ATP-Sensitive Potassium Channels and Efaroxan-Induced Insulin Release in the Electrofusion-Derived BRIN-BD11 β-Cell Line

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The properties of ATP-sensitive K^+ (K_{ATP}) channels were explored in the electrofusion-derived, glucose-responsive, insulin-secreting cell line BRIN-BD11 using patch-clamp techniques. In intact cells, K_{ATP} channels were inhibited by glucose, the sulfonylurea tolbutamide, and the imidazoline compounds efaroxan and phentolamine. Each of these agents initiated insulin secretion and potentiated the actions of glucose. K_{ATP} channels were blocked by ATP in a concentration-dependent manner and activated by ADP in the presence of ATP. In both intact cells and excised inside-out patches, the K_{ATP} channel agonists diazoxide and pinacidil activated channels, and both compounds inhibited insulin secretion evoked by glucose, tolbutamide, and imidazolines. The mechanisms of action of imidazolines were examined in more detail. Pre-exposure of BRIN-BD11 cells to either efaroxan or phentolamine selectively inhibited imidazoline-induced insulin secretion but not the secretory responses of cells to glucose, tolbutamide, or a depolarizing concentration of KCl. These conditions did not result in the loss of depolarization-dependent rises in intracellular Ca^{2+} ($[Ca^{2+}]_i$), K_{ATP} channel operation, or the actions of either ATP or efaroxan on K_{ATP} channels. Desensitization of the imidazoline receptor following exposure to high concentrations of efaroxan, however, was found to result in an increase in SUR1 protein expression and, as a consequence, an upregulation of K_{ATP} channel density. Our data provide *1*) the first characterization of K_{ATP} channels in BRIN-BD11 cells, a novel insulin-secreting cell line produced by electrofusion techniques, and *2*) a further analysis of the role of imidazolines in the control of insulin release. *Diabetes* 48:2349–2357, 1999

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$[Ca^{2+}]_i$, intracellular Ca^{2+}; CDS, clonidine-displacing substance; I_{KATP}, maximal K_{ATP} channel current; K_{ATP} channel, ATP-sensitive K^+ channel; K_D, concentration for half-maximal inhibition.

Ion channels play a key role in the control mechanisms that govern insulin release from pancreatic β-cells. Open ATP-sensitive K^+ (K_{ATP}) channels establish a resting cell membrane potential close to the equilibrium potential for K^+ ions, and their inhibition by glucose metabolism leads to *1*) a depolarization of the cell, causing a change in electrical activity, *2*) the generation of openings from voltage-gated Ca^{2+} channels, *3*) accelerated influx of Ca^{2+}, and *4*) the initiation of exocytosis of insulin-containing secretory granules (1).

The availability of insulin-secreting cell lines has greatly assisted studies of the functional cell biology and biochemistry of β-cells. Although several insulinoma cell lines are available, many lack stability over extended passage numbers or fail to generate adequate responses to glucose within the normal physiologic range. Although existing insulin-secreting cell lines appear to possess functional properties of native β-cells (2–7), each line has inherent deficiencies of insulin biosynthesis or secretion. In many cases, these abnormalities are due to the fact that these cells originate from insulinoma tissue or other genetically altered β-cells. Apart from providing a convenient source of readily available tissue, β-cell lines have also been used to support molecular biological initiatives to isolate and purify proteins relevant to β-cell stimulus-response coupling events and to develop strategies toward the engineering of transplantable β-cells for the treatment of diabetes.

In this context, the β-cell line BRIN-BD11 was generated as an electrofusion product of normal rodent β-cells and the insulinoma cell line RINm5F (8–11). Cellular electrofusion techniques are generally used in the production of hybridoma cells for monoclonal antibody production (13). BRIN-BD11 cells retain features of normal β-cells, in that they are glucose-responsive (8–11) and able to regulate insulin release when challenged with other nutrients and insulinotropic agents (7,10,12), and features of RINm5F cells, in that they are immortalized (8). Here, we describe the electrophysiologic properties of K_{ATP} channels in this novel β-cell line, providing a greater understanding of the ionic basis of insulin release in these cells. In addition, we provide insights into the mechanism of action of imidazolines on insulin-secreting cells.

Imidazoline compounds are insulin secretagogues that mediate their effects on β-cells through K_{ATP} channel–dependent and K_{ATP} channel–independent modes of action (14). In many cells, different imidazoline receptor subtypes mediate the effects of imidazolines. The current description of I-receptors is based solely on functional data using different imidazoline-receptor agonists (14). Two broad classes of I-receptors have been described, but in β-cells, the functional effects of compounds such as efaroxan, idazoxan, and phentolamine (15,16) do not readily relate to any known receptor subtype, leading to the suggestion that insulin-secreting cells possess a "novel" or I_3-receptor subtype (14). This difference adds to the complexity of defining whether the effects of imidazolines on β-cells are mediated through direct interactions with target proteins or receptor-mediated events. One important observation that can be used to elucidate the mechanism of action of imidazolines is that imidazoline receptors can be functionally downregulated by pre-exposure of cells to agonists (17). In β-cells, this procedure has been shown to selectively inhibit imidazoline-induced insulin secretion. We present empirical observations to suggest that imidazoline-induced modulation of K_{ATP} channels is functionally distinct from more distal signaling events.

RESEARCH DESIGN AND METHODS

Maintenance of BRIN-BD11 cells. Experiments were performed using the insulin-secreting cell-line BRIN-BD11. The production and basic characterization of these cells have been described (8). Cells were maintained in culture using RPMI-1640 medium supplemented with 10% (vol/vol) fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere of 5% CO_2 in air at 37°C. The cells were passaged weekly, and all experiments were carried out between passages 24 and 53.

Electrophysiology. All experiments were carried out by patch-clamp techniques (18) using the cell-attached patch and inside-out patch recording configurations (19). For single-channel current records, unless otherwise stated, experiments were carried out under quasi-physiologic cation gradients. Thus, for inside-out patch-clamp experiments, a KCl-rich solution was used to bathe the inner face of the membrane containing (in mmol/l) 140 KCl, 10 NaCl, 1.1 $MgCl_2$, 2.5 glucose, 10 HEPES, and 1 EGTA (pH 7.2 with KOH). The standard extracellular Na^+-rich solution used throughout these experiments contained (in mmol/l) 140 NaCl, 4.7 KCl, 1.1 $MgCl_2$, 2.5 $CaCl_2$, 2.5 glucose, and 10 HEPES (pH 7.4 with NaOH). During cell-attached patch recordings, unless otherwise stated, the NaCl-rich solution was added to the bath and the KCl-rich solution was used to fill the patch pipette. Before experiments, RPMI 1640 medium was removed and replaced with standard extracellular Na^+-rich solution. Data were recorded using a List L/M EPC-7 patch-clamp amplifier (List Electronic, Darmstadt, Germany). Single-channel currents were filtered at 1 kHz low pass, and data were obtained at 0 mV voltage-clamp or by applying a command potential to different voltages. Changes in K_{ATP} channel open-state probability were estimated off-line by analysis of 30-s stretches of continuous data using a CED 1401 interface and designated software (Cambridge Electronic Design, Cambridge, U.K.) (20). All values were then expressed as a fraction of the initial control value. Alternatively, the maximal K_{ATP} channel current (I_{KATP}) under each experimental condition was determined and expressed as a fraction of the initial current value upon inside-out patch formation (20,21). In this manner, we minimized the effects of channel run-down and patch-to-patch variations in the number of operational channels. All experiments were carried out at room temperature (18–22°C) unless otherwise stated.

