

# Imidazoline Compounds Protect Against Interleukin 1 $\beta$ -Induced $\beta$ -Cell Apoptosis

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**Imidazoline compounds have been considered for the treatment of type 2 diabetes. We have now investigated the effects of imidazolines on interleukin (IL)-1 $\beta$ -induced  $\beta$ -cell apoptosis and the signal transduction pathways involved. Inhibition of Ca<sup>2+</sup> influx into  $\beta$ -cells by D-600, a blocker of voltage-gated L-type Ca<sup>2+</sup> channels, suppressed IL-1 $\beta$ -induced apoptosis. Our data show that calcineurin, Ca<sup>2+</sup>/calmodulin-dependent serine/threonine protein phosphatase 2B, is responsible for the effect of Ca<sup>2+</sup> on  $\beta$ -cell apoptosis. We also demonstrate that IL-1 $\beta$ -mediated apoptosis correlates with expression of inducible nitric oxide synthase (iNOS) and the increase in intracellular production of nitric oxide. An inhibitor of cGMP-dependent protein kinase (PKG), KT5823, suppressed IL-1 $\beta$ -induced apoptosis, suggesting the involvement of a PKG-dependent pathway in the apoptotic process. One of the major findings in this study is that imidazoline compounds RX871024 and efaroxan, suggested as prototypes of a new generation of drugs against type 2 diabetes, can protect against IL-1 $\beta$ -induced apoptosis in pancreatic  $\beta$ -cells, possibly by their inhibition of the expression of iNOS, a key element in the IL-1 $\beta$ -induced apoptotic pathway in pancreatic  $\beta$ -cells. These data suggest that imidazoline compounds should be explored as a potential therapeutic agent for the treatment of both type 1 and type 2 diabetes. *Diabetes* 50 (Suppl. 1):S70–S76, 2001**

Compounds with an imidazoline moiety within their structure (imidazoline compounds) are known to promote insulin secretion (1–4) and may therefore represent a new class of drugs in type 2 diabetes; indeed, several imidazoline compounds have already been shown to be antihyperglycemic in humans (5,6).

Previously, we showed that imidazolines stimulate insulin release both by blocking ATP-dependent K<sup>+</sup> channels, with a

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[Ca<sup>2+</sup>]<sub>i</sub>, cytoplasmic free Ca<sup>2+</sup> concentration; FITC, fluorescein isothiocyanate; IL, interleukin; iNOS, inducible nitric oxide synthase; L-NAME, N<sup>G</sup>-nitro-L-arginine methyl ester; NO, nitric oxide; PBS, phosphate-buffered saline; PI, propidium iodide; PKG, cGMP-dependent protein kinase; TUNEL, TdT-mediated X-dUTP nick-end labeling.

subsequent increase in cytoplasmic free Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>), and by directly affecting the exocytotic machinery (3,7). In addition, imidazoline compounds induce release of Ca<sup>2+</sup> from nonmitochondrial thapsigargin-sensitive intracellular stores (8). Whereas short-term intracellular Ca<sup>2+</sup> changes modulate several physiological functions, disturbances in the Ca<sup>2+</sup> homeostatic control can lead to Ca<sup>2+</sup> accumulation and trigger apoptosis (9–12). Because imidazolines increase [Ca<sup>2+</sup>]<sub>i</sub>, it is of interest to study the effects of these compounds on apoptosis of pancreatic  $\beta$ -cells.

Cytokines play an important role in the development of type 1 diabetes (13–15). Among the cytokines, interleukin (IL)-1 $\beta$  seems to be one of the main mediators of  $\beta$ -cell dysfunction (15–18). IL-1 $\beta$  acts on  $\beta$ -cells via IL-1 receptors (19,20), but the signal transduction pathways are not fully understood. IL-1 $\beta$  effects on the  $\beta$ -cell are pleiotropic, including formation of nitric oxide (NO), ceramide, prostaglandins, heat shock proteins, and protease activity (13,15,21). The cytokine also decreases cAMP concentration, energy production, and insulin secretion in the cell (21).

