Sulfonylurea Binding to a Low-affinity Site Inhibits the Na/K-ATPase and the K_{ATP} Channel in Insulin-secreting Cells

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ABSTRACT We have used hamster insulinoma tumor (HIT) cells, an insulin-secreting tumor cell line, to investigate modulation of the Na/K-ATPase and of the ATP-sensitive K channel (K_{ATP}) by the sulfonylurea glyburide. Membrane proteins from cells cultured in RPMI with 11 mM glucose have at least two glyburide receptor populations, as evidenced by high and low binding affinity constants, (K_d = 0.96 and 91 nM, respectively). In these cells K_{ATP} channel activity was blocked by low glyburide concentrations, IC_{50} = 5.4 nM. At 12.5 nM glyburide the inhibition developed slowly, τ = 380 s, and caused reduction of channel activity by 75%. At higher concentrations, however, inhibition occurred at a fast rate, τ = 42 s at 100 nM, and was almost complete. Na/K-ATPase activity measured enzymatically and electrophysiologically was also suppressed by glyburide, but higher concentrations were needed, IC_{50} = 20–40 nM. Inhibition occurred rapidly, τ = 30 s at 50 nM, when maximum, activity was reduced by 40%. By contrast, cells cultured in RPMI supplemented with 25 mM glucose exhibit a single receptor population binding glyburide with low affinity, K_d = 68 nM. In these cells inhibition of the Na/K-ATPase by the sulfonylurea was similar to that observed in cells cultured in 11 mM glucose, but K_{ATP} channel inhibition was markedly altered. Inhibition occurred only at high concentrations of glyburide and at a fast rate; maximum inhibition was observed at ~100 nM. Based on these data, we propose that glyburide binding to the high affinity site affects primarily K_{ATP} channel activity, while interaction with the low affinity site inhibits both Na/K-ATPase and K_{ATP} channel activities. The latter observation suggests possible functional interactions between the Na/K-ATPase and the K_{ATP} channel.

INTRODUCTION

Under physiological conditions the major stimulus for insulin release from the pancreatic β cell originates from uptake and metabolism of circulating nutrients, mainly glucose. Secretion is initiated below 5 mM glucose and increases in a concentration-dependent manner to a maximum level at between 15 to 20 mM (Grodsky et al., 1969; Ashcroft et al., 1972). Initiation of secretion is associated with depolarization of the β cell membrane, which in turn triggers the characteristic bursts of calcium action potentials, the duration of which increases as the secretagogue concentration is raised (Meissner and Schmelz, 1974). Similar initiation and modulation of electrical activity and secretion occurs in response to nonmetabolized secretagogues such as the sulfonylureas, tolbutamide and glyburide (Meissner and Atwater, 1975; Pace, 1984; Panten et al., 1989).

The initial β cell membrane depolarization induced by glucose is attributed to closure of ATP-sensitive K channels (K_{ATP}) by intracellular ATP produced during glycolysis (Ashcroft, 1988). This hypothesis is supported by measurement of intracellular ATP showing that ATP increases as glucose is raised from 0 to 5 mM (Ashcroft et al., 1973; Hellman et al., 1969; Malaisse et al., 1979). However, as glucose is elevated above 5 mM, the cellular level of ATP remains constant, suggesting that a different mechanism must be responsible for the concentration-dependent increase in burst duration and secretion that occur between 5 and 20 mM. Since sulfonylureas mimic the effects of elevating glucose on both the electrical activity and insulin secretion, it had been argued that further closure of K_{ATP} channels was responsible for the effects of elevated glucose, although no mechanism was proposed to explain how elevated glucose may block K_{ATP} channels (Henquin, 1988).

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K_{ATP} channel inhibition by glyburide has been ascribed to interaction of the sulfonylurea with a high-affinity receptor, which may be the K_{ATP} channel itself or a closely associated protein (Aguilar-Bryan et al., 1995). However, the K_d for binding of glyburide to the high-affinity site is below 1 nM, whereas half inhibition of the K_{ATP} channel occurs at concentrations between 6 to 27 nM (Findlay, 1992; Gaines et al., 1988; Sturgess et al., 1988). These observations suggest that modulation of channel activity by glyburide involves binding of the sulfonylurea to a low affinity regulatory site in addition to the high affinity site, the half channel inhibition occurring at a concentration between the two K_d's. This hypothesis is supported by the presence of at least four different types of sulfonylurea binding proteins in insulin-secreting cell membranes with affinity for glyburide ranging from 1 nM to several micromolar (Nelson et al., 1992). While the high-affinity site may be identified with the already described high-affinity sulfonylurea receptor, we postulate that one of the low-affinity sites is associated with the Na/K-ATPase.

Previous biochemical as well as electrophysiological studies have demonstrated that ouabain, a specific blocker of the Na/K-ATPase, increases insulin release in the presence of glucose (Milner and Hales, 1967), and prolongs the bursts of action potentials (Ribalet and Beigelman, 1979; Henquin and Meissner, 1982); more recently, it has also been shown to raise intracellular Ca^{2+} (Grapengiesser et al., 1993). These effects being similar to those exerted by sulfonylureas and elevated glucose, it had been proposed that inhibition of the Na/K-ATPase is involved in secretagogue-induced insulin release (Meissner, 1976; Levin et al., 1978; Ribalet and Beigelman, 1979; Fagin et al., 1987; Gronda et al., 1989); however, this hypothesis remained controversial (Kemmler and Löffler, 1977; Henquin and Meissner, 1982). Subsequent patch-clamp studies successfully demonstrated K_{ATP} channel inhibition by glucose and sulfonylureas, providing a compelling explanation for the initiation of insulin release (Ashcroft, 1988), and the controversy regarding the role of the Na/K-ATPase did not get resolved.

In this study we have determined the binding characteristics of glyburide to HIT cell membrane proteins and correlated these data with enzymatic, as well as electrophysiological, measurements of the effects of glyburide on Na/K-ATPase activity. Our goal was to investigate whether the sulfonylurea glyburide interacts with the Na/K pump, and, if so, whether it involves binding to the high-affinity site or to one with lower affinity. We chose to perform our studies using HIT cells which demonstrate almost complete loss of the high-affinity binding sites at high passage number when cultured in the presence of 25 mM glucose (Aguilar et al., 1992). The data presented here indicate that interaction of glyburide with a low affinity sulfonylurea binding protein, possibly associated with the Na/K pump, causes inhibition of the ATPase as well as of the K_{ATP} channel. The respective role of the Na/K pump and of the K_{ATP} channel in glyburide-evoked insulin release is discussed in view of these findings.