Changes in the intracellular Ca^{2+} concentration. Microfluorimetry with fura-2 was used to monitor changes in cytosolic Ca^{2+} levels (22) in BRIN-BD11 cells using previously described procedures (23). Cells were loaded with 0.5 µmol/l fura-2-AM (Sigma, St. Louis, MO) in 1 ml RPMI 1640 medium for 8 min at 37°C. The composition of basic perifusion medium was (in mmol/l) 137 NaCl, 5.4 KCl, 0.8 $MgSO_4$, 0.3 Na_2HPO_4, 0.4 KH_2PO_4, 4.17 $NaHCO_3$, 10 HEPES, and 1.3 $CaCl_2$. This medium was gassed with air for 20 min, glucose was added at 2 mmol/l, and pH was set to 7.4 with NaOH. Under these experimental conditions, rises in intracellular Ca^{2+} ($[Ca^{2+}]_i$) are monitored by an increase in the fluorescence ratio at 340:380 nm. To quantify changes in $[Ca^{2+}]_i$, an in vitro calibration procedure (22) was used to estimate $[Ca^{2+}]_i$ levels.

Insulin secretion. BRIN-BD11 cells were harvested and seeded at a density of 1.25×10^5 cells in each well of 24-well plates. After culture and attachment overnight at 37°C, the cell monolayers were cultured in RPMI 1640 tissue culture medium with or without additions of efaroxan or phentolamine (as indicated in the table and the figure legends) for a further 20 h. The buffer used in the acute tests of insulin secretion contained (in mmol/l) 140 NaCl, 4.7 KCl, 1.1 $MgCl_2$, 2.0$CaCl_2$, and 10 HEPES (pH 7.4), supplemented with 0.1% (wt/vol) bovine serum albumin together with glucose and other additions as indicated. For measurement of insulin secretion, cells were preincubated in buffer with additions at 37°C for 40 min, after which the buffer was replaced with buffer supplemented with test agents as indicated. At the end of the 20-min incubation period, the buffer was removed from each well, and aliquots were stored at –20°C for subsequent insulin determination by radioimmunoassay as described (8).

Immunoblotting. Cell lysates for SDS-PAGE and Western blotting were prepared using a hypotonic lysis buffer (24). Samples normalized for protein concentration were prepared for electrophoresis as described (25) by adding 2× concentrated loading buffer (250 mmol/l Tris [pH 10.9], 2% 2-mercaptoethanol, 2% SDS, 20% glycerol, 0.01% bromophenol blue). Proteins were subsequently transferred to a nitrocellulose membrane and visualized using an anti-SUR1 antibody (provided by Prof. Susumu Seino, Chiba, Japan) at a concentration of 1:3,000 overnight at 4°C.

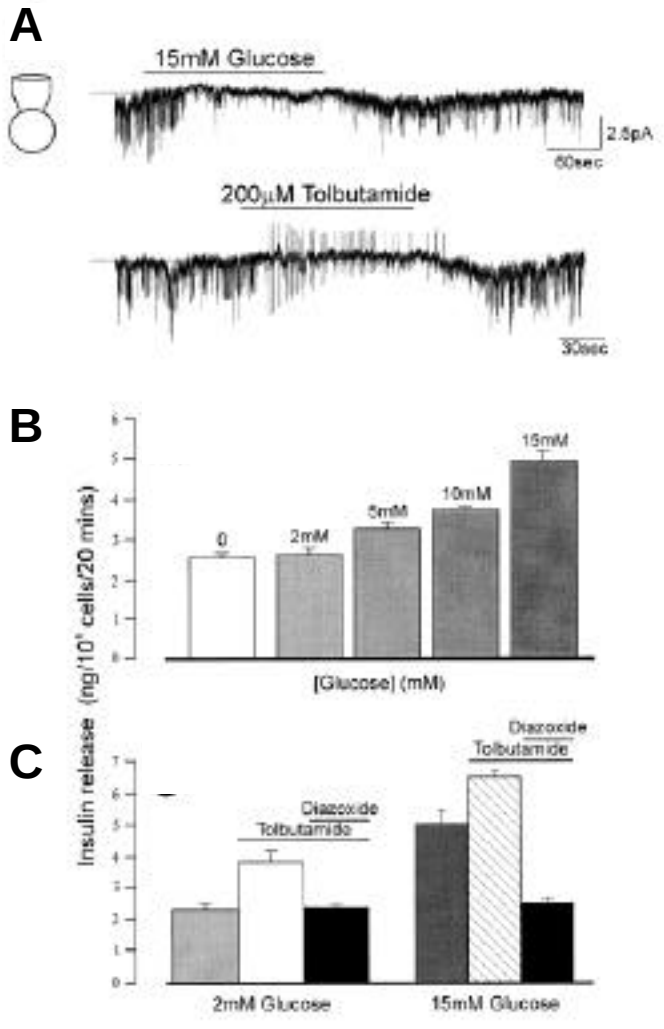

FIG. 1. Effects of glucose and tolbutamide on the ionic control of insulin release from BRIN-BD11 cells. *A*: Intact-cell recordings of K_{ATP} channels and their inhibition by glucose (15 mmol/l) and tolbutamide (200 µmol/l). The data come from separate experiments. Note in the lower figure how closure of channels is also associated with the generation of action potential currents. *B*: Effects of glucose over a range of concentrations (2–15 mmol/l) on insulin secretion (upper section) with a threshold at 5 mmol/l glucose ($P < 0.01$) and further significant increases at 10 and 15 mmol/l ($P < 0.001$). *C*: The lower section shows tolbutamide-induced insulin secretion ($P < 0.01$) at both 2 and 15 mmol/l glucose, which was abolished in each case by the presence of 200 µmol/l diazoxide. Data are means ± SE ($n = 6$).

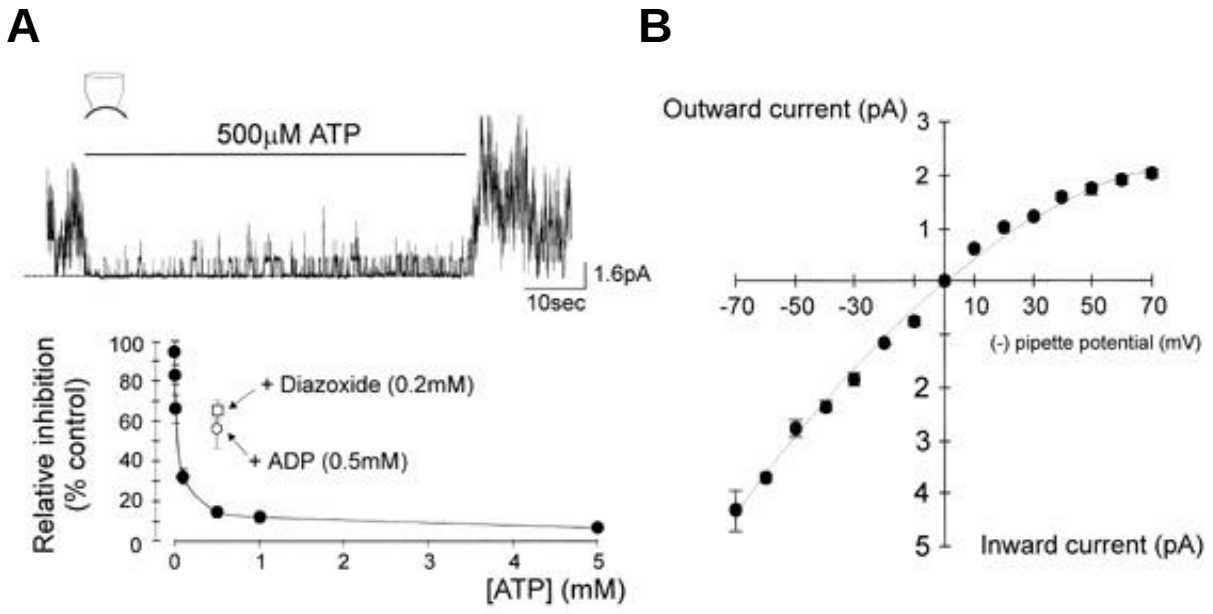

FIG. 2. Properties of K_{ATP} channels in BRIN-BD11 cells. All data were obtained using the inside-out patch configuration. *A*: The upper trace shows sustained block of channels by 500 μmol/l ATP and the refreshment of channels after removal of ATP. The lower panel illustrates the effects of ATP over a range of concentrations on K_{ATP} channel activity, n = 8. Also included are the relative effects of 500 μmol/l ADP (n = 4) and 200 μmol/l diazoxide (n = 4), which, in the presence of ATP, activate channels (see also Fig. 4). *B*: The current (I)-voltage (V) relationship profile for K_{ATP} channels in BRIN-BD11 cells. Average data were obtained from eight experiments in either the intact cell or inside-out patch recording configurations under symmetrical 140 mmol/l KCl-rich solutions. The I-V plot is nonlinear, with an inward current conductance estimated to be ~70 pS. All points are mean values ± SE.