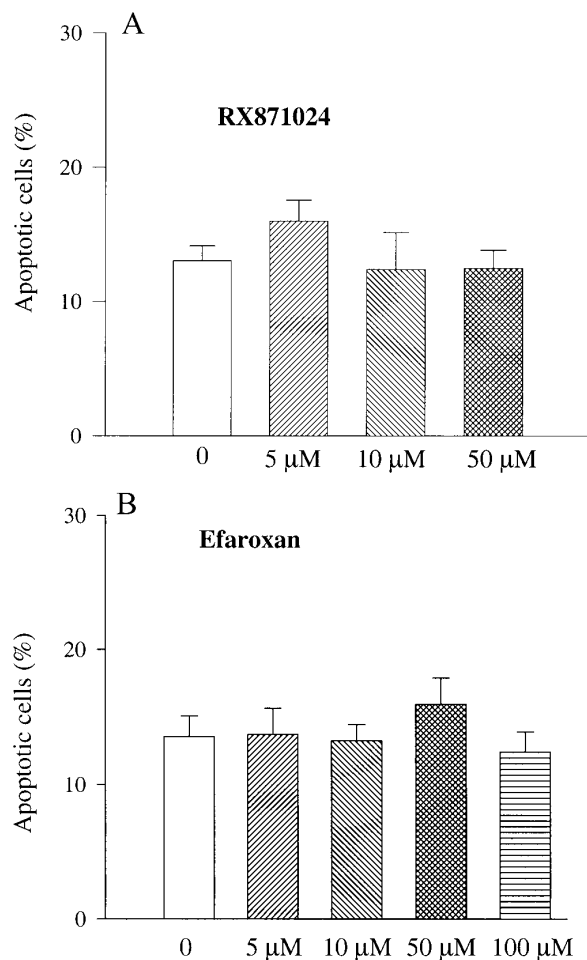
This study was designed to solve two interconnected issues: first, to investigate the mechanism of IL-1 $\beta$ -induced apoptosis in murine pancreatic  $\beta$ -cells (mostly the role of Ca<sup>2+</sup> and NO), and second, to evaluate the effect of imidazoline compounds on IL-1 $\beta$ -induced  $\beta$ -cell apoptosis.

## RESEARCH DESIGN AND METHODS

**Materials.** RPMI-1640 medium and fetal calf serum were obtained from Gibco (Middlesex, U.K.). RX871024 was obtained from Reckitt and Colman (Kingston Upon Hull, U.K.). Efaroxan and bovine serum albumin were purchased from Sigma (St. Louis, MO). Recombinant human IL-1 $\beta$ , N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME), KT5823, and deltamethrin were purchased from Calbiochem (La Jolla, CA). Fura-2 AM, extravidin fluorescein isothiocyanate (FITC) conjugate, and propidium iodide (PI) were purchased from Molecular Probes (Eugene, OR). The kit for TdT-mediated X-dUTP nick-end labeling (TUNEL) reaction was received from Boehringer Mannheim (Mannheim, Germany). All other reagents were of analytical grade and were obtained from Merck (Darmstadt, Germany).

**Isolation of  $\beta$ -cells.** Islets of Langerhans from 10- to 12-month-old obese (*ob/ob*) mice obtained from a local colony were isolated by collagenase digestion. Pancreatic islets were dispersed into small  $\beta$ -cell clusters in Ca<sup>2+</sup>- and Mg<sup>2+</sup>-deficient medium as previously described (22). Cells were cultured on glass coverslips in plastic Petri dishes for 40 h at 37°C in RPMI-1640 medium supplemented with 11 mmol/l glucose, 10% (vol/vol) fetal calf serum, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin.

**TUNEL labeling of  $\beta$ -cells and double-staining for confocal microscopy.** The TUNEL technique was used to detect DNA strand breaks in situ as previously described (12). Cells double-stained with FITC and PI were fixed on glass slides with 50% glycerol in phosphate-buffered saline (PBS). Fluorescence was monitored with a Leica TCS NT laser scanning confocal microscope (Leica Lasertechnik, Heidelberg, Germany), with excitation from the 488-nm line of an argon/krypton laser. Fluorescence emission was detected with a band-pass filter (Chroma Technology, Brattleboro, VT) centered at 530 nm for FITC and above 590 nm for PI. Several confocal images were used for counting the num-



**FIG. 1. RX871024 and efaroxan do not induce apoptosis in pancreatic  $\beta$ -cells.** Pancreatic  $\beta$ -cells were incubated for 40 h in the presence and absence of RX871024 (A) ( $n = 7$ ) and efaroxan (B) ( $n = 3$ ). Statistical significance between means was assessed by Student's  $t$  test for unpaired values.

ber of apoptotic cells. In each condition, a minimum of 1,000 cells from three to seven different isolations were counted.

