

# ATP-Sensitive Potassium Channels and Efaroxan-Induced Insulin Release in the Electrofusion-Derived BRIN-BD11 $\beta$ -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;  $I_{K_{ATP}}$ , maximal  $K_{ATP}$  channel current;  $K_{ATP}$ , channel, ATP-sensitive  $K^+$  channel;  $K_D$ , concentration for half-maximal inhibition.

**I**on channels play a key role in the control mechanisms that govern insulin release from pancreatic  $\beta$ -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  $\beta$ -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  $\beta$ -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  $\beta$ -cells. Apart from providing a convenient source of readily available tissue,  $\beta$ -cell lines have also been used to support molecular biological initiatives to isolate and purify proteins relevant to  $\beta$ -cell stimulus-response coupling events and to develop strategies toward the engineering of transplantable  $\beta$ -cells for the treatment of diabetes.

In this context, the  $\beta$ -cell line BRIN-BD11 was generated as an electrofusion product of normal rodent  $\beta$ -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  $\beta$ -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  $\beta$ -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  $\beta$ -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  $\beta$ -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  $\beta$ -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  $\beta$ -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.

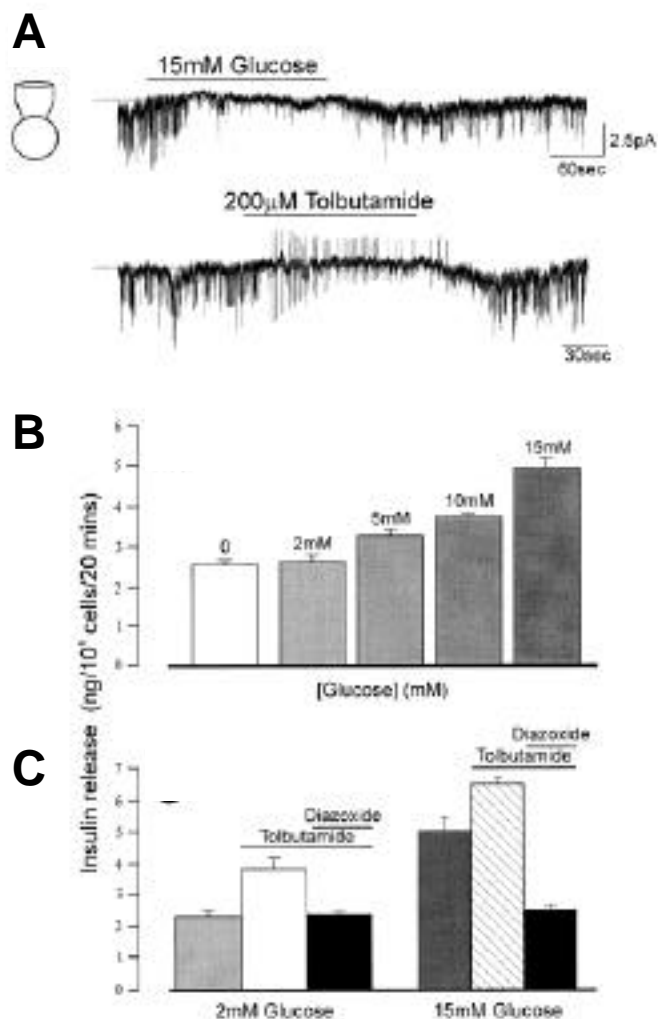
## RESEARCH DESIGN AND METHODS

**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  $\mu$ g/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<sub>2</sub>, 2.5 glucose, 10 HEPES, and 1 EGTA (pH 7.2 with KOH). The standard extracellular Na<sup>+</sup>-rich solution used throughout these experiments contained (in mmol/l) 140 NaCl, 4.7 KCl, 1.1 MgCl<sub>2</sub>, 2.5 CaCl<sub>2</sub>, 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<sup>+</sup>-rich solution. Data were recorded using a List/LM 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<sup>2+</sup> concentration.