Analysis of data. All data are expressed as means ± SE. Statistical comparisons were made using unpaired Student's t test, with values of $P < 0.05$ considered to be significant.

RESULTS

Effects of glucose and sulfonylureas. Figure 1*A* and *B* summarizes the action of glucose and the sulfonylurea tolbutamide on K_{ATP} channels in intact BRIN-BD11 cells. Cell-attached patch recordings typically exhibited regular openings from K_{ATP} channels, with an inward current conductance of ~70 pS under symmetrical 140 mmol/l KCl-rich solutions (Fig. 2*B*). These channels were inhibited by either the addition of glucose (15 mmol/l) to the bath solution at 37°C (n = 11/29 patches; no effects of glucose at 5 mmol/l [n = 9] or 10 mmol/l [n = 7]) (Fig. 1*A*) or exposure to 200 μmol/l tolbutamide (n = 12/14 patches) (Fig. 1*A*). A marked reduction of K^+ channel events was associated with the generation of action potentials, and both glucose (six experiments) and tolbutamide (six experiments) stimulated insulin release from BRIN-BD11 cells (Fig. 1*B*). The actions of glucose on insulin release were concentration-dependent, and tolbutamide was able to both initiate (2 mmol/l glucose) and potentiate glucose-induced insulin secretion from BRIN-BD11 cells (Fig. 1*C*). The effects of both glucose and tolbutamide on secretion were inhibited by the K_{ATP} channel opener diazoxide (200 μmol/l) (Fig. 1*C*; Table 1). Inside-out patch experiments also revealed that tolbutamide reversibly inhibited K_{ATP} channels (see below).

Properties of K_{ATP} channels. The properties of K_{ATP} channels in BRIN-BD11 cells were studied using the inside-out patch clamp configuration (Fig. 2). Under quasiphysiologic cationic gradients, these channels were spontaneously active upon patch formation (Fig. 2*A*), exhibited run-down with time (26), and were inhibited by ATP, which also caused a marked refreshment of K_{ATP} channel activity (Fig. 2*A*). K_{ATP} channels were recorded in 36/36 inside-out patch experiments, and the addition of ATP (1 μmol/l to 5 mmol/l) to the intracellular face of the membrane consistently inhibited channels in a concentration-dependent manner (Fig. 2*A*). The ATP concentration for half-maximal inhibition (K_D) of K_{ATP} channels (in the absence of ADP) was estimated to be 37 ± 0.66 μmol/l (n = 8), and the Hill coefficient for the effects of ATP was ~1 (0.74 ± 0.1, n = 8).

In the continued presence of ATP, both diazoxide (200 μmol/l, n = 15/15) (Fig. 2*A*) and ADP (0.5 mmol/l; n = 8/8) (Fig. 2*A*) caused the activation of K_{ATP} channels. As in native β-cells, the direct addition of 1 mmol/l ADP to the intracellular face of the membrane led to closure of K_{ATP} channels by reducing channel activity to 25.5 ± 10% of the control value (100%) (n = 6/6). Using intact cells and cell-free patch recordings, a current-voltage (I-V) relationship plot for these channels was generated under symmetrical 140 mmol/l KCl-rich cation gradients. From the slope of the inward current, we estimated the single-channel current conductance to be ~70 pS (n = 8) (Fig. 2*B*). Using both recording configurations, there was little difference in the I-V profile for K_{ATP} channels. These channels exhibited inward current rectification, with the outward current conductance of the channels markedly lower than the inward current conductance (Fig. 2*B*).

Effects of diazoxide and tolbutamide on BRIN-BD11 cells. In β-cells, diazoxide is a potent inhibitor of insulin secretion, since it acts as a hyperpolarizing agonist of K_{ATP} channels and prevents voltage-dependent Ca^{2+} influx (27). In BRIN-BD11 cells, diazoxide similarly acted as an agonist of K_{ATP} channels in both intact cells (n = 17/20 patches) (Fig. 3*A*) (see also Fig. 4*A*) and in cell-free patches of membrane (n = 15/15) (Fig. 2*A*). This action was associated with the inhibition of glucose-induced (n = 6) (Fig. 3*B*), sulfonylurea-

TABLE 1
Effects of diazoxide and pinacidil on the response of BRIN-BD11 cells to glucose, tolbutamide, efaroxan, or phentolamine

Addition (200 µmol/l)	Glucose (mmol/l)	Diazoxide (200 µmol/l)	Pinacidil (400 µmol/l)	Insulin release (ng · 10^6 cells^{-1} · 20 min^{-1})	Percent change in insulin release
None	2	–	–	2.33 ± 0.17	100
Tolbutamide	2	–	–	3.82 ± 0.35	164
Efaroxan	2	–	–	3.94 ± 0.39	169
Phentolamine	2	–	–	3.45 ± 0.16	148
None	2	+	–	1.64 ± 0.07*	100
Tolbutamide	2	+	–	2.34 ± 0.08*†	143
Efaroxan	2	+	–	2.24 ± 0.17*‡	137
Phentolamine	2	+	–	2.14 ± 0.17#§	130
None	2	–	+	2.82 ± 0.25	100
Tolbutamide	2	–	+	4.43 ± 0.18†	157
Efaroxan	2	–	+	4.83 ± 0.33†	171
Phentolamine	2	–	+	4.13 ± 0.06*†	146
None	15	–	–	5.02 ± 0.42‖	100
Tolbutamide	15	–	–	6.51 ± 0.18‖	130
Efaroxan	15	–	–	7.63 ± 0.80¶	152
Phentolamine	15	–	–	6.23 ± 0.21‖	124
None	15	+	–	1.59 ± 0.07#	100
Tolbutamide	15	+	–	2.22 ± 0.10#†	140
Efaroxan	15	+	–	2.59 ± 0.23#‡	163
Phentolamine	15	+	–	2.23 ± 0.21#§	140
None	15	–	+	3.20 ± 0.17*	100
Tolbutamide	15	–	+	5.14 ± 0.51**‡	161
Efaroxan	15	–	+	5.24 ± 0.26**†	164
Phentolamine	15	–	+	5.45 ± 0.13**†‖	170

Data for insulin release are means ± SE (n = 6). After a 40-min preincubation, the effects of 200 µmol/l tolbutamide, 200 µmol/l efaroxan, or 200 µmol/l phentolamine were tested during a 20-min incubation period in the continued absence (–) or presence (+) of 200 µmol/l diazoxide or 400 µmol/l pinacidil. Percent change in insulin release is expressed relative to corresponding values in the absence of stimuli (100%, none). *P < 0.01, #P < 0.001, **P < 0.05 compared with the corresponding effects in the absence of diazoxide or pinacidil; †P < 0.001, ‡P < 0.01, §P < 0.05 compared with the effects of either diazoxide or pinacidil alone at the same glucose concentration; ‖P < 0.001, ¶P < 0.01 compared with the corresponding effect at 2 mmol/l glucose.

induced (n = 6) (Fig. 1*C*), and imidazoline-induced insulin release (Table 1). The structurally unrelated channel agonist pinacidil (400 µmol/l) also activated K_{ATP} channels (n = 6/6) (Fig. 4*C*) and inhibited insulin release from BRIN-BD11 cells at elevated glucose concentrations (Table 1).