**Patch-clamp measurements.** Whole-cell  $Ca^{2+}$  currents were recorded by using the perforated-patch variant of the whole-cell patch-clamp recording technique to eliminate the loss of soluble cytoplasmic components. Electrodes were filled with the following (in mmol/l): 76  $Cs_2SO_4$ , 1  $MgCl_2$ , 10  $KCl$ , 10  $NaCl$ , and 5 HEPES (pH 7.35) as well as amphotericin B (0.24 mg/ml) to permeabilize the cell membrane and allow low-resistance electrical access without breaking the patch. The cells were bathed in a solution containing the following (in mmol/l): 138 choline chloride, 10 tetraethylammonium chloride, 10  $CaCl_2$ , 5.6  $KCl$ , 1.2  $MgCl_2$ , 5 HEPES, and 3 glucose (pH 7.4). One set of depolarizing voltage pulses (100 ms) was made from a holding potential of  $-70$  mV to several clamping potentials from  $-70$  to 50 or 80 mV in 10-mV increments at 0.5 Hz. This depolarization protocol was used to evaluate current-voltage relationships in cells bathed in the extracellular solution in the absence or presence of dibutyryl-cGMP (500  $\mu$ mol/l, acute application for 20 min), KT5823 (10  $\mu$ mol/l, acute application for 20 min), and IL-1 $\beta$  (2 ng/ml, incubation for 24 and 48 h). Another set of depolarizing voltage pulses (100 ms, 0.05 Hz) to  $-40$  mV from a set of holding potentials from  $-110$  to  $-60$  mV was used to examine voltage-gated T-type  $Ca^{2+}$  currents in cells incubated with IL-1 $\beta$  for 24 or 48 h. The resulting currents were recorded with an Axopatch 200 amplifier (Axon Instruments, Foster City, CA) and filtered at 1 kHz. All recordings were made at room temperature ( $\sim 22^\circ C$ ). The amplitude of  $Ca^{2+}$  currents was normalized by the capacitance of cells to discriminate between control and IL-1 $\beta$ -treated groups. Acquisition and analysis of data were done using the software program pCLAMP (Axon Instruments).

**Nitrite determination.** Groups of 80–100 islets were incubated in 300  $\mu$ l RPMI-1640 medium (without Phenol Red) with or without IL-1 $\beta$  (2 ng/ml) and

the mixture of IL-1 $\beta$  (2 ng/ml) and RX871024 (50  $\mu$ mol/l) in a 48-well plate (Nunc Delta SI; Nunc, Naperville, IL). After a 40-h incubation, medium was withdrawn and centrifuged for 2 min at 1,500g, and 100  $\mu$ l samples of supernatant were mixed with 20  $\mu$ l of Gries reagent [mixture of equal volumes of 0.5% solution of  $N$ -(1-naphthyl)ethylenediamine in water and 5% solution of sulfanilamide in 25%  $H_3PO_4$ ] as previously described (23). Reaction was carried out for 15 min at room temperature. Nitrite production was determined at 595 nm with reference at 620 nm on a 96-well plate reader. For calibration of results, a standard curve for  $NaNO_2$  in RPMI-1640 medium was established every time.

**Western blotting of inducible nitric oxide synthase.** Groups of 40–60 islets were incubated in 5 ml RPMI-1640 culture medium with or without IL-1 $\beta$  (2 ng/ml) and the mixture of IL-1 $\beta$  (2 ng/ml) and RX871024 (50  $\mu$ mol/l) in Petri dishes (60  $\times$  15 mm; Tamro Lab, Mölndal, Sweden). After a 40 h incubation, islets were picked, washed rapidly with PBS, and put into 1.5 ml Eppendorf tubes, followed by the addition of 20–30  $\mu$ l boiling lysis buffer (1% SDS, 1 mmol/l sodium ortho-vanadate, and 10 mmol/l Tris, pH 7.4) and 5 min of boiling. To reduce viscosity, samples were passed several times through an insulin needle. Then, 2  $\mu$ l aliquots of lysate were taken for protein determination using a bicinchoninic acid protein determination kit (Sigma). Subsequent to electrophoresis in SDS/7.5% PAGE, the gels were blotted onto a polyvinylidene difluoride transfer membrane (Hybond-P; Amersham, Buckinghamshire, U.K.). Membranes were blocked overnight at 4°C with Tris-buffered saline containing 5% dry milk and incubated for 1 h with 1:250 dilution of mouse monoclonal antibodies against inducible nitric oxide synthase (iNOS) (mouse anti-iNOS; Transduction Laboratories, Lexington, KY). The membranes were washed with Tris-buffered saline containing 0.06% Tween 20 (Sigma) and incubated for 1 h with peroxidase-labeled rabbit anti-mouse antibody diluted 1:10,000 (Amersham). The detection step was performed with an enhanced chemiluminescence (ECL) detection kit (Amersham). The membranes were exposed to Hyperfilm-ECL (Amersham).

**Presentation of results.** Confocal images were processed using a Corel Photo-Paint (version 7) and Corel Draw (version 7) program. Selected protocols were tested in  $\beta$ -cells from three to seven preparations from different animals. Data analysis was performed using the program Sigma Plot for Windows (version 4; SPSS, Chicago). All results are expressed as means  $\pm$  SE for the indicated number of experiments.

