsomes or to suspensions at equilibrium. *Main panel*, binding of [^{3}H]glibenclamide (0.1 nM) to microsomes (0.6 mg/ml) was measured in a 2-ml volume in the presence of increasing concentrations of glibenclamide (●), glipizide (▲), gliquidone (△), glisoxepide (■), glibornuride (○), HB699 (▼), gliclazide (▽), tolbutamide and chlorpropamide (□, ◁), and carbutamide (◀). Nonspecific binding represented 15% of total binding (not shown). *Inset*, binding of [^{3}H]glibenclamide (0.1 nM) to cell suspension (5×10^6 cells/ml) was measured in a 2-ml volume in the presence of increasing concentrations of glibenclamide (●), glisoxepide (■), and tolbutamide (□). Abscissa and ordinates are the same as main figure. Nonspecific binding represented 8% of total binding (not shown).

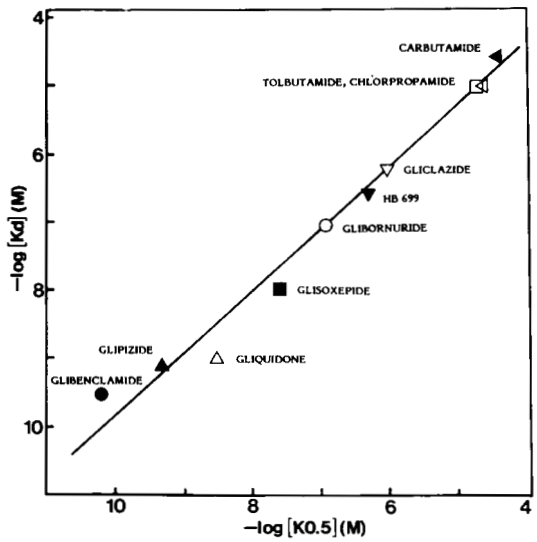

FIG. 6. **Correlation between inhibition by various hypoglycemic drugs of specific [³H]glibenclamide binding and of $^{86}Rb^+$ efflux.** K_d (half-inhibition of [³H]glibenclamide binding by hypoglycemic drugs) was plotted *versus* $K_{0.5}$ (half-inhibition of ATP-sensitive rate of $^{86}Rb^+$ efflux).

nM) at a concentration at which the drug blocks ATP-regulated K^+ channels.

[³H]Glibenclamide has been shown to be a potentially useful ligand for the identification of the sulfonylurea receptor (39). Fig. 5*A* shows equilibrium binding of [³H]glibenclamide to RINm5F microsomes. The specific binding component is high as compared with nonspecific binding. The Scatchard plot of the specific binding component indicates a single type of high affinity binding site with a dissociation constant K_d = 0.3 nM and a maximal binding capacity B_{max} = 150 fmol/mg protein at 4 °C (Fig. 5*A*, *inset*). The same results were obtained at 20 and 37 °C. Specific [³H]glibenclamide binding to RINm5F cell microsomes was inhibited by increasing concentrations of unlabeled glibenclamide and other hypoglycemic drugs (Fig. 5*B*). The rank order of potency of the different hypoglycemic drugs in displacing [³H]glibenclamide was the same as that previously found for inhibition of $^{86}Rb^+$ efflux from ATP-depleted cells (Table I). Other non-sulfonylurea molecules assayed in $^{86}Rb^+$ efflux experiments had no effect on [³H]glibenclamide binding to RINm5F microsomes. One observes in Table I that the most active hypoglycemic drugs in both binding experiments and $^{86}Rb^+$ efflux blockade are also the ones that are given in the lowest therapeutic doses. Moreover, it has been recently shown (22) that glibenclamide was much more potent to release insulin from hamster insulin-secreting tumor insulinoma cells than tolbutamide. This result agrees with the observation that glibenclamide is also much more potent than tolbutamide in blocking ATP-sensitive Rb^+ efflux (Table I). ATP-sensitive K^+ channels can also be recorded from hamster insulin-secreting tumor insulinoma cells using the patch-clamp technique. They are also blocked by sulfonylureas in this cell line (not shown) with properties identical or very similar to those described in this paper for RINm5F cells.

The same $K_{0.5}$ values for inhibition of [³H]glibenclamide binding were obtained with cells in suspension (Fig. 5*B*, *inset*). The rank order of potency for three sulfonylureas was: glibenclamide ($K_{0.5}$ = 0.16 nM), glisoxepide ($K_{0.5}$ = 16 nM), and tolbutamide ($K_{0.5}$ = 10,000 nM). [³H]Glibenclamide binding to intact cells was fully dissociable (not shown), indicating (as do electrophysiological data on excised patches) that receptor sites are localized on the plasma membrane. The total number of receptor sites was calculated to be 5,120 ± 500 sites/cell.

Patch-clamp experiments that have been published while this work was completed have shown that ATP-modulated K^+ channels could be blocked with tolbutamide (40). However, blockade could only be obtained with relatively high tolbutamide concentrations (0.1–1 mM) (12), and the ED_{50} for the drug was 7 μM (40). It turns out that this ED_{50} value corresponds rather well to the $K_{0.5}$ value found in this work for the inhibition of $^{86}Rb^+$ efflux following ATP depletion (22 μM) (Table I) and for the binding constant of tolbutamide at the sulfonylurea receptor site (K_d = 9 μM) (Table I).

The good correlation (slope = 0.94; r = 0.98) between potency to inhibit $^{86}Rb^+$ efflux and affinity for a receptor site measured with [³H]glibenclamide (Fig. 6) strongly suggests that the sulfonylurea receptor is closely associated with the ATP-sensitive K^+ channel.

It is probable that [³H]glibenclamide (or glipizide and gliquidone) will become important molecules in the analysis of the molecular properties of the ATP-modulated K^+ channel in insulin-secreting cells and in other cells having this type of K^+ channel in their membrane, such as cardiac cells (41) and skeletal muscle cells (42).

Acknowledgments—We are grateful to Hoechst, Boehringer Ingelheim, Roche, Schering, and Laboratoires Servier for generous gifts of glibenclamide, HB699, tolbutamide, gliquidone, glibornuride, glisoxepide, carbutamide, and gliclazide. We thank Dr. J. Kitabgi for gifts of chlorpropamide and glipizide. We thank Drs. E. Van Obberghen and R. F. Santerre for the gift of insulinoma cells. Thanks are due to C. Roulinat-Bettelheim and M. T. Bohn for expert technical assistance.

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