Effects of imidazoline insulin secretagogues. The imidazoline-based compounds efaroxan and phentolamine are recognized as inhibitors of K_{ATP} channels in β-cells (15,28) and act as potent insulin secretagogues (16). The pharmacologic modulation of BRIN-BD11 cell function by these compounds

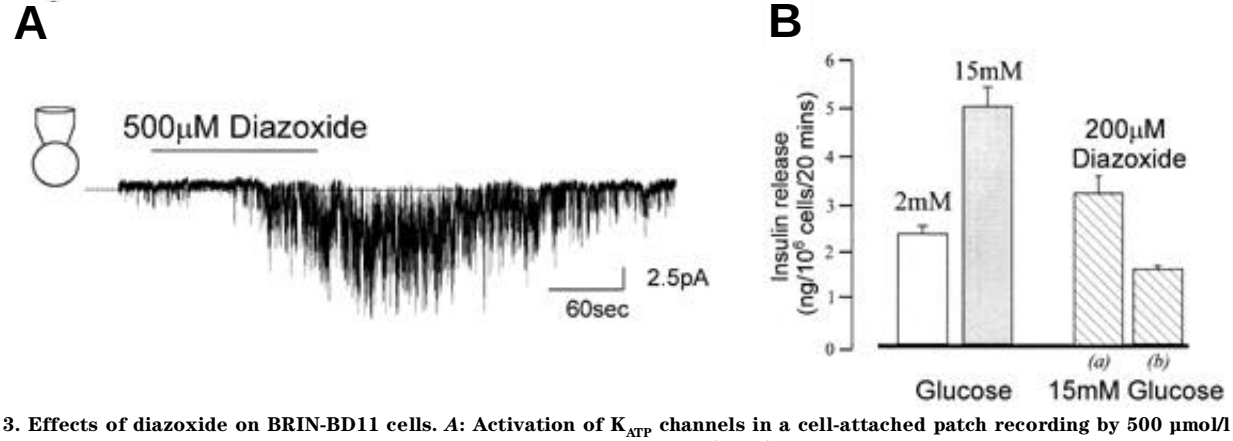

FIG. 3. Effects of diazoxide on BRIN-BD11 cells. *A*: Activation of K_{ATP} channels in a cell-attached patch recording by 500 µmol/l diazoxide. *B*: A summary of the effect of diazoxide on glucose-induced insulin secretion (n = 6). As shown in *B*, the secretory responses to 15 mmol/l glucose (P < 0.001 compared with 2 mmol/l) were significantly inhibited by exposure to diazoxide immediately before the 20-min test incubation (P < 0.01) (a) and continuous exposure to diazoxide during both the 40-min preincubation and the 20-min test incubation (P < 0.001) (b).

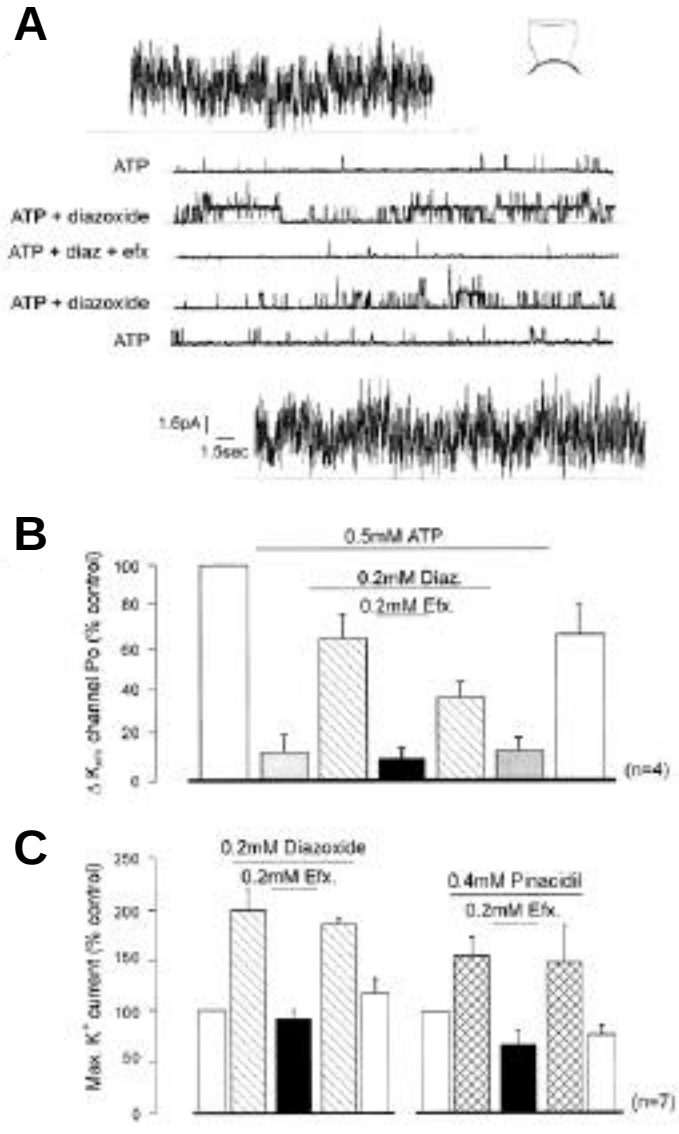

FIG. 4. Actions of diazoxide and efaroxan on K_{ATP} channels in BRIN-BD11 cells. *A*: A typical experiment using the inside-out patch configuration. The records show block of K_{ATP} channels by ATP (500 µmol/l) followed by activation of channels by diazoxide (200 µmol/l). Diazoxide-activated K_{ATP} channels were subsequently inhibited in a reversible manner by 200 µmol/l efaroxan. Open-state probability (Po) values for this experiment: control, 0.48; ATP, 0.002; ATP + diazoxide, 0.08; ATP + diazoxide + efaroxan, 0.0004; ATP + diazoxide, 0.033; ATP, 0.003; and control, 0.47. *B*: Summary data from four similar experiments to examine the effects of diazoxide and efaroxan on K_{ATP} channels in inside-out patches. *C*: A summary of the effects of efaroxan on diazoxide- or pinacidil-induced activation of K_{ATP} channels in intact cell recordings, n = 7 experiments.

was therefore investigated. As in normal β-cells, efaroxan (200 µmol/l) directly inhibited K_{ATP} channels in a reversible manner to an average of 9.9 ± 5.4% (n = 4) of the control values (n = 5/5) (Fig. 5*A*) and promoted insulin secretion (n = 6) (Fig. 5*B*). Efaroxan inhibited both diazoxide- and pinacidil-activated K_{ATP} channels in either intact cells or inside-out patches (diazoxide, n = 13/13; pinacidil, n = 6/7) (Fig. 4) and also partly reversed the inhibition of insulin secretion induced by diazoxide in the presence of 15 mmol/l glucose (Table 1). In a similar manner, K_{ATP} channels were inhibited by phentolamine to 9 ± 4% (n = 4) of the control value (n = 8/8 experiments), and phentolamine was able to both initiate insulin release (Fig. 6*A*) and reverse the actions of diazoxide on either glucose- or tolbutamide-induced insulin secretion (Table 1). Finally, consistent with the modulation of K_{ATP} channels, pinacidil also reversed the effects of tolbutamide or the imidazolines efaroxan and phentolamine on insulin release (Table 1).

Recently, it has been shown in separate studies that long-term pre-exposure of isolated islets of Langerhans to imidazoline receptor agonists leads to the selective downregulation of imidazoline-mediated insulin secretion (17), whereas imidazoline-induced changes in $[Ca^{2+}]_i$ are unaffected (23). We found similar effects in BRIN-BD11 cells. Pre-exposure (20 h) to either efaroxan or phentolamine selectively prevented imidazoline-induced insulin secretion (Fig. 6*A*) but not the responses of cells to glucose (15 mmol/l), tolbutamide (200 µmol/l), or depolarizing concentrations of KCl (25 mmol/l) (Fig. 6*B*).