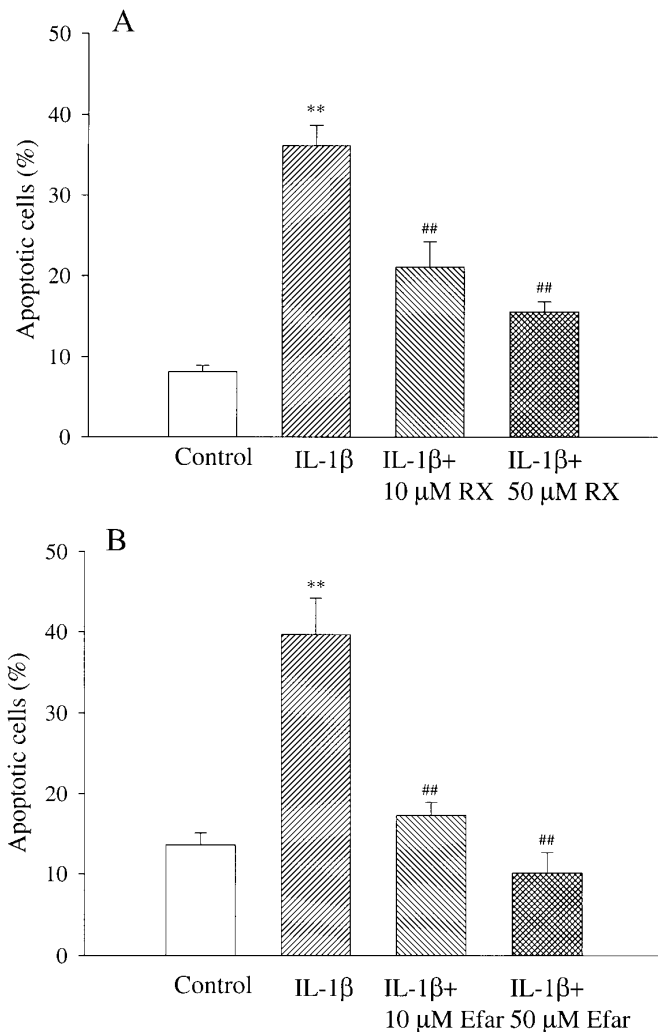
## RESULTS

To investigate the effects of imidazoline compounds on apoptosis, pancreatic  $\beta$ -cells were incubated for 40 h in RPMI-1640 medium containing 11 mmol/l glucose in the presence or absence of various concentrations of RX871024 and efaroxan. This glucose concentration has been shown to be optimal for  $\beta$ -cell survival (12). For evaluation of apoptotic changes, TUNEL labeling and double-staining of pancreatic  $\beta$ -cells for confocal microscopy was performed. Imidazoline compounds RX871024 (5–50  $\mu$ mol/l) and efaroxan (5–100  $\mu$ mol/l) did not trigger apoptosis (Fig. 1).

To study the function of  $\beta$ -cells after long-term treatment with RX871024, cells were incubated for 40 h with or without 50  $\mu$ mol/l RX871024 and stimulated with 17 mmol/l glucose and 25 mmol/l KCl, and  $[Ca^{2+}]_i$  was measured. The results demonstrate no significant difference in increase of  $[Ca^{2+}]_i$  in control and treated cells (data not shown), indicating that RX871024 is not toxic for the  $\beta$ -cells.

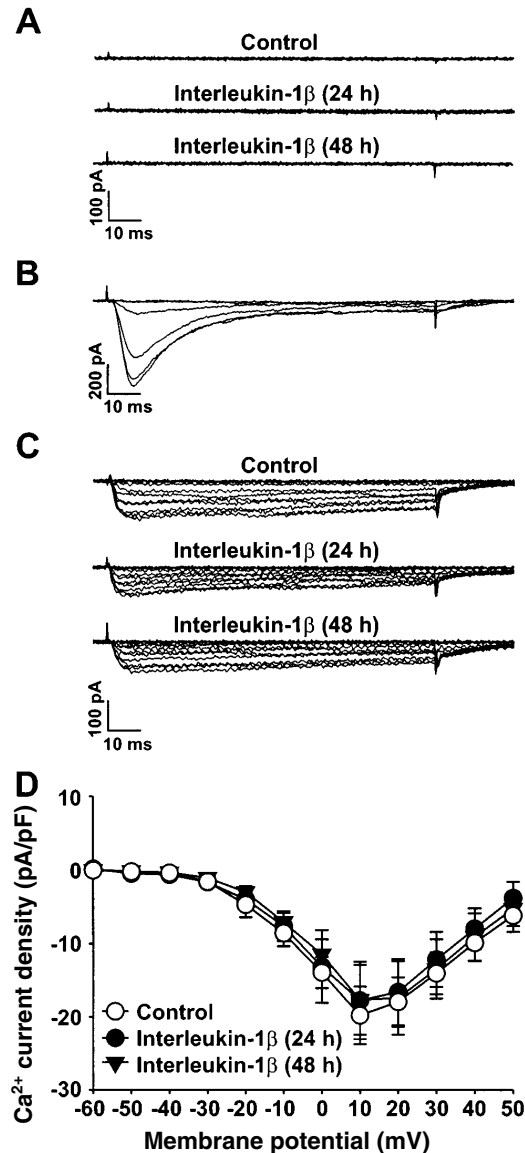
To test whether imidazolines can protect pancreatic  $\beta$ -cells from cytokine-induced apoptosis, the effects of RX871024 and efaroxan were investigated in the presence of IL-1 $\beta$ . Culturing  $\beta$ -cells with 2 ng/ml IL-1 $\beta$  in the presence of 10 and 50  $\mu$ mol/l of the imidazolines suppressed IL-1 $\beta$ -induced apoptosis (Fig. 2).

