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ATP-modulated K⁺ channels sensitive to antidiabetic sulfonylureas are present in adenohypophysis and are involved in growth hormone release

(glipizide/diazoxide)

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ABSTRACT The adenohypophysis contains high-affinity binding sites for antidiabetic sulfonylureas that are specific blockers of ATP-sensitive K⁺ channels. The binding protein has a *M_r* of 145,000 ± 5000. The presence of ATP-sensitive K⁺ channels (26 pS) has been demonstrated by electrophysiological techniques. Intracellular perfusion of adenohypophysis cells with an ATP-free medium to activate ATP-sensitive K⁺ channels induces a large hyperpolarization (≈30 mV) that is antagonized by antidiabetic sulfonylureas. Diazoxide opens ATP-sensitive K⁺ channels in adenohypophysis cells as it does in pancreatic β cells and also induces a hyperpolarization (≈30 mV) that is also suppressed by antidiabetic sulfonylureas. As in pancreatic β cells, glucose and antidiabetic sulfonylureas depolarize the adenohypophysis cells and thereby indirectly increase Ca²⁺ influx through L-type Ca²⁺ channels. The K⁺ channel opener diazoxide has an opposite effect. Opening ATP-sensitive K⁺ channels inhibits growth hormone secretion and this inhibition is eliminated by antidiabetic sulfonylureas.

ATP-sensitive K⁺ (K_{ATP}) channels are present in pancreatic β cells (1) as well as in heart (2, 3), brain (4), skeletal muscle (5), and smooth muscle (5). Their function is particularly well understood in β cells, where they control insulin release in response to extracellular changes of glucose concentrations (1, 6). An increase of the extracellular glucose concentration increases the intracellular ATP/ADP ratio and thereby inhibits K_{ATP} channels inducing a depolarization, the latter leading to activation of Ca²⁺ channels, Ca²⁺ entry, and insulin release.

In the pancreatic β cell, K_{ATP} channels are also regulated by hormones, such as somatostatin and galanin, which inhibit insulin release (7–9). Drugs such as diazoxide, belonging to the family of K_{ATP} channel openers (10, 11), also activate K_{ATP} channels and therefore decrease insulin secretion and provoke hyperglycemia.

One of the important properties of K_{ATP} channels is their high sensitivity to antidiabetic sulfonylureas (12), such as glibenclamide or glipizide, which block these channels at nanomolar concentrations in β cells (12).

To our knowledge, the presence of K_{ATP} channels has not been reported, up till now, in other endocrine systems. For that reason and also because insulin-induced hypoglycemia provokes a drastic decrease of growth hormone (GH) plasma levels in the rat (13), it was important to search for the presence of K_{ATP} channels in adenohypophysis. Other types of K⁺ channels are thought to be important for the regulation of hormone secretion by adenohypophysis cells, such as a delayed rectifier (14), a transient outward I_A type K⁺ channel

(14), and an inwardly rectifying K⁺ channel (15). This paper demonstrates the presence of K_{ATP} channels in adenohypophysis, characterizes their properties and their pharmacology, and indicates that they regulate GH secretion *in vitro*.

MATERIALS AND METHODS

Glibenclamide Binding Assay. For equilibrium binding studies, microsomes prepared from adeno- or neurohypophysis as described (16) (0.1–0.5 mg/ml) were incubated in the presence of increasing concentrations of [³H]glibenclamide (50 Ci/mmol; 1 Ci = 37 GBq) or [¹²⁵I]-labeled hydroxyglibenclamide (¹²⁵I-hydroxyglibenclamide; 2000 Ci/mmol; synthesized as described in ref. 17 for 1 hr at 4°C using previously described techniques (12, 17). Specific [³H]glibenclamide binding and [¹²⁵I]-hydroxyglibenclamide binding were proportional to microsome protein concentrations between 0.1 and 0.5 mg/ml (not shown).