The effects of efaroxan pre-exposure were examined in more detail. In addition to preventing efaroxan-induced insulin secretion, efaroxan failed to potentiate 15 mmol/l glucose-induced secretion under these conditions (insulin release 182 ± 5% [control, n = 6] vs. 113 ± 12% [pre-exposed, n = 6]; values normalized to 15 mmol/l glucose alone, 100%). It was also notable that 200 µmol/l efaroxan was unable to significantly alter the insulin secretion activity of 25 mmol/l KCl or 200 µmol/l tolbutamide after efaroxan pre-exposure (data not shown, n = 6). Figure 7 shows that under conditions known to prevent imidazoline-induced insulin secretion, both efaroxan (n = 13) and KCl (n = 11) caused a marked increase in $[Ca^{2+}]_i$. Pretreatment of BRIN-BD11 cells with efaroxan produced a modest decrease in basal levels of $[Ca^{2+}]_i$ (control value 101 ± 13 nmol/l [n = 17] vs. 78 ± 6 nmol/l [n = 15] in treated cells).

In parallel experiments, pre-exposure of BRIN-BD11 cells for 20 h to 50 µmol/l efaroxan did not lead to loss of K_{ATP} channel numbers or function. (Assessment of K_{ATP} channel density was undertaken by recording the immediate increase in I_{KATP} upon the formation of an inside-out patch from intact cells under quasiphysiologic cationic conditions [18,29].) K_{ATP} channels were found to be operational in intact cells (n = 6) and cell-free patches (n = 17), and there was no impairment of the responses to intracellular nucleotides (n = 14) or imidazolines (n = 7). Thus, in cells pretreated with 50 µmol/l efaroxan, we typically found that the peak K_{ATP} current recorded across inside-out patches (Fig. 8*A*) was similar to controls (26.4 ± 4.6 [n = 7] vs. 26.7 ± 4.2 pA [control; n = 5]). ATP (500 µmol/l) typically reduced the K_{ATP} current in these cells from 22 ± 8 to 3.6 ± 0.8 pA (n = 3) vs. 22 ± 6.8 to 2.2 ± 0.7 pA for controls (n = 5), and efaroxan (200 µmol/l) was also found to reduce K_{ATP} channel activity of pretreated cells to 8.7 ± 5.6% (n = 4) of the control value.

Using Western immunoblotting with a specific SUR1 receptor antibody, we were able to study the effects of efaroxan preincubation on SUR1 protein expression. Compared with untreated cells, 50 µmol/l efaroxan pretreatment led to an increase in SUR1 protein abundance, and similar effects were found at 100 and 200 µmol/l efaroxan (Fig. 8*B*). This increase occurred despite no functional increase in K_{ATP} channel density (Fig. 8*A*). Surprisingly, when the concentration of efaroxan used to preincubate cells was further elevated

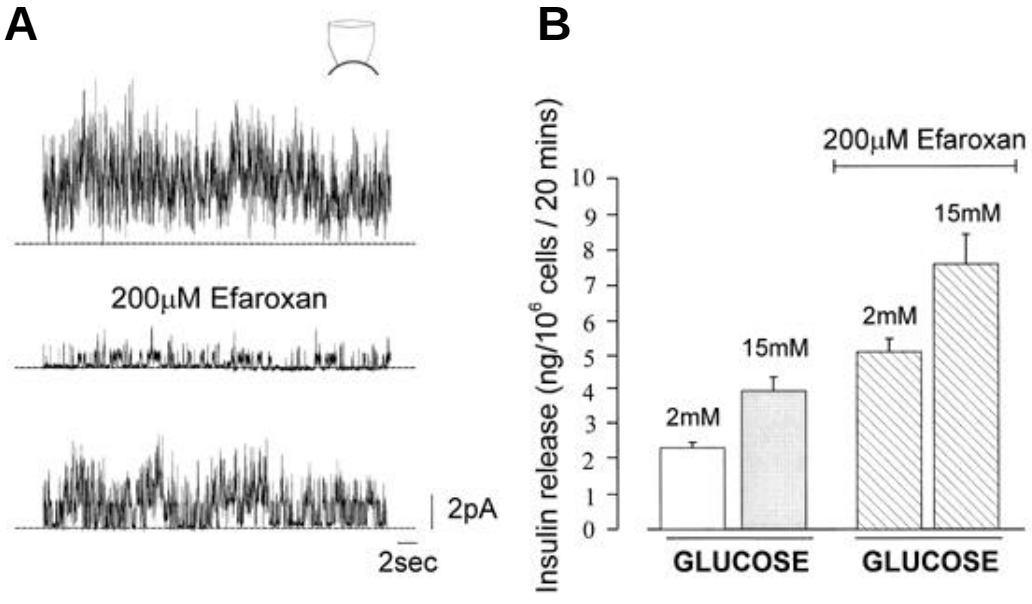

FIG. 5. Imidazoline-induced closure of K_{ATP} channels and insulin secretion. The data shown in *A* come from the same inside-out patch and illustrate reversible block of K_{ATP} channels by 200 μmol/l efaroxan. Open-state probability (Po) values: control, 0.47; efaroxan, 0.037; and control, 0.16. *B*: A summary of the stimulatory actions of 200 μmol/l efaroxan on insulin secretion at 2 mmol/l ($P < 0.01$) and 15 mmol/l ($P < 0.05$) glucose, $n = 6$ in both cases.

to 500 μmol/l, there was a marked increase in the expression of SUR1 protein (Fig. 8*B*) and, as a consequence, an increase in the number of K_{ATP} channels in BRIN-BD11 cells (Fig. 8*A*). Under these conditions, the average peak K_{ATP} channel current was increased from an average of 28 ± 4 ($n = 20$) to 94 ± 39 pA ($n = 7$).

DISCUSSION

In this study, we have provided the first characterization of K_{ATP} channels and their role in the ionic regulation of insulin secretion from the electrofusion-derived BRIN-BD11 cells. We have shown that glucose-, sulfonylurea-, and imidazoline-induced secretory events are associated with the modulation of K_{ATP} channels in a manner similar to that documented in normal β-cells. The actions of glucose occur in association with K_{ATP} channel inhibition (Fig. 1). Because these channels are inhibited by intracellular ATP and activated in the presence of ATP by ADP (Fig. 2), it seems likely that, as in native β-cells, glucose metabolism elevates the intracellular ATP:ADP ratio and initiates K_{ATP} channel closure, the activation of voltage-dependent Ca^{2+} influx (30), and insulin release. In contrast, the actions of sulfonylureas and imidazolines are more direct and bring about the inhibition of K_{ATP} channels in the absence of cytosolic nucleotides (Fig. 5).