MATERIALS AND METHODS

Cell Cultures and Membrane Preparation

The present studies were performed using cells from the insulin secreting cell line hamster insulinoma tumor (HIT)[1] (passage 76-83), provided by the late A. E. Boyd III (Endocrine Division, New England Medical Center, Boston, MA) and CRI-D11 provided by C. N. Hales (Dept. of Clinical Biochemistry, Cambridge, England). The cells were incubated at 37°C in RPMI 1640 medium, supplemented with 10% (vol/vol) FCS, penicillin (100 U/ml), streptomycin (100 U/ml), and 2 mM glutamine. The cells were divided once a week by treatment with trypsin and the medium was changed twice between divisions. The culture was carried out in RPMI medium containing 11 or 25 mM glucose.

Cells were harvested using a cell scraper after rinsing twice with PBS and incubating for 15 min at 37°C in PBS supplemented with 4 mM EDTA and pelleted by centrifugation. The cell pellet was resuspended in Tris/EDTA/PMSF (5, 2.5, and 0.1 mM final concentrations) and allowed to swell on ice for 40 min. The cells were then hand homogenized and centrifuged at 2,500 *g* for 10 min. The supernatant was then centrifuged at 130,000 *g* for 60 min. The resultant pellet was suspended in 50 mM TRIS, and assayed for protein by the method of Bradford.

Binding Studies

Equilibrium binding studies were performed essentially as described by Panten et al. (1989) using 20–30 μg/ml HIT cell membrane proteins. Proteins were incubated in 50 mM Tris, pH 7.4, for 40 min at room temperature in the presence of ^{3}H-glyburide (New England Nuclear, Boston, MA, 50 Ci/mmol) at concentrations ranging from 0.01 to 50 nM. Nonspecific binding was determined by performing the same incubations in the presence of unlabeled K-glyburide (gift from the Upjohn Company, Kalamazoo, MI) at 1 μM. After incubation, separation of bound drug was achieved by filtration through glass microfiber filters (Whatman, Inc., Clifton, NJ) followed by three washes with ice-cold buffer. The filters were put into scintillation vials with instagel scintillation cocktail (Packard Instrument Company, Meriden, CT) and counted in a liquid scintillation counter.

Data analysis. Specific binding was calculated by subtracting nonspecific from total binding. For any given passage number, the assay was done in triplicate for each concentration of radiolabeled glyburide used. The K_d and B_{max} were evaluated from Scatchard plots using Sigmaplot nonlinear curve fitter. The equation used in the fitting program was that derived by Feldman (1972) for a one ligand two-binding site model.

[1] *Abbreviations used in this paper:* HIT, hamster insulinoma tumor. RPMI, Roswell Park Memorial Institute.

Enzyme Assay

Cell storage and membrane preparation. Cells were harvested, as described above, and were placed into 1 ml of a solution containing 0.25 M sucrose, 1 mM EDTA adjusted to pH 7.5 with 0.5 M Tris. One μl of 0.1 M PMSF in 100% ethanol was added and the cells were rapidly frozen over dry ice/acetone, and stored at −70°C. For the enzyme assay cells were thawed on ice, pelleted at 2,500 *g* for 10 min at 4°C, and rinsed twice with the Sucrose-Tris-EDTA solution. The cell pellet was then resuspended in Tris/EDTA/PMSF (5, 2.5, and 0.1 mM final concentrations), and allowed to swell on ice for 45 min. The cells were then hand homogenized and centrifuged at 2,500 *g* for 10 min at 4°C. The supernatant was then centrifuged at 130,000 *g* for 60 min. The resultant pellet was suspended in 1 mM Tris-EDTA, pH 7.4, and stored at −70°C.

Enzyme analysis. The enzyme assay was performed using a method derived from that previously described (Levin et al., 1978). The incubation solution contained 0.5 mM EDTA, 21 mM glycylglycine, 21 mM histidine, 3.6 mM $MgCl_2$, 100 mM NaCl, 20 mM KCl, 10 mM NaN_3, in the presence or absence of ouabain (0.1 mM). One tenth ml of membrane aliquot containing 10–30 μg protein was added to 0.5 ml of the incubation solution, with 10 μl of 0.5 mM KOH alone or with KOH and the desired concentration of glyburide. Samples were first preincubated in a shaker bath at 37°C for 10 min. The reaction was started by adding 50 μl vanadium-free Tris-ATP (final concentration 3 mM) and the samples were incubated again for 10 min at 37°C. The reaction was stopped by immersion of the tubes in ice and addition of 50 μl cold trichloroacetic acid. Samples were centrifuged at 1,100 *g* for 10 min and supernatants were assayed for inorganic phosphate (Pi) by the method of Fiske and Subbarow (1925). Corrections were made for spontaneous hydrolysis of ATP using tubes containing ATP but not tissue.

Na/K-ATPase activity was defined as the difference in Pi liberated from ATP hydrolysis in the presence vs absence of ouabain. Mg-ATPase activity was defined as the amount of Pi liberated from ATP in the presence of ouabain. Enzyme activity was expressed as μM Pi/mg protein/hour or units.

Electrophysiological Studies

Single-channel data recording. 1 h before starting the patch-clamp experiment the RPMI culture medium was replaced with a glucose-free solution containing 135 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.1 mM $MgCl_2$ and 10 mM HEPES, the pH adjusted to 7.4 with NaOH. For outside–out patch experiments, the composition of the bath solution (extracellular type) was 140 mM KCl, 2.5 mM $CaCl_2$, 1.1 mM $MgCl_2$ and 10 mM HEPES, pH adjusted to 7.2 with KOH; that of the pipette solution (intracellular type) was similar, except that Ca^{2+}, EGTA ratio was adjusted to give a pCa of 5 or 6 10^{-7}. ATP (Sigma Chemical Company, St. Louis, MO) and GTP (Boehringer Mannheim Diagnostics, Inc., Houston, TX) were added to give concentrations of 20 μM and 50–100 μM, respectively. Stock solutions of glyburide were made in 0.5 mM KOH, appropriate dilutions were made in saline solution so that the final concentration of KOH in the bath was <5 μM.