** Microfluorimetry with fura-2 was used to monitor changes in cytosolic Ca<sup>2+</sup> levels (22) in BRIN-BD11 cells using previously described procedures (23). Cells were loaded with 0.5  $\mu$ mol/l fura-2-AM (Sigma, St. Louis, MO) in 1 ml RPMI 1640 medium for 8 min at 37°C. The composition of basic perfusion medium was (in mmol/l) 137 NaCl, 5.4 KCl, 0.8 MgSO<sub>4</sub>, 0.3 Na<sub>2</sub>HPO<sub>4</sub>, 0.4 KH<sub>2</sub>PO<sub>4</sub>, 4.17 NaHCO<sub>3</sub>, 10 HEPES, and 1.3 CaCl<sub>2</sub>. 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<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>) are monitored by an increase in the fluorescence ratio at 340:380 nm. To quantify changes in [Ca<sup>2+</sup>]<sub>i</sub>, an in vitro calibration procedure (22) was used to estimate [Ca<sup>2+</sup>]<sub>i</sub> levels.

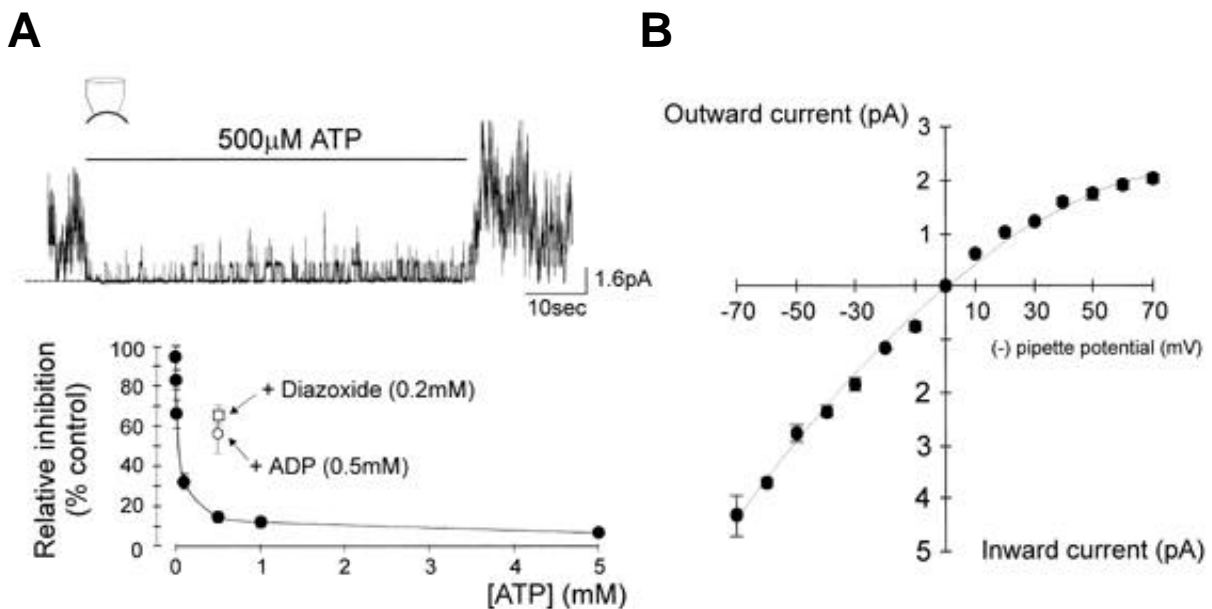
**Insulin secretion.** BRIN-BD11 cells were harvested and seeded at a density of  $1.25 \times 10^5$  cells in each well of 24-well plates. After culture and attachment



**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  $\mu$ mol/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  $\mu$ mol/l diazoxide. Data are means  $\pm$  SE ( $n = 6$ ).

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<sub>2</sub>, 2.0 CaCl<sub>2</sub>, 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  $-20^{\circ}C$  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 $\times$  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. 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  $\mu\text{M}$  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  $\mu\text{M}$  ADP ( $n = 4$ ) and 200  $\mu\text{M}$  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  $\sim 70$  pS. All points are mean values  $\pm$  SE.

