Co-expression of Sulfonylurea Receptors and $K_{\text{ATP}}$ Channels in Hamster Insulinoma Tumor (HIT) Cells

EVIDENCE FOR DIRECT ASSOCIATION OF THE RECEPTOR WITH THE CHANNEL*

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Cell membranes isolated from hamster insulinoma (HIT T15) cells at passages 65–74 contain high and low affinity receptors for a sulfonylurea derivative, 5-['251]iodo-2-hydroxyglyburide ($K_D$ values of ~7 nM and 16 µM). Between passages 75 and 85, the estimated $B_{\text{max}}$ for the high affinity receptor decreases ~10-fold from ~1.6 to 0.16 pmol/mg membrane protein. By contrast, the density of low affinity binding sites, 800–1000 pmol/mg, is unaltered. The drop in high affinity receptors is paralleled by a decrease in the density of $K_{\text{ATP}}$ channels assessed using patch-clamp and "Rh"-efflux techniques. These results strongly support the idea that the high affinity sulfonylurea receptor is an integral part of the $K_{\text{ATP}}$ channel.

The sulfonylurea drugs, particularly tolbutamide and glyburide (glibenclamide), have been widely used to identify ATP-sensitive potassium ($K_{\text{ATP}}$) channels. The rationale behind this usage and the specificity of these drugs has been reviewed by several authors (Ashcroft, 1988; Ashcroft and Ashcroft, 1990; Robertson and Steinberg, 1990). In pancreatic $\beta$-cells a high affinity receptor has been identified which has a similar affinity for binding of various sulfonylureas (Trube et al., 1986; Zinkler et al., 1988a; Ashcroft, 1988), for inhibition of $K_{\text{ATP}}$ channel activity (Schind-Antomarchi et al., 1987) and for stimulation of insulin secretion (Panten et al., 1989; Aguilar-Bryan et al., 1990). A 140-kDa protein can be photolabeled using either [3H]glyburide (Kramer et al., 1988; Bernardi et al., 1988) or a radiodinated derivative of glyburide (Aguilar-Bryan et al., 1990). The photolabeling has displacement properties that exactly parallel binding to high affinity $\beta$-cell receptors (Aguilar-Bryan et al., 1990; Nelson et al., 1992), strongly suggesting that the receptor is the 140-kDa protein. The available evidence suggests a link between this receptor and the channel and is compatible with the receptor being an integral part of the channel or a required regulatory subunit. $\beta$-Cell membranes have additional low affinity glyburide binding sites of undetermined function with $K_D$ values in the micromolar range (Niki et al., 1989; Nelson et al., 1992). These lower affinity sites can also be photolabeled and appear to consist of several protein species (Aguilar-Bryan et al., 1989; Nelson et al., 1992). In this paper we have attempted to determine how “tightly” the coupling is between the high affinity receptor and $K_{\text{ATP}}$ channel activity by examining the density and behavior of the channels and receptors in the membranes of two sublines of HIT T15 cells at different passage numbers. We see a progressive parallel loss of the high affinity receptor and channel activity with increasing passage number in both sublines. The data strongly support the idea that the 140-kDa sulfonylurea receptor is an integral part of the $K_{\text{ATP}}$ channel.

EXPERIMENTAL PROCEDURES

Chemicals

Organic reagents were from Aldrich, tissue culture supplies were obtained from Gibco, and common chemicals were from Sigma.

Cell Lines

HIT T15 cells were derived from dispersed Syrian hamster pancreatic islets following transformation with SV40 (Santerre et al., 1981). Cultures of the original line were obtained from Dr. Santerre. HIT T15-2.2.2 cells are a derivative for T15 cells obtained from Dr. William Rutter's laboratory at the University of California at San Francisco. The T15-2.2.2 subline has been cloned from T15 cells and selected for the ability to be transfected easily by foreign DNA. They have been transfected with the human insulin gene. T15 cells were stored in liquid nitrogen at passage 65; the history of the T15-2.2.2 cells is not as precise, and we have assumed they were stored at approximately passage 75. This estimate is the earliest possible passage at which the T15-2.2.2 cells could have been put into storage. The passage numbers given in the text for the T15-2.2.2 cells are based on this assumption.