In our previous study, we showed that  $\beta$ -cell apoptosis in Swiss-Webster mice induced by cytokine mixture (IL-1 $\beta$  and  $\gamma$ -interferon) is a  $Ca^{2+}$ -dependent process that involves activation of voltage-gated T-type  $Ca^{2+}$  channels (24). To evaluate whether incubation with IL-1 $\beta$  alone activates voltage-gated T-type  $Ca^{2+}$  channels in *ob/ob* mouse  $\beta$ -cells, we measured the activity of these channels before and after 24



**FIG. 2.** Inhibition of IL-1 $\beta$ -induced apoptosis in pancreatic  $\beta$ -cells by RX871024 and efaroxan. RX871024 (RX) (A) ( $n = 5$ ) and efaroxan (Efar) (B) ( $n = 3$ ) at 10 and 50  $\mu$ M significantly decreased apoptosis triggered by 2 ng/ml IL-1 $\beta$ . Statistical significance between means was assessed by Student's  $t$  test for unpaired values. \*\* $P < 0.01$  relative to control; ## $P < 0.01$  relative to 2 ng/ml IL-1 $\beta$ .

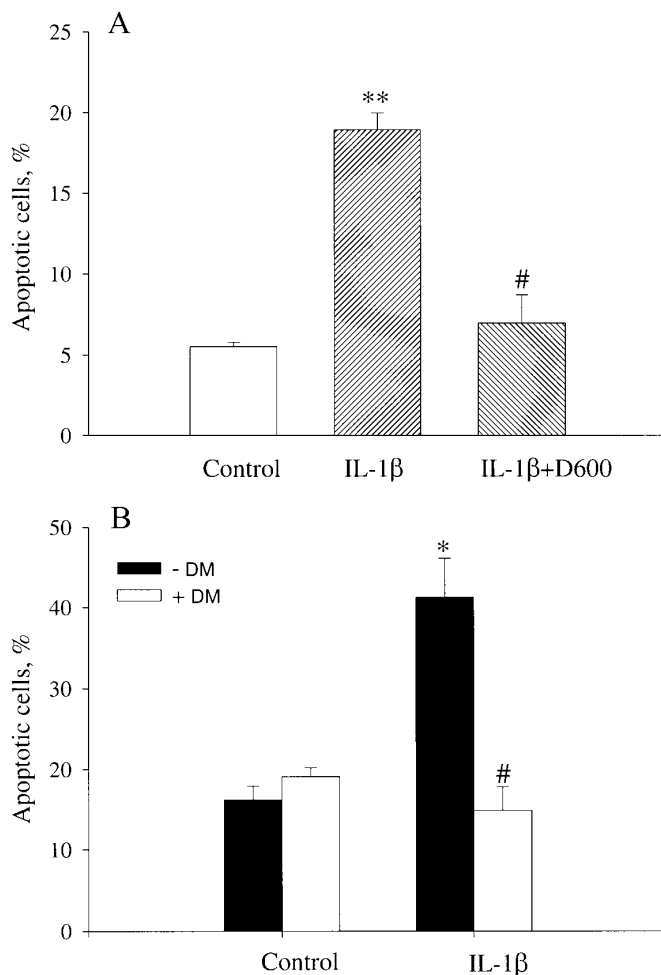
and 48 h of incubation with 2 ng/ml IL-1 $\beta$ . No cells displayed low voltage-gated T-type Ca<sup>2+</sup> currents in either control or IL-1 $\beta$ -treated groups (Fig. 3A). Typical low voltage-gated T-type Ca<sup>2+</sup> currents were observed in hippocampal neurons under the same experimental conditions (Fig. 3B). Nevertheless, IL-1 $\beta$ -induced apoptosis in *ob/ob* mouse  $\beta$ -cells is dependent on [Ca<sup>2+</sup>]<sub>i</sub> because inhibition of Ca<sup>2+</sup> influx by 50  $\mu$ M D-600, a blocker of voltage-gated L-type channels, suppresses apoptosis (Fig. 4A). To determine whether the Ca<sup>2+</sup> dependency of IL-1 $\beta$ -induced apoptosis involves activation of voltage-gated L-type Ca<sup>2+</sup> channels, we compared their activity before and after incubation with IL-1 $\beta$ . As shown in Fig. 3C, there was no marked difference between different treatments in Ca<sup>2+</sup> current traces generated by a set of depolarizing voltage pulses (100 ms) between -60 and 50 mV in 10-mV increments from a holding potential of -70 mV. Compiled data show that treatment with IL-1 $\beta$  did not significantly alter Ca<sup>2+</sup> current density in comparison to controls (Fig. 3D). Thus, the cytokine does