**Analysis of data.** All data are expressed as means  $\pm$  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 1A 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  $\sim 70$  pS under symmetrical 140 mmol/l KCl-rich solutions (Fig. 2B). 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. 1A) or exposure to 200  $\mu\text{M}$  tolbutamide ( $n = 12/14$  patches) (Fig. 1A). 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. 1B). 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. 1C). The effects of both glucose and tolbutamide on secretion were inhibited by the  $K_{ATP}$  channel opener diazoxide (200  $\mu\text{M}$ ) (Fig. 1C; 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. 2A), exhibited run-down with time (26), and were inhibited by ATP, which also caused a

marked refreshment of  $K_{ATP}$  channel activity (Fig. 2A).  $K_{ATP}$  channels were recorded in 36/36 inside-out patch experiments, and the addition of ATP (1  $\mu\text{M}$  to 5 mmol/l) to the intracellular face of the membrane consistently inhibited channels in a concentration-dependent manner (Fig. 2A). The ATP concentration for half-maximal inhibition ( $K_D$ ) of  $K_{ATP}$  channels (in the absence of ADP) was estimated to be  $37 \pm 0.66$   $\mu\text{M}$  ( $n = 8$ ), and the Hill coefficient for the effects of ATP was  $\sim 1$  ( $0.74 \pm 0.1$ ,  $n = 8$ ).

In the continued presence of ATP, both diazoxide (200  $\mu\text{M}$ ,  $n = 15/15$ ) (Fig. 2A) and ADP (0.5 mmol/l;  $n = 8/8$ ) (Fig. 2A) caused the activation of  $K_{ATP}$  channels. As in native  $\beta$ -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  $25.5 \pm 10\%$  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  $\sim 70$  pS ( $n = 8$ ) (Fig. 2B). 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. 2B).

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

TABLE 1

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

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

Data for insulin release are means  $\pm$  SE ( $n = 6$ ). After a 40-min preincubation, the effects of 200  $\mu\text{mol/l}$  tolbutamide, 200  $\mu\text{mol/l}$  efaroxan, or 200  $\mu\text{mol/l}$  phentolamine were tested during a 20-min incubation period in the continued absence (-) or presence (+) of 200  $\mu\text{mol/l}$  diazoxide or 400  $\mu\text{mol/l}$  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. 1C), and imidazoline-induced insulin release (Table 1). The structurally unrelated channel agonist pinacidil (400  $\mu\text{mol/l}$ ) also activated  $K_{\text{ATP}}$  channels ( $n = 6/6$ ) (Fig. 4C) 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  $K_{\text{ATP}}$  channels in  $\beta$ -cells (15,28) and act as potent insulin secretagogues (16). The pharmacologic modulation of BRIN-BD11 cell function by these compounds