Cell Culture

General Cell Culture—T15 and T15-2.2.2 cells were grown as monolayers using T175 flasks in DMEM-HG medium (containing 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 10% fetal bovine serum). Cells were passaged weekly and fed three times per passage. Confluent cells were subcultured as follows: cells were rinsed with 0.05% trypsin/EDTA in phosphate-buffered saline (PBS), then incubated with 0.05% trypsin/EDTA at room temperature to promote detachment. DMEM-HG medium plus serum was added and cells were replated after splitting 1:5.

Large Scale Cell Culture—Roller bottles, 850 cm² area (Becton-Dickinson), were seeded with cells from a T175 flask in 100 ml of

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The abbreviations used are: $K_{\text{ATP}}$, ATP-sensitive potassium; DMEM, Dulbecco's modified Eagle's medium; DMEM-HG, DMEM plus high glucose; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HIT, hamster insulinoma tumor; MOPS, 4-morpholinepropanesulfonic acid; EGTA, ethyleneglycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid.

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DMEM-HG plus 10% fetal bovine serum. Cells were harvested at about 80–90% confluence after approximately three weeks. Medium was changed frequently, approximately every day during the last week.

Cells for Patch-clamping and \(^{36}Rb^+\) Efflux Experiments—Cells were cultured in DMEM-HG media (containing 100 units/ml penicillin, 0.1 mg/ml streptomycin, 10% fetal calf serum) at 37 °C in humidified atmosphere containing 5% CO\(_2\) and 95% air. Medium was changed every 2–3 days before experiments. Cells were plated in DMEM at a density of 2 \(^{10^4}\) cells/well in 12-well plates. For patch-clamp experiments, cells were plated at a density of 2 \(^{10^5}\) cells/dish on plastic coverslips in Falcon Petri dishes (Becton-Dickinson).

Preparation of Iodinated Glyburide Derivatives

The radiolabeled derivative of glyburide, N-[4-[[2-(2-hydroxybenzenecarboxamido)ethyl]benzenesulfonyl]-N' cyclohexyl]urea, was prepared as described previously (Aguilar-Bryan et al., 1990) and used at the concentrations indicated.

Membrane Isolation

Crude membranes were prepared using a procedure worked out for isolation of nuclei and the subsequent purification of transcription factors. The details are as follows: media was removed from roller bottles by aspiration, and the cell layers were quickly rinsed with PBS. Cells were detached by addition of 1 ml of 3.5 mM EDTA, then pelleted and resuspended in sucrose-homogenization buffer containing 25 mM sucrose, 10% glycerol, 25 mM KCl, 10 mM HEPES-KOH, pH 7.5, 1 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine. A homogenate was prepared using a motor-driven Teflon–glass homogenizer. Cellbreakage was monitored using a phase microscope; approximately three passages were required to achieve 90% cell homogeneity. The homogenate was layered over a cushion of the sucrose-homogenization buffer, centrifuged at 3000 rpm for 10 min in a JA14 rotor (Beckman). The resulting pellet was discarded, and the supernatant was re-centrifuged at 40,000 rpm, ~100,000 \(g_x\), for 45 min in the Ti-50.2 rotor (Beckman). The membrane pellet was re-homogenized in 10 mM Tris-HCl, 2 mM EDTA, 150 mM NaCl, and 20% glycerol at pH 7.4 and immediately frozen and stored at -80 °C until used. Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as a standard; receptor levels were monitored by photolabeling as outlined below.

Binding Assays

Equilibrium binding studies were done as described in Aguilar-Bryan et al. (1990), modified as follows. Displacement assays were done in 50 mM MOPS, pH 7.2, in a total volume of 1.0 ml. Crude membranes (250 μg of protein) were incubated with approximately 10\(^6\) cpm of 1 nM \(^{125}\)Iodo-2-hydroxyglyburide for 30 min at room temperature in the presence of increasing concentrations of the unlabeled drug covering the range from 10 μM to 300 μM. Previous kinetic studies have shown that binding is complete within 20 min under these conditions. Binding was terminated by rapid filtration through Whatman GF/F glass fiber filters followed by three 5-ml washes with ice-cold 10 mM MOPS, pH 7.2. Filters were counted in a γ counter; the data were analyzed using Ligand (Munson and Rodbard, 1980). Each point was done in triplicate. A binding model consisting of two sites plus a non-specific binding term was fit to the data using nonlinear regression. The model is given below.