**FIG. 3.** Effects of IL-1 $\beta$  on voltage-gated Ca<sup>2+</sup> currents in the pancreatic  $\beta$ -cell. A: A set of depolarizing voltage pulses (100 ms, 0.05 Hz) to -40 mV from a set of holding potentials from -110 to -60 mV did not evoke any appreciable Ca<sup>2+</sup> current in the  $\beta$ -cell in control and IL-1 $\beta$ -treated groups. B: The same depolarization protocol generated low voltage-gated T-type Ca<sup>2+</sup> currents in the hippocampal neuron. C: The cells in control or IL-1 $\beta$ -treated groups displayed similar whole-cell Ca<sup>2+</sup> current traces evoked by step depolarizing voltage pulses from a holding potential of -70 mV. D: Compiled data illustrated that treatment with IL-1 $\beta$  for 24 or 48 h ( $n = 10$ ) did not affect Ca<sup>2+</sup> current density during depolarizations in the range from -60 to 50 mV in comparison to controls ( $n = 10$ ). Statistical significance was evaluated by one-way analysis of variance followed by a least-significant difference test.

not significantly change the activity of voltage-gated L-type Ca<sup>2+</sup> channels in *ob/ob* mouse  $\beta$ -cells.

It has been shown that an increase in [Ca<sup>2+</sup>]<sub>i</sub> leads to the activation of calcineurin, Ca<sup>2+</sup>/calmodulin-dependent protein serine/threonine phosphatase 2B, which plays an important role in the activation of transcription factors (25,26). The involvement of calcineurin was studied using 20 nmol/l deltamethrin, which at this concentration, specifically blocks the enzyme. Deltamethrin protected cultured  $\beta$ -cells from IL-1 $\beta$ -



**FIG. 4.** Inhibition of apoptosis induced by IL-1 $\beta$  (2 ng/ml) in  $\beta$ -cells by a blocker of voltage-gated L-type channels, D600 (50  $\mu$ mol/l) (A), and an inhibitor of calcineurin/deltamethrin (DM) (20 nmol/l) (B). Statistical significance between means was assessed by Student's *t* test for unpaired values. \**P* < 0.05 and \*\**P* < 0.01 relative to control; #*P* < 0.05 relative to IL-1 $\beta$  (*n* = 3).

induced apoptosis (Fig. 4B), indicating the involvement of calcineurin-mediated signaling pathways.

The reports on the role of the "death receptor" Fas in  $\beta$ -cell apoptosis are controversial (27–30). We investigated whether Fas is involved in the mechanism of IL-1 $\beta$ -induced apoptosis in *ob/ob* mouse  $\beta$ -cells. Cells were treated for 20 h with IL-1 $\beta$  with subsequent incubation in the presence or absence of stimulating anti-Fas antibody (monoclonal antibody CH-11, 250 ng/ml) for an additional 20 h. There was no further increase in apoptosis when anti-Fas antibody was used. Moreover, there was no significant difference in apoptotic rate after culturing with IL-1 $\beta$  and RX871024 in the absence and presence of anti-Fas antibody (data not shown).

It has been shown previously that NO generated by NO synthase within the islet cells mediates many effects of IL-1 $\beta$  on pancreatic islets (17,18,21,31). In the present study, IL-1 $\beta$ -induced apoptosis was suppressed by L-NAME (5 mmol/l), an L-arginine analog inhibitor of NO synthase (Fig. 5A) (32). Furthermore, we measured NO formation by the release of nitrite into culture media during a 40-h incubation. The results are shown in Fig. 5B. Treatment of *ob/ob* islets with IL-1 $\beta$  (2 ng/ml) induced

an approximately threefold increase in the level of nitrite compared with untreated control islets. The increase in nitrite formation was inhibited by RX871024 (50  $\mu$ mol/l), indicating that RX871024 protects against IL-1 $\beta$ -induced apoptosis by decreasing NO production. To clarify the mechanism of this action, we investigated the effect of the compound on iNOS expression. Figures 5C and D show that RX871024 indeed reduces the expression of the enzyme.