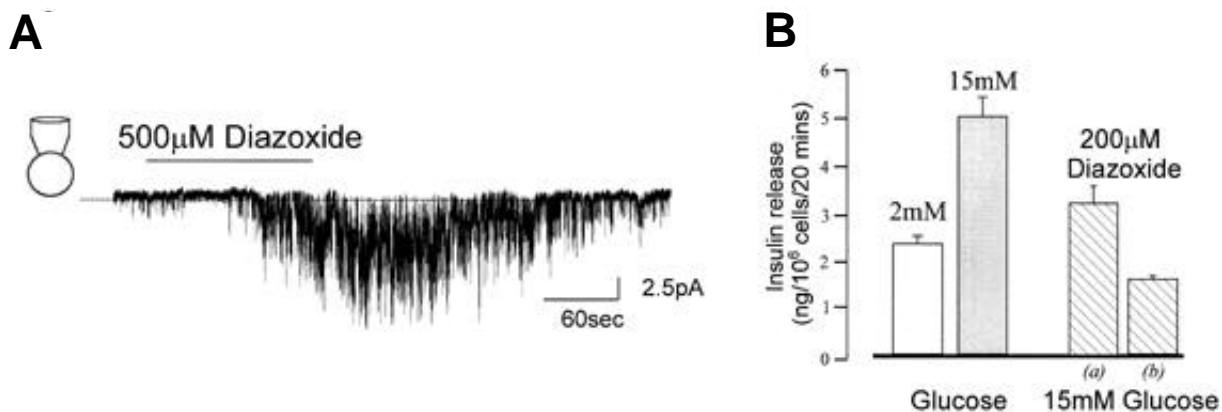
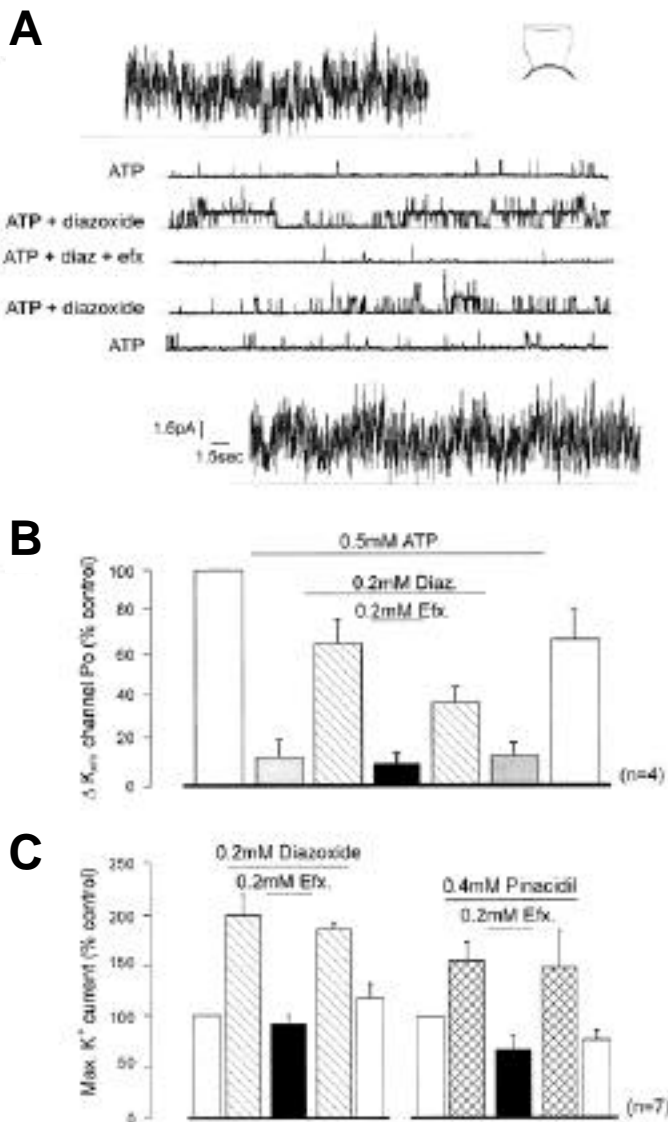


FIG. 3. Effects of diazoxide on BRIN-BD11 cells. **A:** Activation of  $K_{\text{ATP}}$  channels in a cell-attached patch recording by 500  $\mu\text{mol/l}$  diazoxide. **B:** A summary of the effect of diazoxide on glucose-induced insulin secretion ( $n = 6$ ). As shown in **B**, the secretory responses 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  $\mu\text{mol/l}$ ) followed by activation of channels by diazoxide (200  $\mu\text{mol/l}$ ). Diazoxide-activated  $K_{ATP}$  channels were subsequently inhibited in a reversible manner by 200  $\mu\text{mol/l}$  efaroxan. Open-state probability ( $P_o$ ) 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  $\beta$ -cells, efaroxan (200  $\mu\text{mol/l}$ ) directly inhibited  $K_{ATP}$  channels in a reversible manner to an average of  $9.9 \pm 5.4\%$  ( $n = 4$ ) of the control values ( $n = 5/5$ ) (Fig. 5A) and promoted insulin secretion ( $n = 6$ ) (Fig. 5B). 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 phenol-