Photoaffinity Labeling. Adenohypophysis microsomes at a final concentration of 1 mg/ml were incubated for 1 hr at 4°C with 2 nM [¹²⁵I]-hydroxyglibenclamide in 1 ml of buffer containing 50 mM Hepes/NaOH, pH 7.5. A parallel incubation was carried out in the presence of 1 μM glibenclamide to measure the nonspecific labeling. The incubations were irradiated by using an 8-W UV lamp (254 nm; Vilbert Lourmat, Paris) for 6 min at a distance of 20 cm and at 4°C. Samples were washed twice with 1 ml of 50 mM Hepes/NaOH, pH 7.4, and were denatured by addition of Laemmli denaturation buffer (18). After electrophoresis on 7% isocratic polyacrylamide gels under nonreducing and reducing conditions, gels were stained with Coomassie blue, dried, and exposed to Kodak X-Omat AR-5 film with a DuPont Cronex intensifying screen for 3–7 days at –70°C.

Autoradiographic Binding Procedures. After decapitation of rats, their hypophyses were quickly removed, frozen in isopentane at –40°C, and sectioned on a cryostat microtome. Slices of 15-μm thickness were incubated with [³H]glibenclamide for 60 min at 4°C in a 40 mM Hepes/NaOH buffer at pH 7.5. Autoradiographic measurements were carried out as described (19).

Hormone Secretion. Adenohypophysis lobes were rapidly obtained after decapitation of 10 adult female rats. Cell dispersion and culture were carried out according to refs. 20–22. On the fourth day of culture the cells were washed once and incubated for 1.5 hr at 37°C in serum-free Dulbecco's modified Eagle's medium supplemented with 10 mM Hepes/NaOH at pH 7.5. Hormone release was determined

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Abbreviations: GH, growth hormone; K_{ATP}, ATP-sensitive potassium channel.

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by radioimmunoassay (22) after 3 hr of incubation in the presence or absence of diazoxide or glipizide.

Electrophysiology. Current-clamp experiments were carried out using the whole-cell suction-pipette technique (23). Single-channel currents were recorded from inside-out and outside-out membrane patches and their membrane potentials were clamped at 0 mV by a voltage-clamp amplifier (Biologic, Grenoble, France). In all experiments, the intracellular solution contained 150 mM KCl, 1 mM MgCl₂, 2 mM EGTA, and 10 mM HEPES/KOH (pH 7.2). The extracellular solution contained 140 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, and 10 mM HEPES/NaOH (pH 7.3). Experiments were carried out at 25 ± 1°C.

Variations of Intracellular Ca²⁺ Levels. Cytosolic Ca²⁺ in isolated hypophysis cells was measured using indo-1. Cells were incubated for 1–2 hr in the Ringer solution used for electrophysiology with 5 μM of the indo ester. The medium was then replaced by the same solution lacking the indicator. The ratio of the fluorescence signals at 405 and 480 nm measured with a fluorescence microscope (Nikon) was taken as a measure of cytosolic Ca²⁺.

RESULTS

Binding Experiments. Fig. 1A shows that adenohypophysis microsomes have a single type of high-affinity binding site for [³H]glibenclamide, with an equilibrium dissociation constant (K_d) of 0.5 ± 0.1 nM (Fig. 1A *Inset*). Similar results were obtained by using [¹²⁵I]-hydroxyglibenclamide (K_d = 0.7 ± 0.1 nM) (not shown). Maximal binding capacities varied between 60 and 80 fmol/mg of protein depending on microsome preparations. Binding of [³H]glibenclamide or [¹²⁵I]-hydroxyglibenclamide was not detected in neurohypophysis microsomes (not shown). Specific [³H]glibenclamide binding to adenohypophysis microsomes was inhibited by increasing concentrations of unlabeled glibenclamide and other hypoglycemic drugs (not shown). The rank order of potency in inhibiting [³H]glibenclamide binding is glibenclamide, K_d = 0.1 nM > LH33, K_d = 2 nM > glipizide, K_d = 5 nM > glisoxepide, K_d = 110 nM > gliclazide, K_d = 653 nM > tolbutamide, K_d = 12,500 nM. Fig. 1B shows a linear relation between affinities for the different sulfonylureas in adenohypophysis and insulinoma microsomes, respectively.