Data recording and analysis. The techniques to prepare patch electrodes and record single-channel events were similar to those described previously (Ribalet et al., 1988). The data, filtered at 2 kHz with an 8-pole Bessel filter were recorded with either an EPC 7 List (Darmstadt, FRG) or an Axopatch 1A (Axon Instruments, Burlingame, CA) patch amplifier and stored on video cassette at a fixed frequency of 44 kHz after digitization with a digital audio processor. For analysis, the data were acquired on a computer hard disk at a rate of 5.5 kHz; the transfer was carried out with a two-buffer interface allowing continuous acquisition (Bezanilla, 1985). Before measurement of channel activity, amplitude histograms of the current steps were built to determine the half amplitude threshold, and this threshold was used to form an idealized record of the original data. This schematized record was utilized for the subsequent analysis. To construct the concentration–dependence curves the percent open time (NP_o), which is the parameter used to assess the level of channel activity, was determined from data samples of 30-s duration obtained at steady state. Under control conditions, NP_o values varied widely from patch to patch and for this reason averages were used to determine ratios of experimental NP_o's vs control. To produce the time course graphs two experiments were used unless specified, and NP_o values were estimated using 15-s bins and plotted as a function of time.

Whole-cell Na/K-ATPase current recording. Na/K "pump" currents were recorded in HIT cells using the whole-cell clamp configuration, at room temperature, with the pipette solution containing in millimolar: 110 *N*-methyl-D-glucamine (NMDG), 30 NaCl, 10 HEPES, 10 EGTA, 1 $CaCl_2$, and 4 $MgSO_4$ and with a bath solution consisting of 140 TEA, 10 HEPES and 1.2 $MgSO_4$. In addition the pipette solution contained 4–5 mM ATP to obtain maximum Na/K-ATPase activity (Niki et al., 1989*a*). The data, filtered at 0.5 kHz with an 8-pole Bessel filter were recorded using the experimental set up described above and stored on a Hewlett-Packard FM tape recorder. For analysis the data was played back on a chart recorder.

RESULTS

High- and Low-affinity Binding of Glyburide to Proteins of HIT Cells Cultured in 11 mM Glucose

Membranes of insulin-secreting HIT cells possess proteins which bind glyburide with high and low affinity. The present study takes advantage of this observation as well as the fact that culture conditions can affect the level of glyburide binding sites (Aguilar et al., 1992) to elucidate the role of these sulfonylurea-binding proteins in regulating the activity of the Na/K ATPase as well as of the K_{ATP} channel.

The data presented in Fig. 1 illustrate the results of nine experiments carried out to characterize the specific binding of glyburide to HIT cell membrane proteins. The binding data shown in Fig. 1 *A* is well fitted by a two-site model; the K_d's and B_{max}'s deduced from the fit are 0.96 nM and 0.14 pmol/mg, respectively for the high-affinity binding site and 91 nM and 6.1 pmol/mg for the low-affinity binding site. The low K_d value obtained here for glyburide binding to HIT cell membrane proteins is in agreement with previously reported values (Niki et al., 1989*b*; Aguilar-Bryan et al., 1990). When evaluating sulfonylurea binding using very high concentrations of glyburide, we found specific

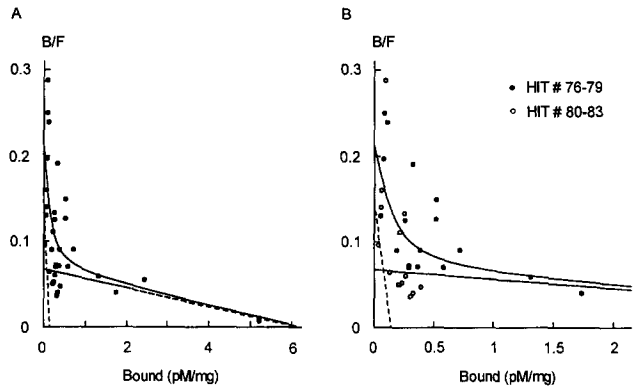

FIGURE 1. Scatchard analysis of ^{3}H glyburide binding to membrane proteins of HIT cells cultured in 11 mM glucose. In *A* and *B*, binding data are plotted as bound/free vs bound (pmol/mg of protein). For each point, the measurements were done in triplicate. The solid line was obtained by fitting all data points using a two site model as described under Materials and Methods. The fit yielded K_d of .96 nM and B_{max} = 0.14 pmol/mg for the high affinity binding site and K_d of 91 nM and B_{max} = 6.1 pmol/mg for the low affinity binding site. These K_d and B_{max} values were then used to draw the straight lines according to the equation: $B/F = -K_a{*}B + K_a{*}B_{max}$ for a one-site model. In *B*, the plot is similar to that in *A* but the scale on the x axis has been expanded to better show the dispersion of the high affinity binding data. Further, the data points have been grouped in two categories as a function of cell passage number. Closed circles are for passage 76 to 79 and open circles are for passage 80 to 83. All fitted lines are the same as in *A*.

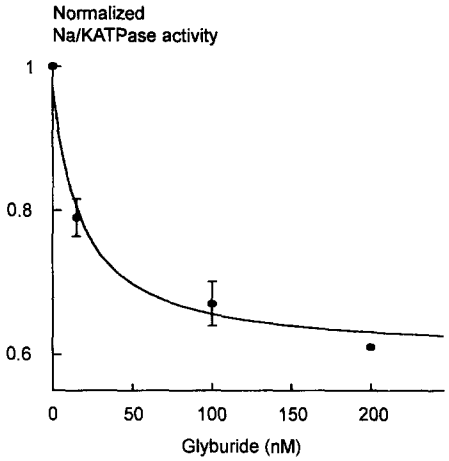

FIGURE 2. Na/K-ATPase inhibition by glyburide in cells cultured with 11 mM glucose. In this plot the normalized enzyme activity (A/A_o) is plotted as a function of glyburide concentration. Each data point represented by a closed triangle is the average of three experiments performed in duplicate. The points were fitted with the following equation: $A/A_o = 1/[1 + (C/K)] + b$. Where C is the concentration of glyburide, K is the IC_{50}. The fit of the data yielded K = 17.5 nM and b = 60.

binding occurring at even lower affinity. Including this data in the analysis revealed a third K_d of ~500 nM. This value may correspond to the μM K_d reported by Aguilar-Bryan et al. (1992). We suggest based on these observations that HIT cell membranes have sulfonylurea binding proteins that may be classified in at least three categories with K_d's close to 1 nM, 100 nM, and 1 μM. The present study focuses on the properties of the two former sites.

In Fig. 1 *B* the data have been plotted as a function of passage number, the filled circles representing data obtained with cells from passages 76–79, the open circles representing data obtained from cells at passages 80–83. Although there appears to be a slight shift to the left, as passage number increases, it should be emphasized that under these culture conditions, the number of binding sites remained relatively high even after passage number 83.