\[
\text{Bound} = \frac{R_{\text{max}}(L)}{K_{D_{\text{hi}}}(L) + 1} + \frac{R_{\text{max}}(L)}{K_{D_{\text{lo}}}(L) + 1} + N_{\text{sp}}(L)
\]

\(R_{\text{max}}(L)\) and \(R_{\text{max}}(L)\) are the maximum concentrations of high and low affinity sites; \(K_{D_{\text{hi}}}(L)\) and \(K_{D_{\text{lo}}}(L)\) are the corresponding dissociation constants, \(N_{\text{sp}}(L)\) is the fraction of total drug which is bound non-specifically, and \(L\) is the concentration of the unbound drug.

Photolabeling

HIT cell membranes at a protein concentration of 5 mg/ml were incubated with 1 or 5 nM \(^{125}\)I-glyburide for 30 min at room temperature. Aliquots (0.5–1 ml) were transferred to parafilm and irradiated using a UV cross-linker (Fisher) equipped with 312 nm bulbs. Irradiation was done in the energy mode using a value of 15,000 μJ/cm² calibrated as described by Nelson et al. (1992). This value was determined to be in the middle of a broad energy optimum (Nelson et al., 1992).

\(^{36}Rb^+\) Efflux Experiments

\(^{36}Rb^+\) efflux measurements were done using modifications of the procedure described by Schmid-Antomarchi et al. (1987). Cells were incubated overnight with 1–2 μCi of \(^{36}Rb^+\) in DMEM in 12-well plates at 37 °C. After equilibration the growth medium was removed, and the cells were washed and incubated for 20 min in Krebs-Ringer bicarbonate buffer (118 mM NaCl, 2.5 mM CaCl\(_2\), 1.2 mM KHPO\(_4\), 4.7 mM KCl, 25 mM NaHCO\(_3\), 1.2 mM MgSO\(_4\), and 10 mM HEPES, pH 7.4) containing \(^{36}RbCl\) (1–2 μCi), 2-deoxy-D-glucose (1 mM) and oligomycin (0.24 μg/ml) to deplete cellular ATP. Parallel incubations were performed with 1 μM glyburide present. After the incubation, the supernatant was aspirated and replaced with the same solution without \(^{36}RbCl^+\) and metabolic inhibitors. At various times the incubation was stopped by washing the cells rapidly with 1 ml of 0.1 M MgCl\(_2\). The \(^{36}Rb^+\) remaining in the cells was determined by scraping them into 0.3% SDS, then measuring radioactivity in a liquid scintillation counter.

Whole-cell and Patch-clamp Experiments

All experiments were done at room temperature, in an oil-gate chamber (Qin and Noma, 1988) as described by Lederer and Nicholls (1989), which allowed the solution bathing the exposed surface of an isolated patch to be changed rapidly (<50 ms). Whole-cell experiments were performed in one channel of the same chamber; solution exchange was achieved within 30 s by a manual switching anterior to the inflow.

Microelectrodes (2–8 MΩ) were pulled from filamented borosilicate glass (1.5 mm, outer diameter, 18100F-6, WPI Inc., New Haven, CT) on a horizontal puller. Micropipettes were “sealed” onto cells by applying light suction to the rear of the pipette. This typically resulted in a seal of 10–100 GΩ. Whole-cell configuration was achieved by further suction on the electrode. Alternatively, inside-out patches were obtained by lifting the electrode slowly then passing the tip through the oil-gate. Frequently, the cell remained attached to the pipette tip on lifting, but was always isolated on passing through the oil-gate, leaving the cell behind at the solution-oil interface. Only rarely were patches ruptured on passing through the oil-solution interface.

The standard extracellular solution used in these experiments had the following composition: 140 mM NaCl, 4 mM KCl, 1 mM MgCl\(_2\), 2 mM CaCl\(_2\), 10 mM Na-HEPES, pH 7.4. The standard intracellular solution contained 140 mM KCl, 10 mM K-HEPES, and 1 mM K-EGTA, pH 7.25. In whole-cell experiments, the pipette solution additionally contained 1 mM MgCl\(_2\) and ATP as described in Fig 4. Solutions of glyburide and \(^{36}Rb^+\) were prepared in dimethyl sulfoxide and added to media such that the concentration of dimethyl sulfoxide was less than 0.1%. Other additions to solutions are described in the figure legends.