The biophysical and regulatory properties of K_{ATP} channels in BRIN-BD11 cells are similar to those reported in native

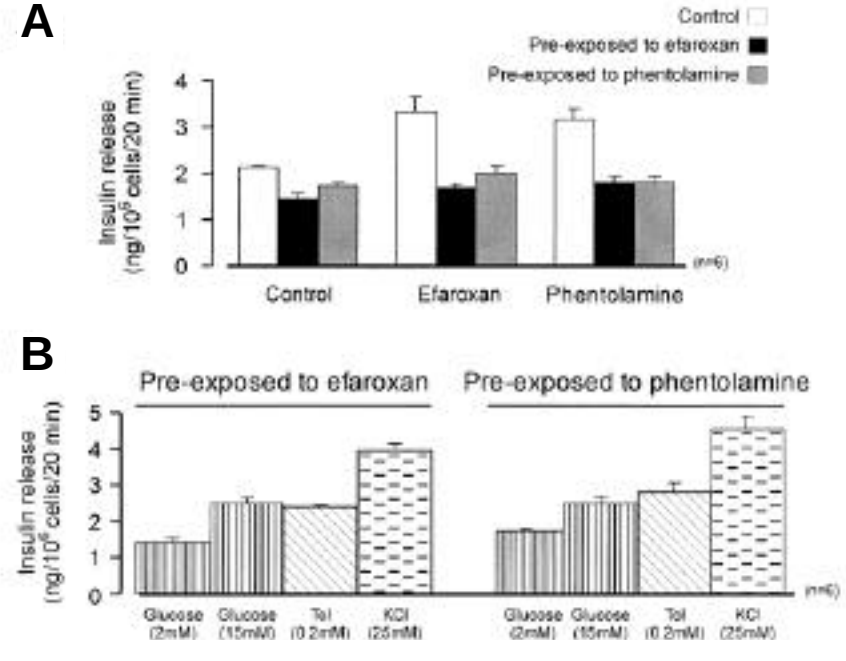

FIG. 6. Effects of pre-exposure of BRIN-BD11 cells to imidazolines and their responses to a variety of insulin secretagogues. BRIN-BD11 cells were cultured for 20 h in standard RPMI-1640 tissue culture medium or medium supplemented with 50 μmol/l of either efaroxan or phentolamine (*A*). After a 40-min preincubation, effects of efaroxan or phentolamine were tested during a 20-min incubation period ($n = 6$) in each case. In the presence of 2 mmol/l glucose, efaroxan and phentolamine evoked significant ($P < 0.01$) secretory responses after culture in standard culture medium (control). Culture of BRIN-BD11 cells in the presence of either imidazoline effectively removed the ability of each of the agents to evoke insulin-secretory responses at 2 mmol/l glucose. As illustrated in *B*, however, after 20-h exposure to either imidazoline, BRIN-BD11 cells still showed demonstrable responses ($P < 0.001$) to 15 mmol/l glucose, 200 μmol/l tolbutamide, or 25 mmol/l KCl over those observed with 2 mmol/l glucose, $n = 6$ in each case.

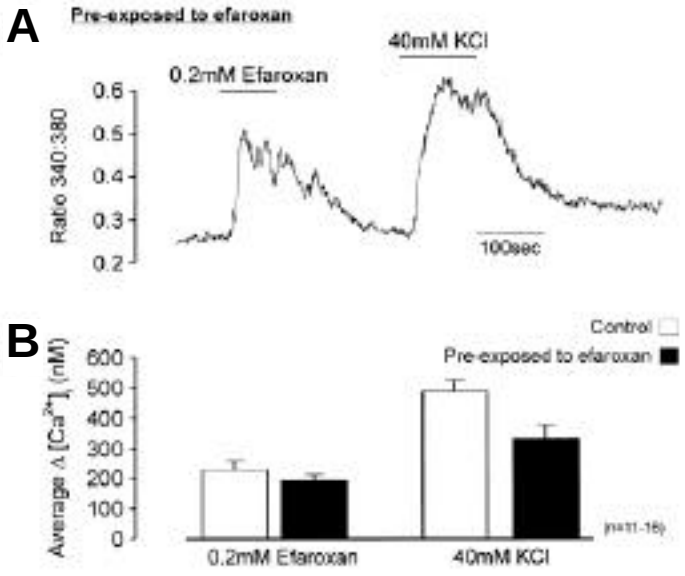

FIG. 7. Effects of pre-exposure of BRIN-BD11 cells to efaroxan and modulation of depolarization-dependent Ca^{2+} influx. *A*: A typical experiment in which both efaroxan and KCl induce a marked increase in $[Ca^{2+}]_i$ in BRIN-BD11 cells pre-exposed to efaroxan for 24 h. Changes in $[Ca^{2+}]_i$ are represented by an increase in the ratio of 340:380 nmol/l in an individual cell loaded with fura-2. *B*: A summary of the effects of efaroxan and KCl on control cells (efaroxan, *n* = 16; KCl, *n* = 11) and downregulated cells (efaroxan, *n* = 13; KCl, *n* = 11).

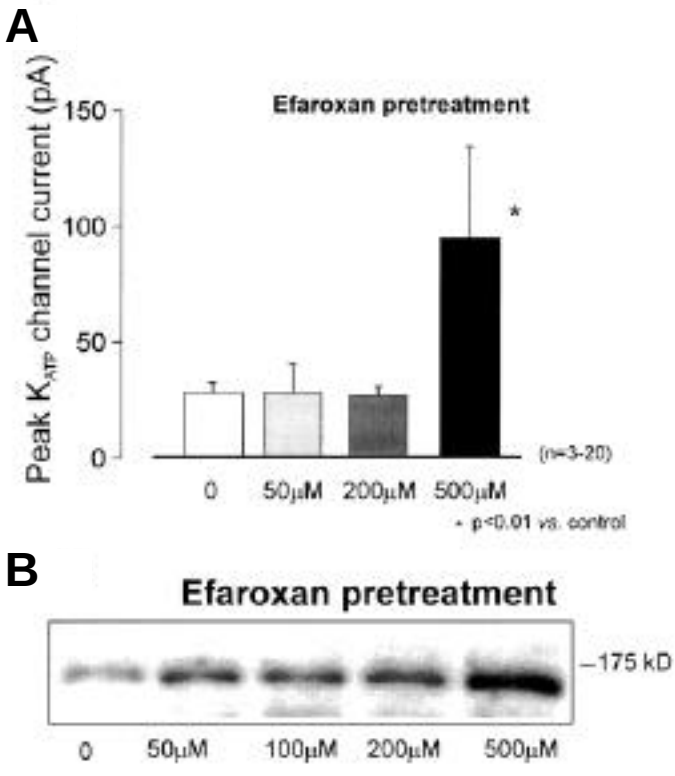

FIG. 8. K_{ATP} channels and SUR1 protein expression in BRIN-BD11 cells pre-exposed to efaroxan. All data were obtained from parallel studies carried out on the same batches of cells exposed for 24 h to efaroxan over a range of different concentrations (50 μmol/l to 500 μmol/l). *A*: Peak K_{ATP} current density was measured by isolating inside-out patches from intact cells under quasiphysiologic cation gradients. Overall, there was no significant increase in channel density between 50 and 200 μmol/l efaroxan, but exposure to 500 μmol/l led to a marked increase in channel numbers. *B*: A typical experiment (*n* = 4) to assess the effects of imidazoline receptor desensitization on SUR1 protein abundance. Note that compared with control levels of expression, there was a marked increase in SUR1 protein at all concentrations tested, particularly at 500 μmol/l efaroxan.

insulin-secreting cells (31,32). The channels *1*) have a nonuniform current-voltage relationship profile, *2*) exhibit inward current rectification, *3*) undergo spontaneous rundown in isolated patches that is reversed by intracellular action of ATP, *4*) have a half-maximal inhibition by ATP of approximately 37 μmol/l (Fig. 2), and *5*) are modulated by both intracellular ADP and ATP. From recombination studies, the molecular architecture of these channels has recently been described as a heteromultimeric complex of the ATP-binding cassette (ABC) protein, SUR1, and the weak inward rectifier K^+ channel, Kir6.2 (33,34). The channel appears to be formed from an obligatory octameric complex consisting of four Kir6.2 subunits and four SUR1 subunits in a 1:1 stoichiometry $(Kir6.2/SUR1)_4$. Kir6.2 gates the ionic conductance of K^+ and bestows biophysical properties on the complex. In addition, Kir6.2 appears to control the ATP-sensitivity of the channel complex (35), while SUR1 endows both diazoxide- and sulfonylurea-sensitivity on Kir6.2 as well as the control of the ADP-induced modulation of the complex (36–38).