Na/K-ATPase Inhibition by Glyburide (Cells Cultured in 11 mM Glucose)

Enzymatic activity. We tested the effects of glyburide on Na/K-ATPase activity using enzymatic as well as electrophysiological approaches. Assay of the enzyme activity showed clear inhibitory effect of the sulfonylurea, as illustrated in Fig. 2. Fitting the experimental values obtained with concentrations of glyburide between 12.5 and 200 nM yielded an IC_{50} of 17.5 nM. There was no apparent correlation between the potency of glyburide to inhibit Na/K-ATPase activity and the cell passage number. For plotting purposes the data points have been normalized; the absolute enzyme activity obtained in three experiments performed in duplicate was 6.66 ± 1.42 Units. In these experiments Mg^{2+}-ATPase activity was not influenced by glyburide.

Whole-cell recording of the current elicited by activation of the Na/K-ATPase. Intracellular microelectrode recording of β cell membrane potential has demonstrated the presence of an ouabain-sensitive Na/K pump current that contributes to β cell electrical activity (Ribalet and Beigelman, 1979). Using the whole-cell configuration of the patch-clamp technique we have characterized the current elicited by activation of the Na/K-ATPase in insulin-secreting cells, and studied the effects of glyburide on this current. As illustrated in Fig. 3 (*top*) recordings of Na/K pump currents were started with no K^+ in the bath; in these experiments the intracellular solution contained 30 mM Na^+. Upon addition of 2 mM K^+ to the bath an outward current developed that was probably associated with a net outward movement of positive charges. This current developed within <1 min. After removal of external K^+, the current returned to its basal value. The return to baseline was slower than the onset, an effect most likely due to the time required for washing K^+ from the bath. In the presence of 4–5 mM intracellular MgATP the K^+-evoked currents could be as large as 5 to 6 pA and as

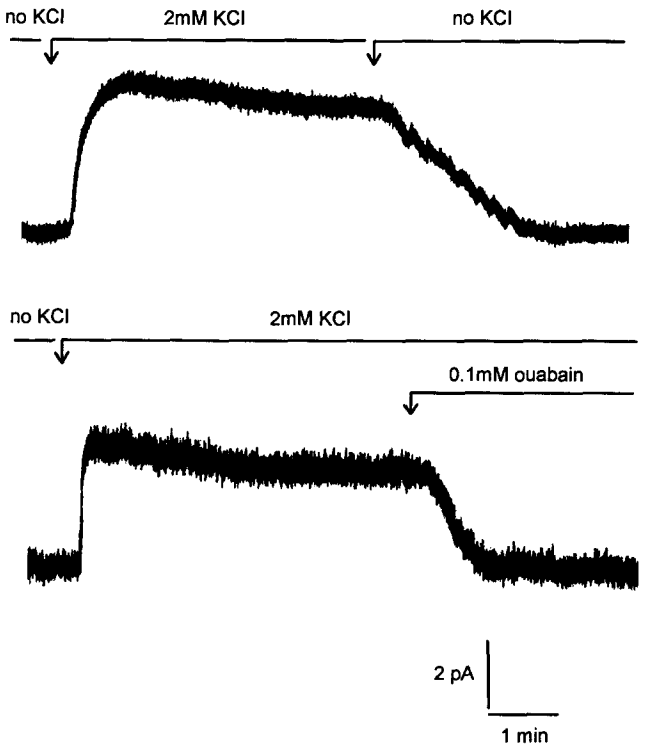

FIGURE 3. Recording of whole-cell current elicited by K^+-induced Na/K-ATPase activation. The upper trace is a typical recording of K^+-evoked Na/K pump current. To activate the pump, K^+ was directly added to the bath and the solution was stirred. Removal of K^+ was performed by perfusing a 0.75 ml bath with 5 ml of a solution without K^+. The return of the current trace to baseline after K^+ removal indicates that the exchange of the solution was satisfactory under these conditions. The lower trace illustrates the inhibitory effect of ouabain. In this type of experiment K^+ was first added to the bath to activate the pump current and remained present for the rest of the experiment. Ouabain was also directly added to the bath.

small as 1 pA. In the presence of intracellular MgATP concentrations <1 mM, the currents were dramatically reduced being often <0.5 pA.

To investigate whether the K^+-activated currents could be ascribed to activation of the Na/K-ATPase activity we examined the effects of ouabain, a specific Na/K pump blocker, on these currents. At bath concentrations below 10 μM ouabain had almost no effect, but at 500 μM it almost completely blocked K^+-evoked currents within 1 min. Blockage was reversible upon removal of ouabain, and the current returned to 80% of its steady state initial value within 5 min. These results are compatible with the K^+-activated current being primarily due to activation of the Na/K-ATPase. The inhibition by a high level of ouabain and the fast reversibility of this effect are comparable to those observed with intracellular recordings of membrane potential in pancreatic β cells in which addition of ouabain at concentrations >100 μM caused a membrane depolarization which was rapidly reversible upon washing the inhibitor from the bath, and in which low levels of ouabain had no effect (Ribalet and Beigelman, 1979).

Inhibition of the Na/K-ATPase current by glyburide. Having established that K^+-evoked currents result primarily from activation of the Na/K pump we investigated the effect of the sulfonylurea glyburide. Fig. 4 (*top*) illustrates the effects of 25 and 125 nM glyburide. Addition of 25 nM caused a gradual decrease in current amplitude, a 20% diminution occurring within 2 min after addition of the drug. Further elevation of bath glyburide resulted in a further 20% decrease in current amplitude. In this case the inhibition occurred rapidly within <1 min. Upon removal of extracellular K^+, the current returned to baseline within a minute. This effect of glyburide was partly reversible, but the current elicited by subsequent applications of K^+ showed different time courses, the initial phase of the evoked current becoming transient. Thus, after glyburide, K^+-evoked currents peaked rapidly within 30 s and decayed towards a plateau within 3–5 min. At steady state the current amplitude was often 10–20% lower than that measured before application of glyburide.

Fig. 4 (*bottom*) shows the dose-dependent inhibition of K^+-activated Na/K-ATPase current by glyburide. Fitting the averaged data points obtained from five experiments yielded an IC_{50} of 35.5 nM, a value comparable to that obtained for Na/K-ATPase inhibition using the enzyme assay (17.5 nM).