Patch-clamp currents were measured using an Axopatch 1C Patch Clamp (Axon Instruments, Inc., Burlingame, CA) with a 1 or 10GΩ headstage, and filtered at 1–5 kHz. Signals were digitized at 22 kHz (VR-10 digital recorder, Instrutech Corp.) and stored on video tape. Experiments were replayed through an 8-pole Bessel filter (at 0.5–2 kHz) and digitized at 20 kHz into a microcomputer using PC clamp software (Axon Instruments) for subsequent analysis using Lotus 1-2-3 (Lotus Development Corp., Cambridge, MA).

RESULTS

Early Passage HIT Cells Have More High Affinity Sulfonylurea Receptors than Later Passage Cells—A survey of the sulfonylurea-binding proteins identifiable by photolabeling in HIT T15 cell membranes isolated at increasing passage number is given in Fig. 1. The 140-, ~65-, ~55-kDa and a 30-kDa species are the most prominent bands (see Aguilar-Bryan et al. (1990) and Nelson et al. (1992) for a detailed discussion of these components). The 140-kDa band has been identified with the high affinity receptor; we note that the intensity of labeling in this band drops markedly with increasing passage number. We have quantitated this by cutting out the 140-kDa
bands and determining the radioactivity. The data were normalized to picomoles per mg of membrane protein using a value of 0.4% for the efficiency of photolabeling (Nelson et al., 1992). The results are given in Fig. 2. There is approximately a 10-fold drop in receptor content as the passage number increases. Note: these are not $B_{\text{max}}$ values, since the photolabeling reactions are not carried out at saturating concentrations of the drug. We observe the same qualitative results with T15-2.2.2 cells (data not shown), a loss of the high affinity receptor with continuous passaging. The filled symbols in Fig. 2 are insulin secretion data taken from a paper by Zhang et al. (1989) studying the loss of glucose-sensitive insulin release in HIT cells as a function of passage number. These data are considered further under “Discussion.”

The binding parameters for the receptors in early and late passage cells were determined by a filtration binding assay. This was done using T15 cells at passages 70 and 90 and T15-2.2.2 cells at estimated passage numbers of 75 and 90. Both sets of cells exhibit a drop in receptor number with continuous passage. The loss is more profound in the T15 cells which start with a higher initial number. The results are consistent with our earliest available passage of T15-2.2.2 cells being comparable with T15 cells in the later stages of transition shown in Fig. 2. A comparison of the binding properties of receptors in early and late passage membranes is given in Fig. 3. The comparison was done using membranes from passage 70 T15 cells as the early passage “baseline” and averaged data from membranes isolated from passage 90 T15 and T15-2.2.2 cells as late passage. The early passage curve was calculated using the binding parameters averaged from three experiments done in triplicate using T15 membranes; the late passage curve is the average of six experiments, three with T15 membranes plus three with T15-2.2.2 membranes, each done in triplicate. The top panel illustrates the full displacement curves. The bottom panel gives partial Scatchard plots which have been plotted to show the loss of high affinity binding sites. For illustration, one set of early passage T15 cell data points is given along with one set of data points from passage 90 T15-2.2.2 cells. The data points are the average of triplicate assays. Passage 90 T15 cells would show equivalent behavior. Both the early and late passage cells show two classes of binding sites, one with high affinity and a second low affinity class. The high affinity class is reduced in the late passage cell membranes, but the low affinity sites remain. A summary of the binding parameters derived from the data and used to calculate the curves is given in Table I. The estimates for the $K_D$ values are in good agreement for both classes of sites in both sets of membranes. The estimated $K_D$ values are approximately 10-fold weaker (6.9 and 3.8 nM versus 0.36 nM) than we reported earlier (Aguilar-Bryan et al., 1990). The reason for this is unclear but may reflect either the differences in the assay method or in the new membrane isolation procedure. Nonspecific binding is essentially the same in both preparations. The binding data are consistent with the presence of qualitatively similar high and low affinity receptors in both cell sublines and, as shown in Fig. 1, there are no new species of receptor(s) identified during continuous culture. The single significant difference is in the $B_{\text{max}}$ values; membranes isolated from early passage cells have more high affinity receptors in agreement with the photolabeling results.