To evaluate whether cGMP-dependent protein kinase (PKG) is involved in IL-1 $\beta$ -induced apoptosis, we used KT5823 (1  $\mu$ mol/l), a selective inhibitor of the enzyme (Fig. 6A). The results show that KT5823 suppresses  $\beta$ -cell apoptosis. To test for possible modulation of voltage-gated Ca<sup>2+</sup> currents by PKG,  $\beta$ -cells were acutely exposed to a PKG activator (membrane-permeable cGMP analog dibutyryl-cGMP) or to a PKG inhibitor (KT5823) for 5, 10, and 20 min. The results show that there is no significant difference in the amplitude of Ca<sup>2+</sup> currents between controls and the different treatments (Fig. 6B and C).

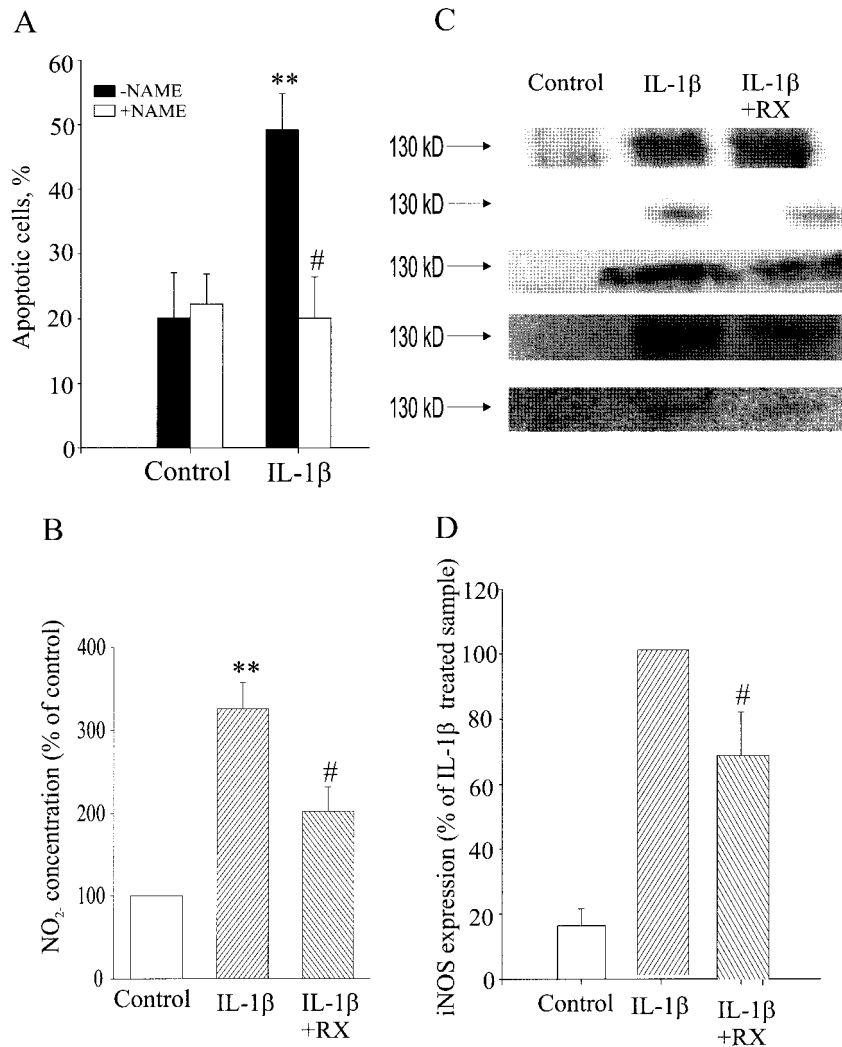
## DISCUSSION

Proinflammatory cytokines, especially IL-1 $\beta$ , play an important role in  $\beta$ -cell destruction, mainly by apoptosis, in type 1 diabetes (13–18). In this study, we show that the imidazoline compounds RX871024 and efaroxan promote survival of  $\beta$ -cells by inhibition of IL-1 $\beta$ -induced apoptosis. In addition, and in contrast to the sulfonylurea tolbutamide (12), these imidazolines did not by themselves induce  $\beta$ -cell apoptosis. In the present investigation, we have not been able to demonstrate that IL-1 $\beta$ -induced apoptosis in  $\beta$ -cells from *ob/ob* mice involved the death receptor Fas. These data are in agreement with reports that IL-1 $\beta$  alone did not induce Fas expression in  $\beta$ -cells from nonobese diabetic (NOD) mice (29,30).

It is known that  $\beta$ -cells contain both of the two major types of NO synthase: constitutive (cNOS) and inducible (iNOS) isoforms (31). When expressed, the latter is the major determinant of NO concentration in cells (33). Our data show that IL-1 $\beta$ -induced  $\beta$ -cell apoptosis in *ob/ob* mice involves expression of iNOS and a subsequent increase in NO concentration in the cell. These results are in full agreement with the majority of the data obtained in other  $\beta$ -cells and in vivo using inhibitors of NO production (13,15,21).