Study of K_{ATP} Channel Inhibition by Glyburide in Outside–Out Patches

It is generally accepted that blockage of K_{ATP} channel activity by sulfonylureas involves binding of the drug to a high affinity receptor site very likely associated with a recently cloned protein of 140,000 mol wt (Aguilar-Bryan et al., 1995). Binding of sulfonylurea to this high-affinity site (K_d <1 nM) accounts at least in part for the low IC_{50} value (between 6 and 27 nM) that characterizes channel block by glyburide (Findlay, 1992; Gaines et al., 1988; Sturgess et al., 1988). These values are lower than those obtained in the present study for Na/K pump inhibition (IC_{50} = 20–40 nM). To determine if indeed a difference exists between the dose-dependent block of the K_{ATP} channel and of the Na/K-ATPase, experiments were performed to test the effect of glyburide on K_{ATP} channel activity, and to compare the result with that obtained for the Na/K-ATPase in the same cells under similar experimental conditions. K_{ATP} channel response to glyburide was investigated using outside-out patches with concentrations ranging from 2.5 to 12.5 nM. Examination of the data presented in Fig. 5 (*A–C*) indicates that K_{ATP} channel inhibition was partial at 6 nM and much more pronounced at 12.5 nM. In Fig. 5 *D*, average responses from five experi-

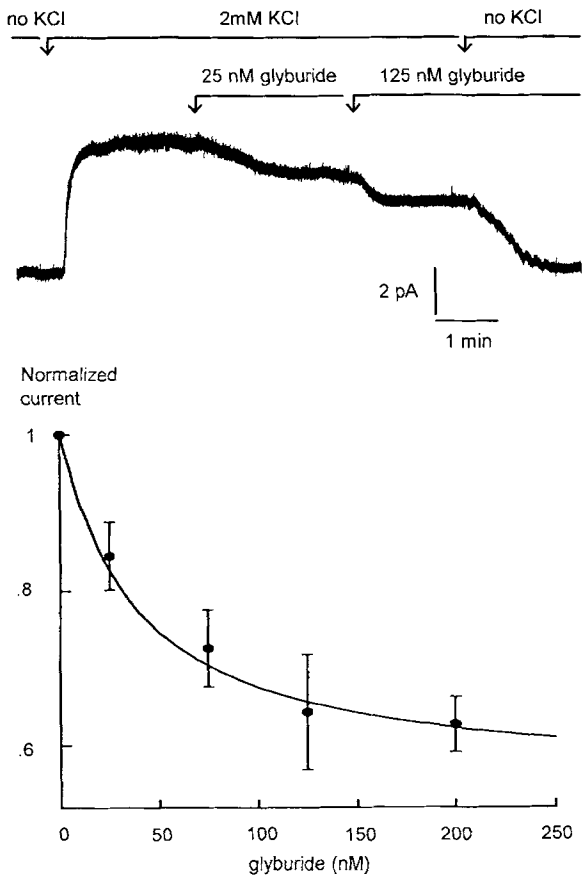

FIGURE 4. Glyburide-induced inhibition of whole-cell current elicited by K^+-induced Na/K-ATPase activation. The upper trace illustrates the inhibitory effects of increasing concentrations of glyburide. In these experiments glyburide was directly added to the bath and the solution stirred. The graph in the lower panel is a plot of normalized Na/K pump current. The closed circles which represent averaged data were fitted with the following equation: $I/I_o = 1/[1 + (C/K)] + b$. The fit of the data yielded $K = 35.5$ nM and $b = 55$.

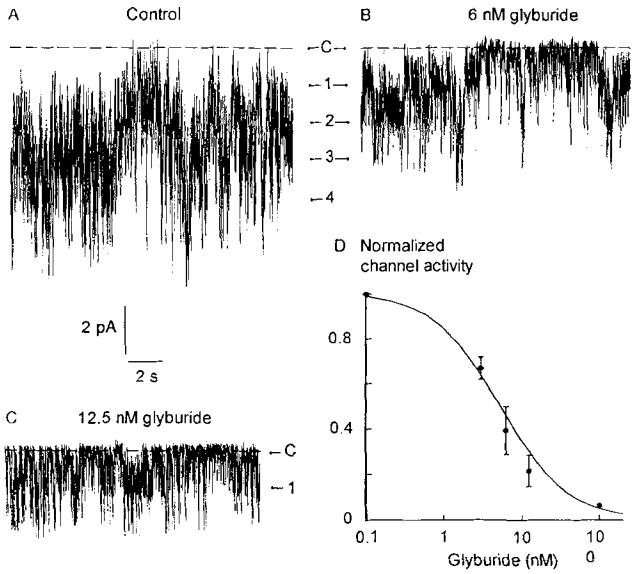

FIGURE 5. Concentration-dependent effects of glyburide on K_{ATP} channel activity in the outside–out membrane patch of HIT cells cultured in 11 mM glucose. Recordings in *A–C* are from the same patch and depict the inhibitory effect of bath applied glyburide. The downward current steps represent inward current in symmetrical 140 mM KCl for a pipette holding potential of −40 mV. The numbers close to the current traces indicate the number of open channels. The pipette solution contained 50 μM GTP and 20 μM ATP. In *D*, the channel activity normalized to the activity measured in the absence of drug was plotted as a function of glyburide concentration (Log scale). The closed circles which represent averaged data were fitted with the equation: $I/I_o = 1/1 + (C/K)$. The fit of the data yielded $K = 5.4$ nM.

ments are normalized and plotted as a function of glyburide concentration to illustrate the dose-dependent effect of this agent. The fit to the data yielded an IC_{50} of ~5.4 nM. This plot also demonstrates that glyburide concentrations as high as 100 nM were required for channel inhibition to reach near completion.

In this set of experiments there was no clear correlation between the potency of glyburide to block channel activity and the passage number of the cell culture. Thus, the slight decrease in the number of high affinity sites illustrated in Fig. 1 *B* did not appear to affect the inhibitory effect of glyburide.

The results shown in Fig. 6 (*top*) illustrate K_{ATP} channel activity behavior in response to addition of 12.5 nM glyburide in two patches. In one patch, channel activity declined steadily after addition of glyburide. Fitting of the data indicated that the decay occurred exponentially with a time constant of ~400 s. In the other patch, channel activity oscillated before the addition of the sulfonylurea, and this behavior remained unchanged after addition of glyburide even though the overall level of activity decreased with a time course similar to that observed in the other experiment. The right side of this panel illustrates the rate of recovery of channel activity after glyburide removal.