Autoradiography. The hypophysis presents a heterogeneous distribution of glibenclamide binding sites. High receptor densities were found in adenohypophyses (49 ± 2 fmol/mg of protein) (Fig. 2). Conversely, the neurohypophysis and the intermediate lobe contained low levels of glibenclamide binding sites (14 ± 3 fmol/mg of protein) (Fig. 2).

Photoaffinity Labeling. [¹²⁵I]-hydroxyglibenclamide was used to affinity label adenohypophysis microsomes. Typical results presented in Fig. 3 indicate that radioactivity was specifically incorporated into a polypeptide of M_r 145,000 ± 5000. Gel patterns were identical in the presence and absence (not shown) of the reducing agent 2-mercaptoethanol, indicating a monomeric structure.

Electrophysiology. Membrane potentials were recorded from adenohypophysis cells in primary cultures using the whole-cell suction-pipette technique. The average potential was -37.0 ± 11.3 mV (n = 114). About one-third of the cells (30 of 85 cells), intracellularly perfused with an ATP-free solution, hyperpolarized from -35.7 ± 10.5 mV to -64.6 ± 7.3 mV. The hyperpolarization was accompanied by a reduction in membrane resistance, indicating the activation of a K⁺ conductance. The K_{ATP} channel blocker glipizide (1 μM) reversed these effects (n = 9, Fig. 4A). When 2 mM ATP was included in the dialyzing pipette, membrane potentials remained at an average value of -37.5 ± 11.8 mV (n = 18).

These results suggest that a fraction of hypophysis cells possesses ATP- and sulfonylurea-sensitive K⁺ channels.