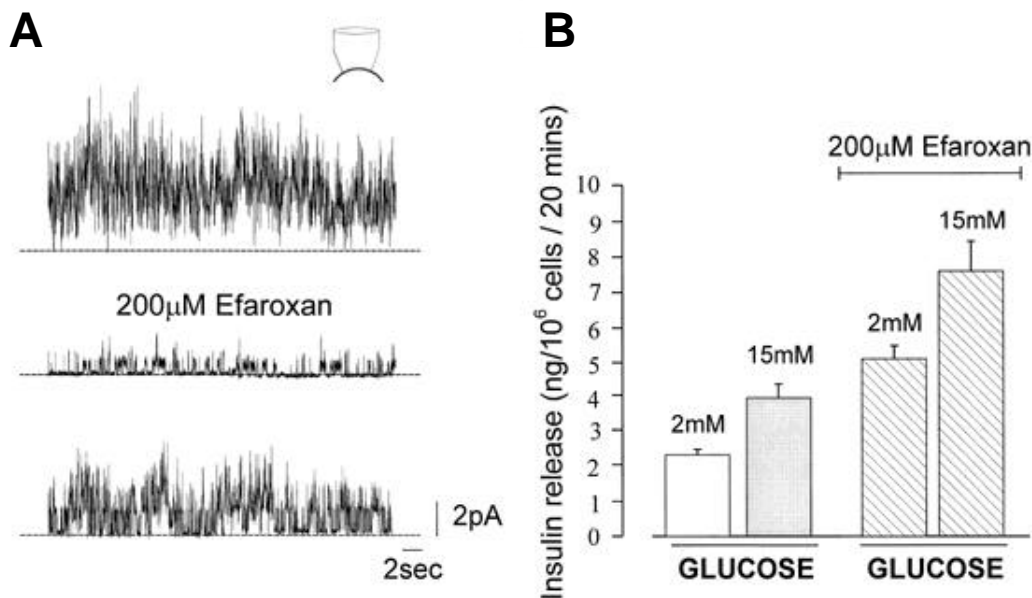
amine to  $9 \pm 4\%$  ( $n = 4$ ) of the control value ( $n = 8/8$  experiments), and phentolamine was able to both initiate insulin release (Fig. 6A) and reverse the actions of diazoxide on either glucose- or tolbutamide-induced insulin secretion (Table 1). Finally, consistent with the modulation of  $K_{ATP}$  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  $[\text{Ca}^{2+}]_i$  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. 6A) but not the responses of cells to glucose (15 mmol/l), tolbutamide (200  $\mu\text{mol/l}$ ), or depolarizing concentrations of KCl (25 mmol/l) (Fig. 6B).

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  $182 \pm 5\%$  [control,  $n = 6$ ] vs.  $113 \pm 12\%$  [pre-exposed,  $n = 6$ ]; values normalized to 15 mmol/l glucose alone, 100%). It was also notable that 200  $\mu\text{mol/l}$  efaroxan was unable to significantly alter the insulin secretion activity of 25 mmol/l KCl or 200  $\mu\text{mol/l}$  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  $[\text{Ca}^{2+}]_i$ . Pretreatment of BRIN-BD11 cells with efaroxan produced a modest decrease in basal levels of  $[\text{Ca}^{2+}]_i$  (control value  $101 \pm 13$  nmol/l [ $n = 17$ ] vs.  $78 \pm 6$  nmol/l [ $n = 15$ ] in treated cells).

In parallel experiments, pre-exposure of BRIN-BD11 cells for 20 h to 50  $\mu\text{mol/l}$  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  $\mu\text{mol/l}$  efaroxan, we typically found that the peak  $K_{ATP}$  current recorded across inside-out patches (Fig. 8A) was similar to controls ( $26.4 \pm 4.6$  [ $n = 7$ ] vs.  $26.7 \pm 4.2$  pA [control;  $n = 5$ ]). ATP (500  $\mu\text{mol/l}$ ) typically reduced the  $K_{ATP}$  current in these cells from  $22 \pm 8$  to  $3.6 \pm 0.8$  pA ( $n = 3$ ) vs.  $22 \pm 6.8$  to  $2.2 \pm 0.7$  pA for controls ( $n = 5$ ), and efaroxan (200  $\mu\text{mol/l}$ ) was also found to reduce  $K_{ATP}$  channel activity of pretreated cells to  $8.7 \pm 5.6\%$  ( $n = 4$ ) 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  $\mu\text{mol/l}$  efaroxan pretreatment led to an increase in SUR1 protein abundance, and similar effects were found at 100 and 200  $\mu\text{mol/l}$  efaroxan (Fig. 8B). This increase occurred despite no functional increase in  $K_{ATP}$  channel density (Fig. 8A). Surprisingly, when the concentration of efaroxan used to preincubate cells was further elevated