The data presented in Fig. 6 (*bottom*) demonstrates that K_{ATP} inhibition occurred rapidly at 100 nM, with a decay time constant of 42 s. This observation is in sharp contrast with the slow rate of channel blockage (400 s) recorded for low (12.5 nM) glyburide concentration. These findings are very similar to those reported in cardiac cells where K_{ATP} channel block by glyburide also occurs at two different rates. In this preparation two rates with values of 611 s and 112 s are evident at 10 nM glyburide (Findlay, 1992). In HIT cells, the fast decay in channel activity is usually difficult to detect at low glyburide concentrations (~10 nM); however, in two occasions, fast initial decrease in channel activity of ~10% could be observed at 12.5 mM glyburide (result not shown). In addition, in cardiac cells, the response

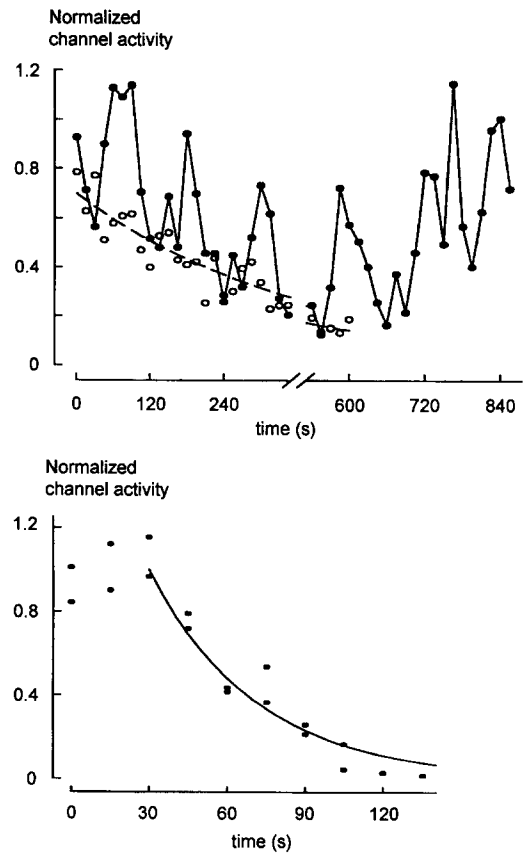

FIGURE 6. Time course of K_{ATP} channel inhibition by glyburide. The top illustrates the results of two experiments carried out with outside–out membrane patches of HIT cells cultured with 11 mM glucose. The closed and open circles correspond to normalized NP_o values recorded from two different patches after addition of 12.5 nM glyburide to the bath. NP_o's were estimated using data samples of 15-s duration and plotted as a function of time. The dotted line represents a fit of the open circles using a single exponential function that yielded a τ = 380 s. The solid line drawn through the closed circles illustrates the inhibitory effect of glyburide as well as the reversibility of this effect. The right side of the panel shows the recovery of channel activity following removal of glyburide at t = 660 s. The lower panel illustrates the effect of 100 nM glyburide. The experiment was carried out with an outside–out membrane patch. Glyburide was added to the bath at t = 20 s. The closed circles correspond to normalized NP_o values. NP_o's were estimated using data samples of 15-s duration and plotted as a function of time. The solid line represents a fit of the data points starting at t = 30 s and using a single-exponential function that yielded a τ = 42 s.

to 100 nM glyburide is similar to that recorded with HIT cells in that the slow component becomes negligible and only fast decay in channel activity is observed, τ = 55 s (Findlay et al., 1992).

The data presented thus far indicate that membranes of the insulin secreting cells HIT possess high and low affinity sites for glyburide. Inhibition of K_{ATP} channel activity occurs with an IC_{50} of 5.4 nM, consistent with channel block involving glyburide binding to the high-affinity site. By contrast, requirement for higher concentrations of glyburide to block the Na/K pump is indicative of a different mechanism involving a lower affinity binding site.

Binding of Glyburide to Membrane Proteins of HIT Cells Cultured in 25 mM Glucose

In cells cultured in the presence of 25 mM glucose, there is almost complete loss of the high-affinity binding of glyburide to HIT cell membrane proteins (Aguilar-Bryan et al., 1992). Data are presented in Fig. 7 that corroborate this observation. The experimental points obtained from six different cell batches taken between passage 76 and 82 are well fitted using a one-binding site model (*solid line*), thus, demonstrating the existence of a single population of glyburide binding proteins. Under these conditions the K_d value of 68 nM is similar to that obtained for the low-affinity binding sites, 91 nM, of cells cultured in the presence of 11 mM glucose.

We have used this property of HIT cells to assess whether the high affinity sulfonylurea receptor controls the activity of Na/K-ATPases and K_{ATP} channels and if so to what extent.

Inhibition of Na/K-ATPase enzyme activity by glyburide (cells cultured in 25 mM glucose). In cells cultured with 25 mM glucose, the absolute enzyme activity was 6.73 ± 2.47 U. This value is not significantly different from

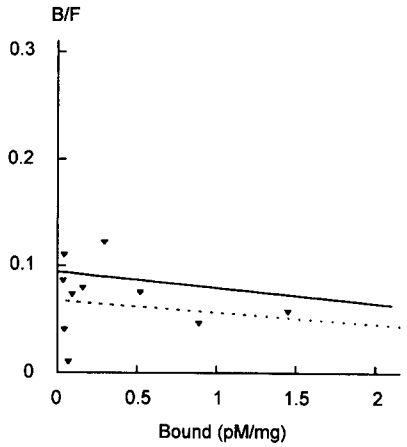

FIGURE 7. Scatchard analysis of ^3H glyburide binding to membrane proteins of HIT cells cultured in 25 mM glucose. Binding data are plotted as bound/free vs bound (pmol/mg of protein). Each point represents measurements done in triplicate. Fitting of the data with a two-site model gave only a single value for K_d and B_{max} indicative of a single population of binding sites. The solid line represents a fit to the data performed using a single binding site model. This fit yielded a K_d of 68 nM and a B_{max} of 6.4 pmol/mg of protein. The dotted line taken from the plot in Fig. 1 represents the binding of glyburide to the low-affinity site in cells cultured with 11 mM glucose and is shown for comparison.

that measured with cells cultured in the presence of 11 mM glucose, suggesting that cell culture with high glucose does not affect the activity of the Na/K-ATPase. We investigated the dose-dependent inhibition of the Na/K pump by glyburide using cells cultured in the presence of 25 mM glucose as well as insulin secreting cells, CRI-D11, which also lack high-affinity sites for the sulfonylurea (Khan et al., 1993). The two cells lines responded to glyburide similarly. Normalized data obtained in two experiments carried out with HIT cells and one with CRI-D11 are plotted in Fig. 8. Measurements were made in duplicate for each experiment. Fitting of the data yielded an IC_{50} of 26.5 nM, a value close to that obtained with cells cultured in the presence of 11 mM glucose (17.5 nM). There was no significant difference between the effects of glyburide under the two culture conditions. Accordingly, whole-cell recording of the Na/K pump current demonstrated that glyburide had similar inhibitory effects when cells were cultured in the presence of 11 or 25 mM glucose (result not shown).