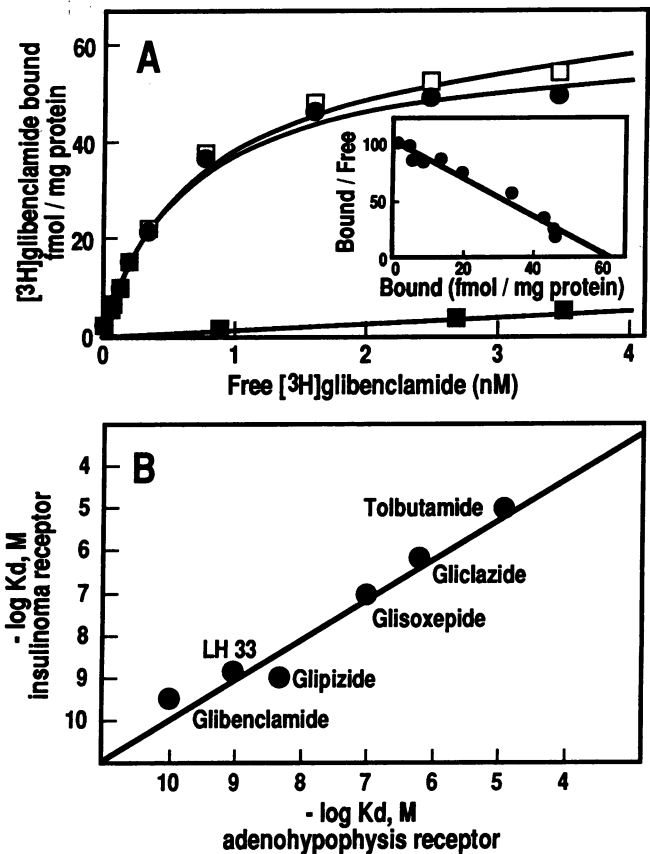


FIG. 1. Equilibrium binding of [³H]glibenclamide to adenohypophysis microsomes and inhibition of [³H]glibenclamide binding to adenohypophysis microsomes by increasing concentrations of sulfonylureas. (A) Equilibrium binding to adenohypophysis microsomes was measured using increasing concentrations of [³H]glibenclamide at pH 7.5 and at 4°C. □, Total binding; ■, nonspecific binding in the presence of 1 μM glibenclamide; ●, specific binding. (*Inset*) Scatchard plots for the specific binding component. (B) K_d values of different sulfonylureas for binding to adenohypophysis microsomes were plotted against K_d values (taken from ref. 12) of different sulfonylureas for binding to insulinoma cell (RINm5F) microsomes (slope = 1.3; r = 0.99). The true dissociation constant (K_d) was calculated by $K_{0.5} = K_d [1 + ([^3\text{H}]\text{glibenclamide})/K_i(^3\text{H}]\text{glibenclamide})]$, where [³H]glibenclamide] is the concentration of [³H]glibenclamide (0.5 nM) used in the experiment and (K_i [³H]glibenclamide) is the equilibrium dissociation constant of the [³H]glibenclamide receptor complex.

This fraction of cells generally displayed burst-like electrical activity and, when voltage-clamped, showed a voltage-dependent sodium current, a delayed rectifier, and a rapidly inactivating K⁺ (I_A) current, which are characteristics of GH-secreting cells (14).

It was found that 8 of 19 outside-out membrane patches, excised from cells that hyperpolarized if they were intracellularly dialyzed with an ATP-free solution, contained glipizide-sensitive K⁺ channel activity (Fig. 4B). The frequency of channel opening was voltage-independent. The current-voltage relation (Fig. 4C, ○) was linear, indicating a unitary conductance of 26 pS and, as the intercept with the abscissa was close to the K⁺ equilibrium potential (-85 mV), a selectivity for K⁺ over Na⁺ and Cl⁻ ions. In other experiments, inside-out membrane patches were excised in a medium devoid of ATP and containing 150 mM KCl and 2 mM EGTA. The pipette contained a Na⁺-rich solution. Fig. 4D shows that K⁺ channel activity was completely and reversibly inhibited by application of 2 mM ATP to the intracellular face of the membrane patch, as expected for a K_{ATP} channel.

Diazoxide is the best-known K_{ATP} channel opener for pancreatic β cells (10, 11). It requires the presence of

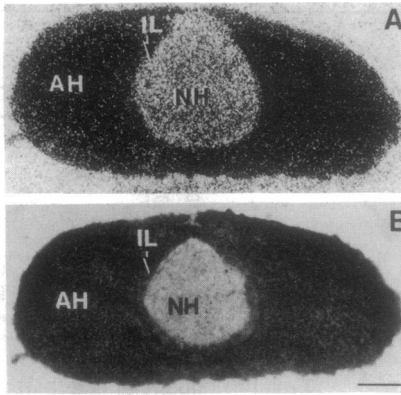


FIG. 2. Distribution of glibenclamide binding sites in rat hypophysis. (A) Autoradiogram of hypophysis sections obtained after incubation with 2.5 nM [^3H]glibenclamide. The dark areas indicate the high grain densities—i.e., high binding site densities. (B) Corresponding Giemsa-stained tissue section. AH, adenohypophysis; IL, intermediate lobe; NH, neurohypophysis. (Bar = 500 μm .)

intracellular MgADP to activate K_{ATP} channels (24). Similar results were obtained in adenohypophysis cells. It was found that 4 of 11 adenohypophysis cells, intracellularly perfused with 1 mM ATP, 250 μM ADP, and 2.5 μg of oligomycin per ml to prevent the conversion of ADP to ATP (25), hyperpolarized by 30–35 mV upon extracellular application of 0.3 mM diazoxide. When ADP was absent from the intracellular perfusate, none of 15 cells hyperpolarized under diazoxide. Fig. 4E shows that the diazoxide-induced hyperpolarization was antagonized by glipizide.