In native insulin-secreting cells, K_{ATP} channels are activated by the agonists diazoxide and pinacidil (27,32), and similar effects were also found in BRIN-BD11 cells (Figs. 2–4), with diazoxide acting as a more potent agonist of these channels than pinacidil (Fig. 4) (39). Both pinacidil and diazoxide inhibit insulin secretion induced by glucose, sulfonylurea, or imidazolines in BRIN-BD11 cells (Figs. 1 and 3; Table 1), and consistent with their relative actions on K_{ATP} channels, pinacidil was only effective at elevated glucose concentrations. The data also show the relative effects of diazoxide and pinacidil on the K_{ATP} channel-independent pathway of regulated secretion (40). In the presence of tolbutamide, efaroxan, or phentolamine, K_{ATP} channels will be tonically inhibited, and potentiation of insulin secretion by glucose occurs via the augmentation pathways. Under these conditions, diazoxide inhibited insulin release to near basal values, whereas pinacidil had only a modest effect (Table 1).

The ionic basis for the actions of imidazolines on BRIN-BD11 cells has also been characterized. These agents are thought to represent a novel class of insulin secretagogues that act on a discrete biological receptor, the imidazoline receptor (14). Several reports have also posited the existence of an endogenous ligand for these receptors that is thought to be a component of clonidine-displacing substance (CDS) (41). CDS, like efaroxan and phentolamine, is a potent β-cell agonist (41). The mode of action of imidazoline receptor agonists occurs through inhibition of K_{ATP} channels by an interaction with the Kir6.2 subunit rather than SUR1 and a depolarization of the cell membrane potential, leading to an elevation of the cytosolic Ca^{2+} concentration and initiation of Ca^{2+}-dependent exocytosis (15,16,23,42). In addition, some data suggest that the imidazoline compound RX871024 augments Ca^{2+} and distal signaling events from β-cells (43,44). Here, we have shown that efaroxan and phentolamine inhibit K_{ATP} channels and that the diazoxide- and pinacidil-induced activation of these channels can be reversed by efaroxan (Figs. 4 and 5). These effects provide an explanation for our

observations that imidazolines promote insulin release in both the absence and presence of glucose and how these compounds reverse the inhibition of glucose-induced secretion by diazoxide and pinacidil (Fig. 6; Table 1).

We have also shown that effects of imidazolines on insulin release can be downregulated by pre-exposure of BRIN-BD11 cells to either efaroxan or phentolamine. As in previous studies, these actions occurred without any significant modulation of the secretory responses of BRIN-BD11 cells to glucose, tolbutamide, or KCl (Fig. 6) or depolarization-induced rises in $[Ca^{2+}]_i$ (Fig. 7). We now show that these conditions do not prevent K_{ATP} channels from operating, nor do they lead to any significant loss of channel sensitivity to either imidazolines or ATP. That K_{ATP} channels operate in BRIN-BD11 cells after preexposure to imidazolines provides a new insight into the mechanisms of action of imidazolines on β-cells—namely, that the loss of imidazoline-induced secretion under these conditions is not attributed to K_{ATP} channel function. This finding is consistent with data showing that, under the same conditions, *1*) glucose and sulfonylureas act as insulin secretagogues (Fig. 6) (17) and *2*) glucose-, sulfonylurea-, and imidazoline-induced cytosolic Ca^{2+} signaling events are operational (23). Thus, preincubation conditions result in the selective loss of a key component of imidazoline-mediated insulin release that is independent of $[Ca^{2+}]_i$ signaling and K_{ATP} channel function. The identity of this component, however, is not immediately obvious, since tolbutamide, glucose, and KCl all still effectively operate under the same conditions. One implication of these findings is that the imidazoline receptor is more intimately associated with the events of exocytosis than previously thought. Indeed, modulation at this site appears to be obligatory for the actions of efaroxan but not other insulin secretagogues. Alternatively, these findings may suggest that efaroxan pretreatment results in the upregulation of an imidazoline receptor associated with the inhibition of insulin release. However, our experiments to test whether efaroxan can inhibit secretion stimulated by glucose, tolbutamide, and KCl in pre-exposed cells do not support such a possibility. In addition, ongoing studies indicate that similar pre-exposure to tolbutamide in culture abolishes sulfonylurea-induced insulin secretion without affecting the insulinotropic actions of efaroxan (N.H.M., A.J. Ball, P.R.F., unpublished observations).

As an additional complexity, we also show that the effects of pre-exposure to efaroxan are associated with an increase in SUR1 protein abundance and that this increase can lead to an increase in the number of functional K_{ATP} channels (Fig. 8). An increase in K_{ATP} channel numbers might have accounted for the loss of imidazoline-induced secretion (Fig. 6), but this possibility is not supported by the fact that sulfonylureas and glucose effectively promote insulin release under these conditions. The data do, however, suggest the possibility that there is a functional negative correlation between a putative I-receptor in β-cells and SUR1, although further studies are needed to clarify this relationship in more detail.

In summary, we have documented the properties of K_{ATP} channels in the control of insulin release from BRIN-BD11 cells. These cells conserve many of the electrophysiologic, pharmacologic, and regulatory properties of native β-cells and therefore present a valuable model cell for future studies of the actions of imidazolines and stimulus-response coupling of insulin secretion. These observations, together with secretory and metabolic characteristics of the cell line (8–11), indicate that electrofusion represents a powerful tool for engineering novel β-cell lines for potential gene therapy of diabetes.

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REFERENCES

1. Dunne MJ, Aynsley-Green A, Lindley KJ: Nature's K_{ATP} channel knock out. *News Physiol Sci* 12:197–203, 1997
2. Gazdar AF, Chick WL, Oie HK, Sims HL, King DL, Kitchen KC: Continuous, clonal, insulin- and somatostatin-secreting cell lines established from a transplantable rat islet-cell tumor. *Proc Natl Acad Sci U S A* 77:3519–3523, 1980
3. Santerre RF, Cook RA, Crisel RMD, Shar JD, Schmidt RJ, Williams DC, Wilson CP: Insulin synthesis in a clonal cell line of simian virus 40-transformed hamster pancreatic beta-cells. *Proc Natl Acad Sci U S A* 78:4339–4343, 1981
4. Efrat S, Linde S, Koford H, Spector D, Delannoy M, Grant S, Hanahan D, Baekkeskov S: B-cell line derived from transgenic mice expressing a hybrid insulin gene. *Proc Natl Acad Sci U S A* 85:9037–9041, 1988
5. Miyazaki JI, Araki K, Yamato E, Ikegama H, Asano T, Shibasaki Y, Oka Y, Yamamura KI: Establishment of a pancreatic B-cell line that retains glucose-inducible insulin secretion: special reference to expression of glucose transporter isoforms. *Endocrinology* 127:126–132, 1990
6. Asfari M, Janjic D, Meda P, Li G, Halban PA, Wollheim CB: Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. *Endocrinology* 130:167–178, 1992
7. McClenaghan NH, Flatt PR: Engineering cultured insulin-secreting pancreatic B-cell lines. *J Mol Med* 77:235–243, 1999
8. McClenaghan NH, Barnett CR, Ah-Sing E, Abdel-Wahab YH, O'Harte FP, Yoon TW, Swanston-Flatt SK, Flatt PR: Characterization of a novel glucose-responsive insulin-secreting cell line, BRIN-BD11, produced by electrofusion. *Diabetes* 45:1132–1140, 1996
9. McClenaghan NH, Gray AM, Barnett CR, Flatt PR: Hexose recognition by insulin-secreting BRIN-BD11 cells. *Biochem Biophys Res Commun* 223:724–728, 1996
10. McClenaghan NH, Barnett CR, O'Harte FP, Flatt PR: Mechanisms of amino acid-induced insulin secretion from the glucose-responsive BRIN-BD11 pancreatic B-cell line. *J Endocrinol* 151:49–57, 1996
11. Rasschaert J, Flatt PR, Barnett CR, McClenaghan NH, Malaisse WJ: D-glucose metabolism in BRIN-BD11 islet cells. *Biochem Mol Med* 57:97–105, 1996
12. McClenaghan NH, Flatt PR: Glucose and non-glucidic nutrients exert permissive effects on 2-keto acid regulation of pancreatic B-cell function. *Biochim Biophys Acta* 1426:110–118, 1999
13. Lo MMS, Tsong TYT, Conrad MK, Strittmatter SM, Hester LD, Snyder SH: Monoclonal antibody production by receptor-mediated electrically induced cell fusion. *Nature* 310:792–794, 1984
14. Eglen RM, Hudson AL, Kendall DA, Nutt DJ, Morgan NG, Wilson VG, Dillon MP: 'Seeing through a glass darkly': casting light on imidazoline 'I' sites. *Trends Pharmacol Sci* 19:381-390, 1998
15. Dunne MJ, Harding EA, Jaggar JH, Squires PE, Liang R, Kane C, London NJM, James RLF: Potassium channels, imidazolines and insulin-secreting cells. *Ann N Y Acad Sci* 763:243–262, 1995
16. Morgan NG, Chan SL, Brown CA, Tsoli E: Characterization of the imidazoline binding site involved in regulation of insulin secretion. *Ann N Y Acad Sci* 763:361–373, 1995.
17. Chan SL, Brown CA, Morgan NG: Stimulation of insulin secretion by the imidazoline alpha 2-adrenoceptor antagonist efaroxan is mediated by a novel, stereoselective, binding site. *Eur J Pharmacol* 230:375–378, 1993
18. Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ: Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch* 391:85–100, 1981
19. Kane C, Shepherd RM, Squires PE, Johnson PRV, James RFL, Milla PJ, Aynsley-Green A, Lindley KJ, Dunne MJ: Loss of functional K_{ATP} channels in β-cells causes persistent hyperinsulinaemic hypoglycaemia of infancy. *Nature*