**FIG. 5.** Imidazoline-induced closure of K<sub>ATP</sub> channels and insulin secretion. The data shown in *A* come from the same inside-out patch and illustrate reversible block of K<sub>ATP</sub> channels by 200 μmol/l efaroxan. Open-state probability (P<sub>o</sub>) 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<sub>ATP</sub> channels in BRIN-BD11 cells (Fig. 8*A*). Under these conditions, the average peak K<sub>ATP</sub> channel current was increased from an average of 28 ± 4 (*n* = 20) to 94 ± 39 pA (*n* = 7).

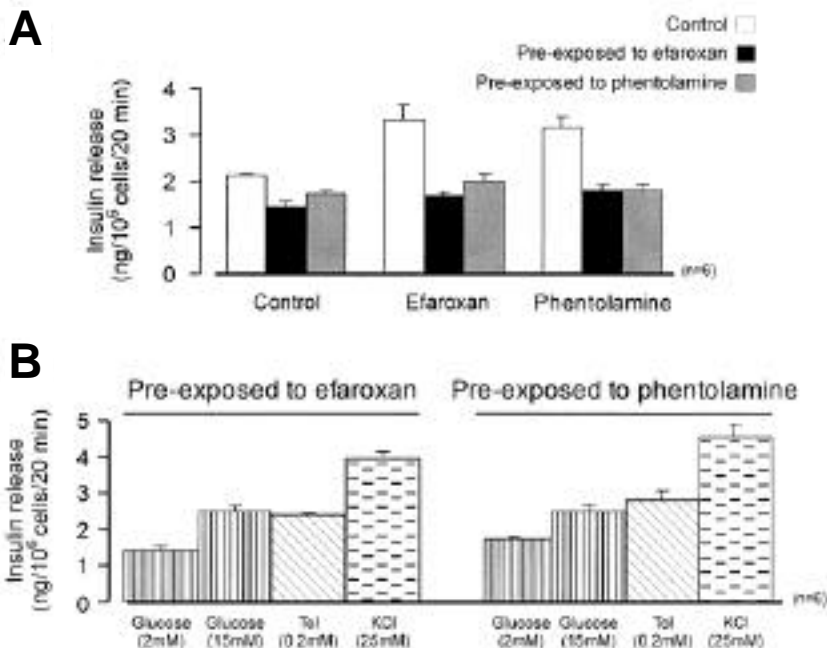
**DISCUSSION**

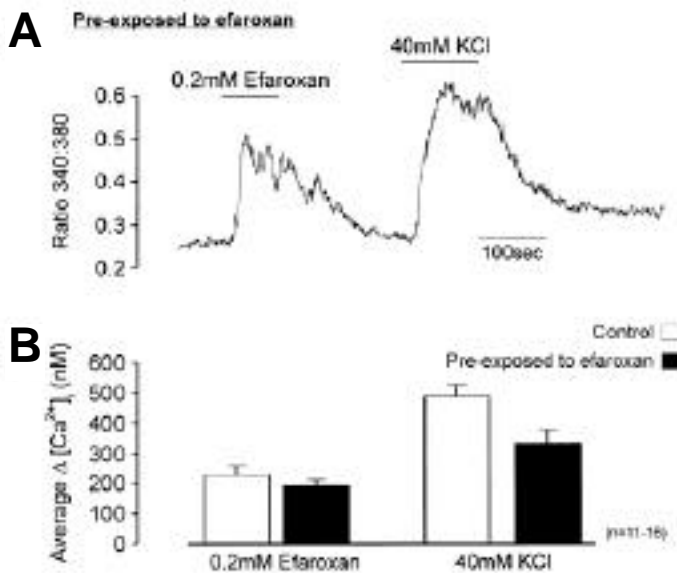
In this study, we have provided the first characterization of K<sub>ATP</sub> channels and their role in the ionic regulation of insulin secretion from the electrofusion-derived BRIN-BD11 cells. We have shown that glucose-, sulfonylurea-, and imidazoline-induced secretory events are associated with the modulation of K<sub>ATP</sub>