Intracellular Ca^{2+} Measurements. If K_{ATP} channels are important for the function of adenohypophysis cells, their blockade should result in depolarization, opening of L-type Ca^{2+} channels, and intracellular accumulation of Ca^{2+} , whereas their activation by K^+ channel openers such as diazoxide should have inverse effects. Indeed, exposure of the cells to 20 mM glucose, which is expected to increase the ATP/ADP ratio and therefore to inhibit the K_{ATP} channel, induced an increase of intracellular Ca^{2+} , which was eliminated by the L-type Ca^{2+} channel inhibitor PN200-110 (Fig. 5A). The sulfonylurea glipizide, which also blocks the K_{ATP} channels, had the same effect as glucose (Fig. 5B). Intracellular Ca^{2+} fluctuations induced by glucose were also suppressed by diazoxide (Fig. 5C and D) and the inhibitory effect of diazoxide was eliminated by glipizide (Fig. 5C).

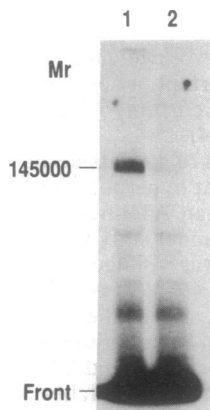


FIG. 3. Photoaffinity labeling of adenohypophysis microsomes with ^{125}I -hydroxyglibenclamide. The autoradiographic pattern of photoaffinity labeling with 2 nM ^{125}I -hydroxyglibenclamide carried out with adenohypophysis microsomes in the absence (lane 1) and presence (lane 2) of 1 μM glibenclamide. Samples were treated with 2% 2-mercaptoethanol.