Med 2:1344–1347, 1996
20. Dunne MJ, Findlay I, Petersen OH: The effects of pyridine nucleotides on the gating of ATP-sensitive K^+ channels in insulin-secreting cells. *J Membrane Biol* 102:205–216, 1988
21. Lebrun P, Antoine M-H, Ouedraogo R, Dunne MJ, Kane C, Hermann M, Herchuelz A, Masereel B, Delarge J, de Tullio P, Pirotte, B: Activation of ATP-dependent K^+ channels and inhibition of insulin release: effect of BPDZ-62. *J Pharmacol Exp Ther* 277:156–162, 1996
22. Grynkirwicz G, Poenie M, Tsien RY: A new generation of Ca^{2+} indicators with greatly improved properties. *J Biol Chem* 260:3440–3450, 1985
23. Shepherd RM, Hashmi MN, Kane C, Squires PE, Dunne MJ: Elevation of cytosolic calcium by imidazolines in mouse islets of Langerhans: implications for stimulus-response coupling of insulin release. *Br J Pharmacol* 119:911–916, 1996
24. Walker J, Watson J, Holmes C, Edelman A, Banting G: Production and characterisation of monoclonal and polyclonal antibodies to different regions of the cystic fibrosis transmembrane conductance regulator (CFTR): detection of immunologically related proteins. *J Cell Sci* 108:2433–2444, 1995
25. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685, 1970
26. Findlay I, Dunne MJ: ATP maintains ATP-inhibited K^+ channels in an operational state. *Pflugers Arch* 407:238–240, 1986
27. Dunne MJ, Harding EA, Jaggar JH, Ayton BJ, Squires PE: Endogenous and chemical activators of ATP-regulated potassium channels in insulin secreting cells: possible mechanisms and physiological significance. In *Frontiers of Insulin Secretion and Pancreatic B-Cell Research*. Flatt PR, Lenzen S, Eds. London, Smith-Gordon Press, 1995, p. 153–159
28. Dunne MJ: Block of ATP-regulated potassium channels by phentolamine and α-adrenoceptor antagonists. *Br J Pharmacol* 103:1847–1850, 1991
29. Dunne MJ, Findlay I, Petersen OH, Wollheim CB: ATP-sensitive K^+ channels in an insulin-secreting cell-line are inhibited by D-glyceraldehyde and activated by membrane permeabilization. *J Membr Biol* 93:271–279, 1986
30. Salgado AP, Santos RM, Tome AR, Fernandes AP, Flatt PR, Rosario LM: Glucose-mediated Ca^{2+} signalling in the novel insulin-secreting cell-line BRIN-BD11 (Abstract). *Diabetologia* 39 (Suppl. 1):A117, 1996
31. Ashcroft FM, Proks P, Smith PA, Ämmälä C, Bokvist K, Rorsman P: Stimulus-secretion coupling in pancreatic beta cells. *J Cell Biochem* 55 (Suppl.):54–65, 1994
32. Dunne MJ, Petersen OH: Potassium selective ion channels in insulin-secreting cells: physiology, pharmacology and their role in stimulus secretion coupling. *Biochim Biophys Acta* 1071:67–82, 1991
33. Inagaki N, Gonoi T, Clement JP 4th, Namba N, Inazawa J, Gonzalez G, Aguilar-Bryan L, Seino S, Bryan J: Reconstitution of IKATP: an inward rectifier subunit plus the sulphonylurea receptor. *Science* 270:1166–1170, 1995
34. Sakura H, Ämmälä C, Smith PA, Gribble FM, Ashcroft FM: Cloning and functional expression of the cDNA encoding a novel ATP-sensitive potassium channel expressed in pancreatic B-cells, brain, heart and skeletal muscle. *FEBS Lett* 377:338–344, 1995
35. Tucker SJ, Gribble FM, Zhao C, Trapp S, Ashcroft FM: Truncation of Kir6.2 produces ATP-sensitive K^+ channels in the absence of the sulphonylurea receptor. *Nature* 387:179–183, 1997
36. Ämmälä C, Moorhouse A, Gribble F, Ashfield R, Proks P, Smith PA, Sakura H, Coles B, Ashcroft SJH, Ashcroft FM: Promiscuous coupling between the sulphonylurea receptor and inwardly rectifying potassium channels. *Nature* 379:545–548, 1996
37. Nichols CG, Shyng SL, Nestorowicz A, Glaser B, Clement JP 4th, Gonzalez G, Aguilar-Bryan L, Permutt MA, Bryan J: Adenosine diphosphate as an intracellular regulator of insulin secretion. *Science* 272:1785–1787, 1996
38. Ashcroft FM, Gribble FM: Correlating structure and function in ATP-sensitive K^+ channels. *Trends Neurosci* 21:288–294, 1998
39. Dunne MJ: Effects of pinacidil, RP 49356 and nicorandil on ATP-sensitive potassium channels in insulin-secreting cells. *Br J Pharmacol* 99:487–492, 1990
40. Aizawa T, Komatsu M, Asanuma N, Sato Y, Sharp GWG: Glucose action 'beyond ionic events' in the pancreatic beta cell. *Trends Pharm Sci* 19:496-499, 1998
41. Chan SLF: Clonidine displacing substance and its putative role in control of insulin release. *Gen Pharmacol* 31:525–529, 1998
42. Proks P, Ashcroft FM: Phentolamine block of K_{ATP} channels is mediated by Kir 6.2. *Proc Natl Acad Sci U S A* 94:11716–11720, 1998
43. Zaitsev SV, Efanov AM, Efanova IB, Larsson O, Ostenson CG, Gold G, Berggren PO, Efendic S: Imidazoline compounds stimulate insulin release by inhibition of K_{ATP} channels and interaction with the exocytotic machinery. *Diabetes* 45:1610–1618, 1996
44. Efanova IB, Zaitsev SV, Brown G, Berggren P-O, Efendic S: RX871024 induced Ca^{2+} mobilisation from thapsigargin-sensitive stores in mouse pancreatic β-cells. *Diabetes* 47:211–218, 1998