channels in a manner similar to that documented in normal β-cells. The actions of glucose occur in association with K<sub>ATP</sub> 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<sub>ATP</sub> channel closure, the activation of voltage-dependent Ca<sup>2+</sup> influx (30), and insulin release. In contrast, the actions of sulfonylureas and imidazolines are more direct and bring about the inhibition of K<sub>ATP</sub> channels in the absence of cytosolic nucleotides (Fig. 5).

The biophysical and regulatory properties of K<sub>ATP</sub> 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.

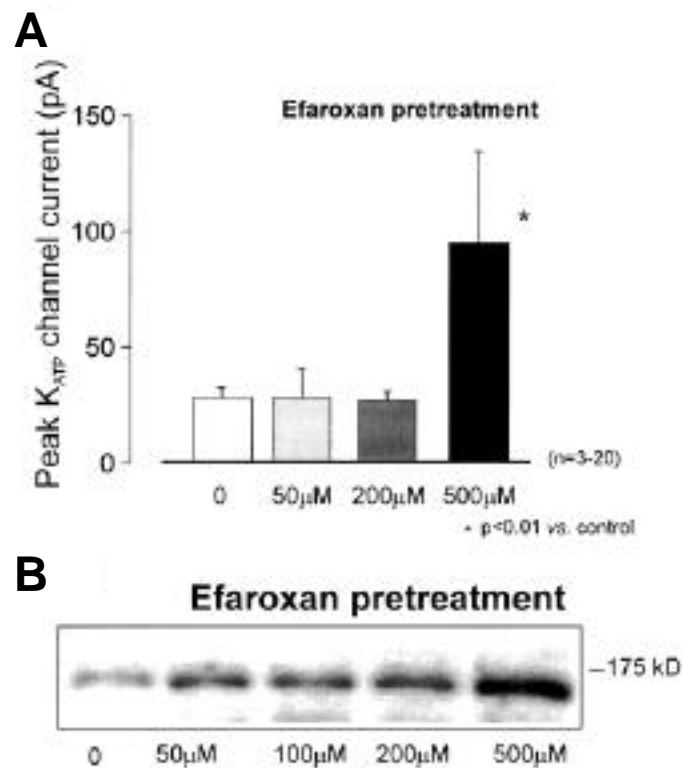




**FIG. 7.** Effects of pre-exposure of BRIN-BD11 cells to efaroxan and modulation of depolarization-dependent  $Ca^{2+}$  influx. **A:** A typical experiment in which both efaroxan and KCl induce a marked increase in  $[Ca^{2+}]_i$  in BRIN-BD11 cells pre-exposed to efaroxan for 24 h. Changes in  $[Ca^{2+}]_i$  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,  $n = 11$ ).

insulin-secreting cells (31,32). The channels 1) have a nonuniform current-voltage relationship profile, 2) exhibit inward current rectification, 3) undergo spontaneous run-down in isolated patches that is reversed by intracellular action of ATP, 4) have a half-maximal inhibition by ATP of approximately 37  $\mu\text{mol/l}$  (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)<sub>4</sub>. 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 glu-



**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  $\mu\text{mol/l}$  to 500  $\mu\text{mol/l}$ ). **A:** Peak  $K_{ATP}$  current density was measured by isolating inside-out patches from intact cells under quasiphsyologic cation gradients. Overall, there was no significant increase in channel density between 50 and 200  $\mu\text{mol/l}$  efaroxan, but exposure to 500  $\mu\text{mol/l}$  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  $\mu\text{mol/l}$  efaroxan.

cose 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  $\beta$ -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  $\beta$ -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 pre-exposure to imidazolines provides a new insight into the mechanisms of action of imidazolines on  $\beta$ -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  $\beta$ -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  $\beta$ -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), indi-

cate that electrofusion represents a powerful tool for engineering novel  $\beta$ -cell lines for potential gene therapy of diabetes.

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