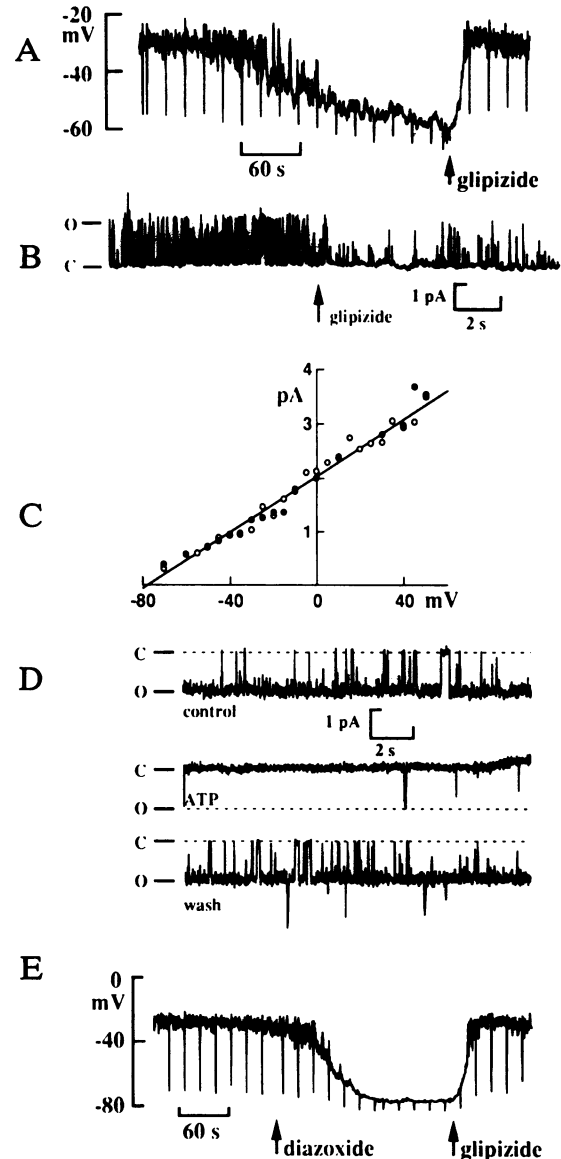


FIG. 4. Intracellular ATP depletion induces K_{ATP} channel opening and membrane hyperpolarization. (A) Adenohypophysis cells were intracellularly dialyzed with a K^+ -rich and ATP-free solution and bathed in a Na^+ -rich Ringer medium. The record in A starts right after breaking into the whole-cell configuration. After 1–2 min of dialysis, the membrane hyperpolarizes and membrane resistance decreases. The change in membrane resistance is indicated by the downward deflections in the trace, which were induced by 300-ms, 10-pA current pulses. Upon extracellular application of 1 μM glipizide, the membrane depolarized and resistance increased. (B) Outside-out membrane patches were excised with a pipette containing a K^+ -rich and ATP-free solution, bathed in Na^+ -rich Ringer solution and clamped at 0 mV. Channel activity was inhibited by 1 μM glipizide. (C) The current–voltage relations of the unitary currents measured before (○) or after (●) partial inhibition by glipizide are identical but not completely inhibited by the sulfonylurea. (D) Inside-out patches were excised in medium containing a K^+ -rich and ATP-free medium. The pipette contained the Na^+ -rich solution. Channel activity observed in the absence of ATP (upper trace) was inhibited by 2 mM ATP applied to the intracellular face of the membrane patch (second trace). Channel activity reappeared after wash-out of ATP. In the records, the closed and open states of the channel are marked “C” and “O,” respectively. (E) Cells that were intracellularly perfused with 1 mM ATP and 250 μM ADP hyperpolarized upon application of 0.3 mM diazoxide. The hyperpolarization was antagonized by 1 μM glipizide.

Effect of Diazoxide and of Glipizide on GH Secretion. After showing that K_{ATP} channels were present in adenohypophy-

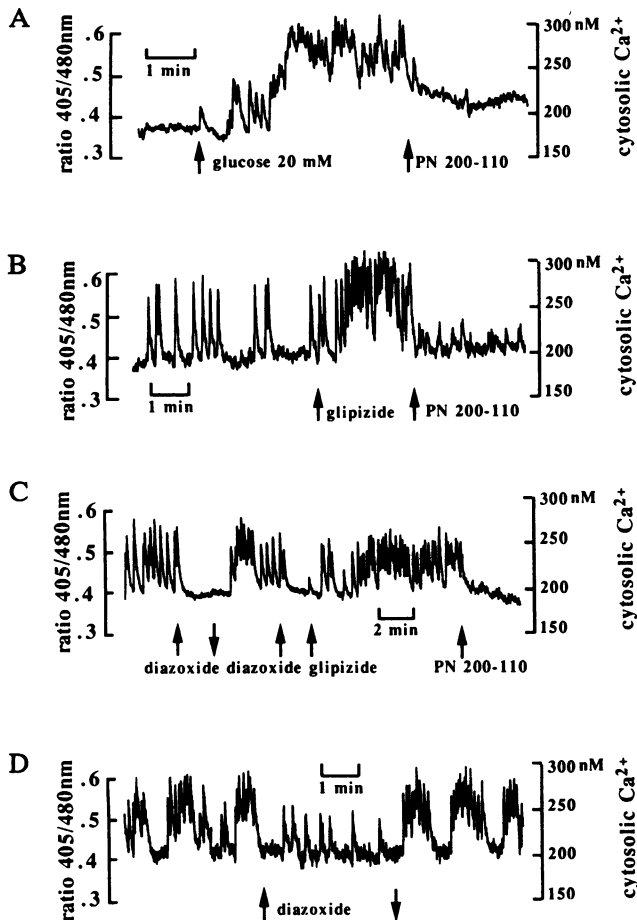


FIG. 5. Cells were incubated in Ringer solution containing 3 mM (A–C) or 20 mM (D) glucose. If incubated in 3 mM glucose, an increase of the extracellular glucose concentration to 20 mM (A) or application of 1 μ M glipizide (B) increased the frequency of spontaneous Ca^{2+} fluctuations that were inhibited to a large extent by subsequent application of 200 nM Ca^{2+} channel blocker PN 200-110. The frequency of spontaneous cytosolic Ca^{2+} fluctuations in 3 mM glucose (C) or 20 mM glucose (D) was inhibited by 0.3 mM diazoxide.