Thus a loss of high-affinity sites does not affect Na/K-ATPase inhibition by glyburide suggesting that this effect of the sulfonylurea is mediated via binding to a low affinity site, which is independent of the high-affinity sulfonylurea receptor.

K_{ATP} Channel Inhibition by Glyburide in Cells Cultured in the Presence of 25 mM Glucose

The data presented in *A–C* of Fig. 9 are representative of six outside–out patch-clamp experiments performed to test the effect of glyburide on K_{ATP} channel activity in cells cultured in the presence of 25 mM glucose. As expected, due to the loss of high-affinity sites, K_{ATP} channels became much less sensitive to glyburide at concentrations below 12.5 nM. Surprisingly however elevated levels of glyburide caused channel inhibition. Examination of the data presented in Fig. 9, *A* and *C*, demonstrates almost complete blockage of channel activity by 100 nM. In Fig. 9 *D*, average responses from the six experiments are normalized and plotted as a function of glyburide concentration to illustrate the dose-dependent effect of glyburide on channel activity under these conditions. The fit to the data yielded an IC_{50} of 29 nM which was greatly different from that obtained for cells cultured in the presence of 11 mM glucose (5.4 nM) but was very similar to that recorded for the inhibition of the Na/K ATPase by glyburide (20–40 nM). Further, the rate of inhibition of the K_{ATP} channel and of the Na/K-ATPase were similar. At high glyburide concentration (100 nM) K_{ATP} channel activity decayed rapidly at a rate similar to that observed in cells cultured in 11 mM glucose (Fig. 6, *bottom*), approaching the rate of Na/K-ATPase inhibition.

Assessment of the level of single channel activity recorded from membrane patches of cells cultured in the presence of 11 mM glucose (n = 5) or 25 mM glucose

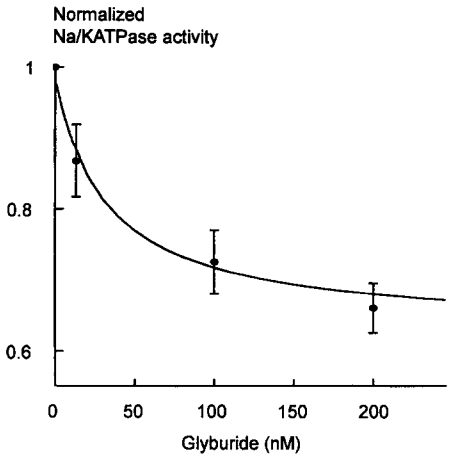

FIGURE 8. Na/K-ATPase inhibition by glyburide in cells cultured with 25 mM glucose. In this plot the data was normalized and plotted as a function of the concentration of glyburide. Each data point represented by closed circle is the average of three experiments performed in duplicate. The points were fitted with the equation: $A/A_o = 1/[1 + (C/K)] + b$. The fit of the data yielded K = 26.5 nM and b = 67.

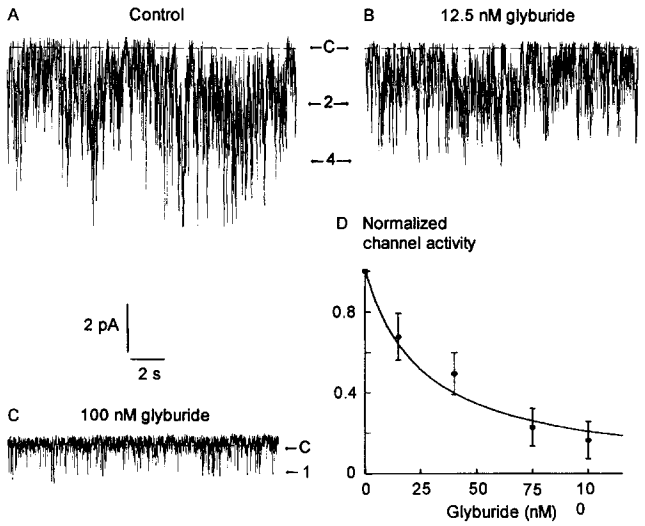

FIGURE 9. Concentration-dependent effects of glyburide on K_{ATP} channel activity in outside-out patch of HIT cells cultured with 25 mM glucose. Recordings in *A–C* are from the same patch and depict the inhibitory effect of bath applied glyburide. The downward current steps represent inward current in symmetrical 140 mM KCl for a pipette holding potential of −40 mV. The numbers close to the current traces indicate the number of open channels. The pipette solution contained 50 μM GTP and 20 μM ATP. In *D*, the channel activity normalized to the activity measured in the absence of drug was plotted as a function of glyburide concentration. The closed circles which represent averaged data were fitted with the following equation: $I/I_o = 1/1 + (C/K)$. The fit of the data yielded K = 29 nM.

(n = 6), does not show any significant difference. Assuming that the density of high-affinity sites has substantially decreased in the latter case it may be postulated that the K_{ATP} channel and the high-affinity receptor are two different entities. Similar conclusions have been reached using the insulin secreting cells, CRI-D11, which also lack high-affinity binding sites for the sulfonylurea (Khan et al., 1993).

In summary, membrane proteins of HIT cells cultured in the presence of 25 mM glucose display greatly reduced level of high-affinity glyburide binding sites. Under these culture conditions the characteristics of K_{ATP} channel inhibition by the sulfonylurea are similar to those of Na/K pump inhibition.

DISCUSSION

Glyburide Binding to HIT Cell Membrane Proteins

In insulin secreting cells at least four types of membrane proteins bind sulfonylureas (Nelson et al., 1992), but while these proteins have similar affinity for tolbutamide, their affinity for glyburide varies. Thus, the recently cloned high-affinity sulfonylurea receptor (Aguilar-Bryan et al., 1995) has a K_d of the order of 1 nM for glyburide, while the others have K_d's close to or >100 nM (Nelson et al., 1992). It is believed that the high-affinity receptor is the primary target for sulfonylureas, causing K_{ATP} channel closure and insulin release (Panten et al., 1989). However, published data demonstrating glyburide-induced closure of K_{ATP} channels and stimulation of insulin release have indicated that these effects occur between 10 and 100 nM (Gaines et al., 1988; Sturgess et al., 1988; Panten et al., 1989), concentrations significantly greater than the K_d for glyburide binding to the high-affinity site. This suggests that modulation of channel activity and insulin release by glyburide may involve binding of the sulfonylurea to a second, lower affinity regulatory site in addition to the high-affinity site. The identity and function of the low-affinity binding receptor sites remain uncertain; our data indicate that one of these sites may be associated with the Na/K-ATPase.