Estimates of ATP Sensitivity and $K_{\text{ATP}}$ Channel Numbers from Patch Clamp Data—We have estimated the numbers of $K_{\text{ATP}}$ channels and their ATP sensitivity from membrane patches and whole-cell currents. Fig. 4 illustrates the $K_{\text{ATP}}$
The ATP sensitivity of the channels in the two sublines was estimated from a more complete data set. The $K_i$ values for ATP inhibition of channel activity, approximately 5 $\mu$m, were comparable for both. Using late passage T15-2.2.2 cells (passage 90) we were able to find single channel activity in only 3 out of 17 patches and estimated a $K_i = 2 \mu$m; a value of 6 $\mu$m was estimated from 21 patches of T15 cells between passages 67 and 72. The results suggest there is little difference in ATP sensitivity between the channels detectable in early and late passage cells, although the analysis is difficult because of the small number of usable patches that can be obtained after passage 75.

A comparison of whole cell currents in an early passage cell (a T15 cell at passage 70, upper panel) and a late passage cell (a passage 90 T15-2.2.2 cell, lower panel) is given in Fig. 5. Dialysis begins at the arrows. An outward K+ current develops in the T15 cells after a short lag period, but is not observed in the late passage cells. As a measure of cells with significant KATP channel activity, we have scored the frequency of cells which develop an outward current > 10 pA. The results are given in Table II along with the patch-clamp data. The patch-clamp data confirm there are fewer KATP channels in our earliest available T15-2.2.2 cells when compared with our earliest passage T15 cells, 1.5 versus 4.25 channels/pool. The data also show that the number of channels in T15-2.2.2 cells drop with increasing passage number. Similarly, the number of T15 cells that develop an outward current > 10 pA shows a marked drop with passage number.

Comparison of 86Rb Efflux from Early and Late Passage HIT T15 Cells—In an effort to complement the electrophysiological data and estimate differences in KATP number by another strategy, 86Rb efflux studies were done on early and late passage T15 cells. The results of one set of experiments are given in Fig. 6. In the early passage cells, reducing the ATP level increases 86Rb efflux; glyburide, which blocks KATP channels, slows this efflux. This is easily seen by comparison of the open and the filled squares. There is a residual flux in the presence of glyburide that is presumably due to non-glyburide-sensitive K+ channels. We have designated this a “leak” current. The late passage T15 cells show no significant differences in efflux either with or without glyburide (compare the open and filled triangles). The result indicates that we are not able to detect the expected increase in 86Rb efflux through the smaller numbers of KATP channels present in the late passage cells against the background leak flux. We have attempted to evaluate the sensitivity of our 86Rb efflux experiments using a simple model to explain the efflux kinetics. Rubidium efflux from the early passage cells appears to

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**Table I**

**Summary of receptor binding data**

The model being fitted to the data is given under “Experimental Procedures.” $B_{max(0)}$ and $B_{max(16)}$ are the maximum concentrations of high and low affinity sites; $K_{D(0)}$ and $K_{D(16)}$ are the corresponding dissociation constants; $N_{SP}$ is the fraction of total drug which is bound nonspecifically. The ± S.E. values are approximate standard errors as discussed by Munson and Rodbard (1980) and by Feldman (1972). The early passage values were derived from three data sets; the late passage values were derived from six data sets as discussed under “Results” and the legend to Figure 3.

|           | Early passage cells | Late passage cells |
|-----------|---------------------|--------------------|
| $K_{D(0)}$ (nM) | 6.9 ± 1.1            | 3.8 ± 2.5          |
| $K_{D(16)}$ (nM) | 16.1 ± 4.2          | 14.0 ± 2.8         |
| $N_{SP}$ | 0.01 ± 0.001        | 0.012 ± 0.001      |
| $B_{max(0)}$ (pmol/mg) | 1.64 ± 0.24      | 0.16 ± 0.08        |
| $B_{max(16)}$ (pmol/mg) | 800 ± 200         | 960 ± 200          |

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Sulfonylurea Receptors and K\textsubscript{ATP} Channels

FIG. 4. ATP dependence of K\textsubscript{ATP} channels from early and late passage HIT cells. The top trace gives an example of channel activity in a membrane patch taken from a passage 70 T15 cell. The bars mark periods when the patch is in the oil-gate; the ATP concentrations are given above each trace. Compare the lower trace obtained with a patch from a passage 79 T15-2.2.2 cell. The single channel conductance is the same in both, but the channel density is reduced in the lower panel. Note that the frequency of cells that we obtain positive patches from in late passage cells is small; the trace given is a relatively infrequent event. The holding potential was 0 mV; experiments were done at room temperature.