sis, it was important to check whether their pharmacological regulation could alter hormone secretion. Fig. 6A shows that the K^+ channel opener diazoxide (500 μ M) decreased the basal level of GH secretion by about 50%. The $K_{0.5}$ value for diazoxide-induced inhibition was $85 \pm 10 \mu\text{M}$. Fig. 6B (Inset) shows that inhibition of GH secretion by diazoxide was fully reversed by 1 μ M glipizide. The half-maximum effect for glipizide was at $18 \pm 3 \text{ nM}$ (Fig. 6B), a value similar to the K_d value for glipizide binding to its receptor site ($K_d = 5 \text{ nM}$, Fig. 1B).

DISCUSSION

Ca^{2+} is essential for GH secretion by somatotrophs. A part of Ca^{2+} required for secretion comes from the spontaneous electrical activity of the cells and penetrates through L-type Ca^{2+} channels (26, 27) as for the pancreatic β cell (1). Since K^+ channels are well known to be involved in the regulation of the resting potential and of the spontaneous activity, their role in GH secretion by the somatotrophs has of course been extensively studied. Three main types of K^+ channel currents important for secretion by rat somatotrophs have been previously identified: a delayed rectifier current (I_K), a transient outward current (I_A), and an inward rectifier current (14, 15).

The work presented in this paper shows that in addition to these K^+ channels, adenohipophys cells have K_{ATP} chan-

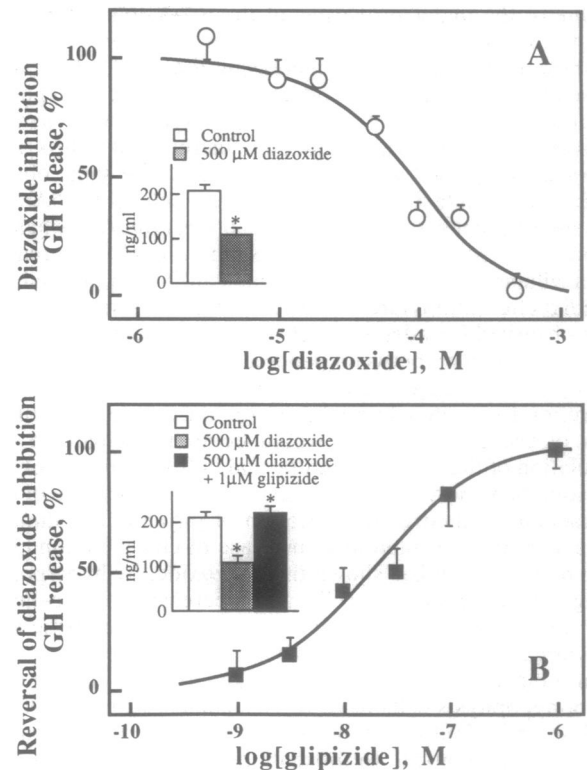


FIG. 6. Inhibition of basal GH secretion from cultured rat female adenohipophys cells by diazoxide and reversal by increasing concentrations of glipizide. (A Inset) Values represent mean \pm SEM of 12 samples. *, $P < 0.01$ versus corresponding control group. Main curve, values represent mean \pm SEM of 12 samples. One-hundred percent GH, $200 \pm 20 \text{ ng/ml}$; 0% GH, $100 \pm 10 \text{ ng/ml}$. (B Inset) Values represent mean \pm SEM of 12 samples. *, $P < 0.01$ versus corresponding control group. Main curve, values represent mean \pm SEM of 12 samples. Zero percent GH, $90 \pm 10 \text{ ng/ml}$; 100% GH, $205 \pm 22 \text{ ng/ml}$. Diazoxide, 400 μM .