Inhibition of the Na/K-ATPase Activity Is Mediated via Sulfonylurea Binding to a Low-affinity Site

Membrane proteins of HIT cells cultured in 11 mM glucose exhibit two major glyburide binding affinities with K_d's of 0.96 and 91 nM (Fig. 1). In these cells, both enzymatic assay and whole-cell Na/K pump current recording show that glyburide has potent inhibitory effects at concentrations ranging from 10 to 100 nM (Figs. 2 and 4). These concentrations far exceed those required for occupation of the high-affinity site, indicating that modulation of Na/K-ATPase activity involves binding of glyburide to the site with lower affinity.

Additional studies were carried out on late passages of HIT cells cultured with 25 mM glucose and with CRI-D11 cells both of which exhibit loss of high-affinity glyburide binding sites (Aguilar et al., 1992; Khan et al., 1993; see also present study). Despite the absence of the high-affinity sulfonylurea receptor, the ability of glyburide to suppress Na/K-ATPase activity persisted (Fig. 8). These observations indicate that pump inhibition by glyburide is mediated via a low-affinity binding protein which is independent of the high-affinity sulfonylurea receptor.

Given that the effects of glyburide on the Na/K pump current were demonstrated under conventional whole-cell voltage clamp, it is unlikely that a soluble second messenger is involved in this process, and it is suggestive of a direct effect of the sulfonylurea on the pump. Supporting this hypothesis we have found that the Na/K-ATPase β_1 subunit of HIT cells has an apparent molecular weight close to 50 kD (data not shown), which is comparable to the molecular mass of one of the low affinity sulfonylurea binding proteins (Nelson et al., 1992). These observations suggest that the β subunit may be a low affinity sulfonylurea binding protein.

K_{ATP} Channel Inhibition Is also Mediated via Sulfonylurea Binding to a Low-affinity Site

In cells cultured with 11 mM glucose, K_{ATP} channel activity recorded in outside-out patches is slowly inhibited by ~75% at 12.5 nM glyburide (Fig. 5), an effect which can be attributed, at least in part, to occupation of the high-affinity sulfonylurea receptor. By contrast, the K_{ATP} channel of cells cultured in 25 mM glucose, therefore lacking this high-affinity site, was not affected by low concentrations of glyburide, but high concentrations (10–100 nM) had potent and rapid inhibitory effects on the channel (Fig. 9).

Thus low concentrations of glyburide have a slow inhibitory effect on channel activity (τ = 400 s at 12.5 nM), whereas high concentrations cause rapid inhibition (τ = 42 s at 100 nM). Similar results have been reported with cardiac cells, and in this case both the fast and slow rates (τ = 611 and 112 s) can be distinguished at 10 nM (Findlay, 1992). Based on these findings it may be concluded that fast and slow channel block by glyburide reflect two separate regulatory mechanisms.

Nature of the Low-affinity Sulfonylurea Binding Protein that Regulates K_{ATP} Channel Activity

Thus, K_{ATP} channel inhibition by sulfonylureas may involve glyburide binding to the high-affinity sulfonylurea receptor, as well as to a low-affinity binding protein that remains to be identified. It may be argued that the low-affinity site is on the channel itself or on a protein directly associated with it. Our data demonstrating similarities of concentration dependence and

kinetics data of Na/K pump and K_{ATP} channel inhibition by high glyburide in cells cultured with 25 mM glucose, suggest that the low-affinity sulfonylurea binding protein which is associated with the Na/K pump may also interact with the K_{ATP} channel.

Thus, binding of glyburide to both the high-affinity sulfonylurea receptor ($K_d \sim 1$ nM) and to a low-affinity site, possibly associated with the Na/K-ATPase ($K_d \sim 30$ nM), may account for half-channel inhibition occurring at concentrations between 6 and 27 nM (Findlay, 1992; Gaines et al., 1988; Sturgess et al., 1988).

Can Coupling between the Na/K-ATPase and the K_{ATP} Channel Account for K Channel Inhibition by High Glyburide Concentrations?

Interactions between the Na/K-ATPase and the K_{ATP} channel have already been proposed, suggesting that ATP serves as a link between the two moieties. One scenario assumes that inhibition of Na/K-ATPase activity leads to elevation of ATP which then blocks K_{ATP} channels (Tung et al., 1990; Tsuchiya et al., 1992; Graspengiesser et al., 1993; Hurst et al., 1993). However, such a mechanism should not apply in our experiments since the intracellular ATP level is approximately the same as the pipette solution, that is 10–20 μM, and is well below the Na/K-ATPase K_m of 500 μM. We propose instead that direct interaction of the low-affinity glyburide binding protein, associated with either an active or an inactive pump, with the K_{ATP} channel accounts at least in part for K_{ATP} channel inhibition by glyburide.

Is Na/K-ATPase Inhibition Responsible for Sulfonylurea-induced Insulin Release?

Since inhibition of the Na/K pump by ouabain causes burst elongation and stimulation of insulin release (Meissner, 1976; Ribalet and Beigelman, 1979; Milner and Hales, 1967; Levin et al., 1978) it may be argued, in view of our results, that the effects of sulfonylureas on electrical activity and insulin release also involves Na/K pump inhibition. Such a hypothesis is supported by studies of the dose-dependent effect of glyburide on secretion in islets as well as cells from the insulinoma cell lines HIT and CRI-C1 (Gaines et al., 1988; Sturgess et al., 1988; Panten et al., 1989) indicating that stimulation of release occurs at glyburide concentrations similar to those evoking Na/K pump inhibition (10–40 nM).

This observation suggests that Na/K pump inhibition may play a role in stimulation of insulin release by glyburide. However, our data indicate that glyburide at these concentrations interact with a low-affinity site which may inhibit both the Na/K pump and the K_{ATP} channel. Therefore, it remains to be determined whether these effects of glyburide on secretion and electrical actviity may be due to Na/K pump inhibition or to concomitant blockage of K_{ATP} channels.

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