FIG. 5. Whole cell currents in early and late passage HIT cells. The top trace gives an example of the developing whole cell current in a HIT T15 cell at passage 70. A high resistance seal was obtained and dialysis was started by breaking the membrane (arrow). Current developed over several minutes as the ATP level dropped. A sustained current was maintained for several minutes, but declined as the K\textsubscript{ATP} channels inactivated at low ATP concentration. Compare the bottom trace from a passage 90 T15-2.2.2 cell; the current rise was barely visible under the same conditions. The holding potential was −50 mV. Depolarizing pulses (1 s) were applied at the times indicated by the bars. The experiments were done at room temperature.

| Cells   | Passage no. | No. of channels/patch | Mean no. of channels open | Frequency of whole cell currents |
|---------|-------------|------------------------|---------------------------|---------------------------------|
| T15     | 67-72       | 4.25 ± 0.3             | 2.0 ± 0.2                 | 17/18                           |
|         |             | (n = 21)               |                           |                                 |
| T15     | 81          | ND                     | ND                        | 2/4                             |
| T15-2.2.2 | 77-81      | 1.5 ± 0.1              | 0.42 ± 0.1               | ND                              |
|         |             | (n = 21)               |                           |                                 |
| T15-2.2.2 | 90-98      | −0.4                   | <0.1                      | 2/6                             |
|         |             | (n = 17)               |                           |                                 |

TABLE II
Summary of channel data

The ± values are standard errors of the mean. The frequency of whole cell currents was scored as the number of cells which developed a current > 10 pA upon dialysis without ATP divided by the total number of trials. ND, not determined.

be biphasic with a fairly rapid initial loss followed by a slow component that has the same kinetics as the glyburide insensitive leak current. We assume that following incubation with metabolic inhibitors an initial number of K\textsubscript{ATP} channels, N\textsubscript{ATP}, are open, but then close at a constant rate. The mechanism for closing is not critical, but two obvious candidates are either “rundown” in the absence of ATP, as has been described for these channels in membrane patches (Trube and Hescheler, 1984; Ohno-Shosaku et al., 1987), or reclosure following an increase in the ATP concentration. Three differential equations describe the model.

\[
\frac{d[Rb]}{dt} = -k\text{ATP}N\text{ATP}[Rb] + k\text{leak}[Rb]
\]

(1)

\[
\frac{dN\text{ATP}}{dt} = -k\text{leak}N\text{ATP}
\]

(2)

\[
\frac{d[Rb]}{dt} = -k\text{leak}[Rb]
\]

(3)

Where [Rb] is the concentration of rubidium in cells, k\text{ATP} is the rate constant for rubidium efflux per channel, k\text{leak} is the rate constant for closing or inactivation of K\textsubscript{ATP} channels and k\text{leak} is the rate constant for "Rb" efflux through non-glyburide-sensitive K¹ channels. The model was fit to the data in Fig. 6 using nonlinear fitting procedures. The lines in Fig. 6
DISCUSSION

There has been some controversy regarding the existence of multiple populations of glyburide (glibenclamide) binding sites in insulinoma and cardiac cell membranes (Ashcroft, 1988; Ashcroft and Ashcroft, 1990; Misler et al., 1986). Our HIT cell data clearly display multiple binding sites which can be adequately represented by two populations. The binding parameters of these populations are in reasonable agreement with those reported by others (Schmid-Antomarchi et al., 1987; French et al., 1990; Posset et al., 1988; Niki et al., 1989) and are in good agreement with our initial report on the iodinated derivative of glyburide (Aguilar-Bryan et al., 1990). The estimated KΘ value for the iodo-drug is approximately 7 nM, somewhat higher than we reported previously, but in general agreement with the values generally reported. Glyburide is approximately a 10-fold better displacer than the iodinated derivative indicating the KΘ for glyburide is near 0.5–1 nM. Our current evidence, from photolabeling studies done using a similar concentration series of unlabeled displacing drug, indicates that the 140-kDa species is the sole labeled high affinity receptor (Nelson et al., 1992). These studies also show that we can identify several low affinity binding proteins with somewhat different affinity constants grouped around the values, 15–20 μM, obtained by filtration assays.