nels. [^3H]Glibenclamide binding sites, which are usually considered to be good indicators of the presence of K_{ATP} channels, are localized almost exclusively in adenohipophys, suggesting a role for this class of channels in the endocrine function of this organ. The level of glibenclamide binding sites is two to three times lower in adenohipophys than in insulinoma cells (12), and about seven times lower than in substantia nigra (19), the richest brain region for these drug binding sites.

The affinity of glibenclamide for its binding sites ($K_d = 0.5 \text{ nM}$) and the rank order of affinities of various other sulfonylureas are very similar in pancreatic β cells and in adenohipophys cells. Electrophysiological properties of the K_{ATP} channel in adenohipophys cells are also very similar, if not identical, to those found in pancreatic β cells. Affinity labeling experiments have shown that the sulfonylurea binding component has a M_r of 145,000, very similar again to the molecular weight found for insulinoma cells (17, 28, 29) [and for pig brain (16)].

The K_{ATP} channel is probably important in adenohipophys cell function since its opening abolishes spontaneous electrical activity. Diazoxide, the classical opener of K_{ATP} channels in pancreatic β cells (10, 11), also opened K_{ATP} channels in adenohipophys cells and, like intracellular ATP depletion, induced large hyperpolarizations (30–35 mV) that were antagonized by sulfonylureas. Again, diazoxide had the same type of effects on membrane excitability and worked in the same range of concentrations in adenohipophys cells and in pancreatic β cells.

One of the major properties of the K_{ATP} channel in pancreatic β cells is to control intracellular Ca^{2+} concentration. In these cells, glucose, by increasing the ATP/ADP ratio, closes K_{ATP} channels and consequently provokes a depolarization of the β cell that leads to L-type Ca^{2+} channel activation and to intracellular Ca^{2+} increase, which is suppressed by L-type Ca^{2+} channel blockers. The same observation was made for adenohypophysis cells (Fig. 5A). In pancreatic β cells, the K_{ATP} channel opener diazoxide has an opposite effect; it hyperpolarizes and thereby decreases intracellular Ca^{2+} , a decrease that can be suppressed by antidiabetic sulfonylureas. Once again, the same situation was observed for adenohypophysis cells (Fig. 5C).

The parallelism between pancreatic β cells and adenohypophysis cells suggests a role for K_{ATP} channels in hormone secretion from adenohypophysis. As in the β cell, the function of the K_{ATP} channel would be to participate in the regulation of the resting potential and in the regulation of the bursting behavior. It has been shown that an increase of the extracellular glucose concentration of rat anterior pituitary cells in culture resulted in an increase of GH secretion (30). The present work has shown that diazoxide, by hyperpolarizing adenohypophysis cells and decreasing intracellular Ca^{2+} , inhibited GH secretion, whereas the antidiabetic sulfonylurea glipizide reversed this inhibitory effect.

In summary, all of these results taken together suggest that adenohypophysis cells have K_{ATP} channels with pharmacological and biophysical properties that are very similar, maybe even identical, to those in the pancreatic β cells and that these K_{ATP} channels probably play the same role in hormone secretion (GH for adenohypophysis cells, insulin for β cells).

The significance of the present results in relation to diabetes deserves active investigation. In diabetic rats and in normal rats with hypoglycemia, GH levels are suppressed (13, 31), but this response in rats is in sharp contrast to that in humans, where hypoglycemia results in a prompt increase in GH secretion. One possibility is that GH response in humans is primarily due to regulation from the hypothalamus, and that if human somatotrophs were isolated, they might behave similarly to those of the rat.

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