We find a parallel decline in both the numbers of high affinity sulfonylurea binding sites and \( K_{ATP} \) channels in HIT cells with increasing passage number without a corresponding decline in the number of low affinity binding sites. These observations may explain, in part, the variation in estimated numbers of receptors in these cells and stress the importance of using closely passage-matched cells in doing receptor/channel comparisons. We note, for example, that beyond passage 80–85 it is difficult to detect photolabeling of the 140-kDa protein although the lower molecular mass, low affinity species are readily identifiable. Passage number, or the number of corresponding cell divisions, is the determining factor in these differences. This conclusion is based on the fact that the drug binding studies and ATP sensitivity data indicate the high affinity receptors, from early or late passages from either subline, are quantitatively similar in terms of association and inhibition constants and we see no evidence for new receptor species by photolabeling. The historical record on passage numbers in T15-2.2.2 cells is incomplete so we cannot precisely age-match the two sublines. However, although our T15-2.2.2 cells start from a lower level of receptor, we still see a loss with continuous passage. Since the historical record is incomplete, we cannot determine whether there are other differences between the two sublines.

A passage-dependent loss of glucose-sensitive insulin release between passages 70 and 90 has been described (Santerre et al., 1981; Ashcroft et al., 1986; Zhang et al., 1989). We have included, for comparative purposes, the insulin release data of Zhang et al. (1989) and note that this measure of specific \( \beta \)-cell function declines at the same passage numbers as the sulfonylurea receptor. An explanation for the loss of glucose-sensitive insulin release has not been provided, but our data suggest that it could result from the loss of \( K_{ATP} \) channel activity over the same passages. A decrease in numbers of \( K_{ATP} \) channels, with no change in their ATP sensitivity, would lead to a decreased maximal effect of glucose on membrane potential, without change in the relative glucose sensitivity of the response. A further extrapolation from these results predicts that glibenclamide-sensitive insulin secretion would be similarly decreased between passages 70 and 90.

The reason(s) for the loss of the high affinity receptor are unclear, but provide an opportunity to assess the coupling between receptor number and channel activity. The results strongly support the idea that the high affinity receptor is an integral component of the channel. We find a parallel drop in receptor number and channel activity as determined by \( \text{"Rb" efflux} \), by estimates of channel density from patch-clamp experiments and by whole cell currents. Where channel ATP sensitivity could be assessed in the later passages, it did not differ substantially from early passage cells, and the single channel conductance was unaffected. In the most extreme examples where we see barely detectable levels of the high affinity receptor, i.e. \(<0.1\ \text{pmol/mg membrane protein} \), we are unable to resolve a glyburide-sensitive \( \text{"Rb" efflux} \) from the “background” leak current, are unable to find channels in most of the patches examined, and have difficulty detecting a developing \( K^+ \) current during whole cell dialysis without ATP.
Without molecular cloning and co-expression of binding activity and channel activity, it is not possible to determine with certainty whether the sulfonylurea receptor is sufficient to form a K\textsubscript{ATP} channel or is an essential element in a heteromeric channel. The presence of sulfonylurea binding and the glyburide inhibition of K\textsuperscript{+} fluxes have been taken as indicators of K\textsubscript{ATP} channels (Bernardi et al., 1988; Fosset et al., 1988; Sturgess et al., 1985; Zunkler et al., 1988b). In the absence of evidence to the contrary, there seems to be an increasing consensus that the receptor is a constitutive part of the channel. This is supported by the recent observation that K\textsubscript{ATP} channels from smooth muscle can be re-incorporated into planar lipid bilayers (Kovacs and Nelson, 1991) and retain glyburide sensitivity. The present demonstration is the first to show, in a controlled way, that the expression of sulfonylurea receptors and K\textsubscript{ATP} channels is regulated in parallel within the same cell type. Considering all of these results, we conclude that the high affinity sulfonylurea receptors are indeed structural components of K\textsubscript{ATP} channels.

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