Our data, which indicate that a selective inhibitor of PKG (KT5823) abolished IL-1 $\beta$ -induced apoptosis, provide evidence that the pathway controlling IL-1 $\beta$ -induced  $\beta$ -cell death involves activation of PKG. These results are in line with the suggestion that PKG may be involved in NO-induced apoptosis in insulin-secreting HIT T15 cells based on the data showing that the NO donor S-nitrosoglutathione and the permeable analog of cGMP stimulated apoptosis, an effect that could be inhibited by KT5823 (34). Mechanisms involving PKG in  $\beta$ -cells are poorly understood; our data show that they do not involve activation of Ca<sup>2+</sup> channels. It has been demonstrated that PKG can regulate gene expression, and among others, it activates *c-fos*, an early gene that is often expressed in cells undergoing apoptosis (35). Data showing that IL-1 $\beta$  induces expression of *c-fos* in rat islets and in purified rat  $\beta$ -cells (36,37) favor this suggestion.

We have shown here that IL-1 $\beta$ -induced apoptosis in *ob/ob* mouse  $\beta$ -cells is a Ca<sup>2+</sup>-dependent process. This is in accordance with our previous observation that  $\beta$ -cell apoptosis in Swiss-Webster mice induced by cytokine mixture (IL-1 $\beta$  and  $\gamma$ -interferon) is Ca<sup>2+</sup>-dependent and involves voltage-gated

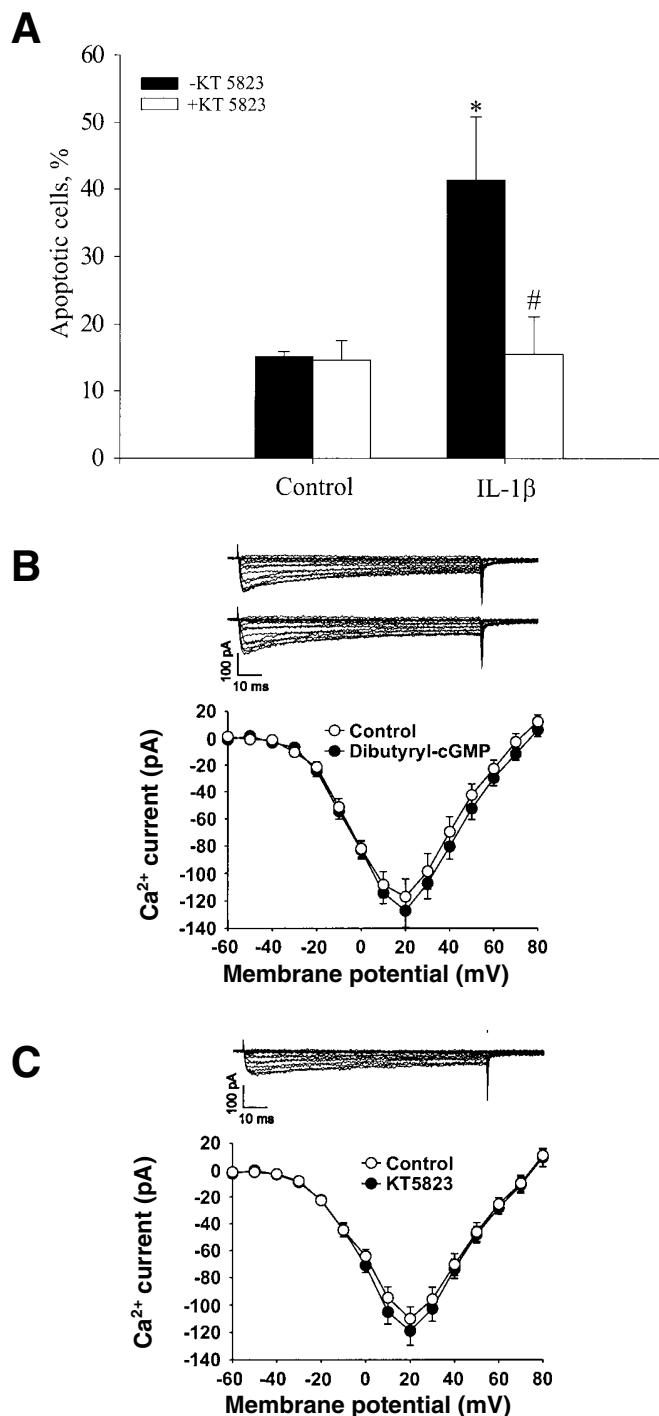


**FIG. 5. Protective effect of RX871024 against IL-1 $\beta$ -induced apoptosis in  $\beta$ -cells involves inhibition of NO formation by suppressing iNOS expression.** *A:* Inhibitor of NO synthase L-NAME (5 mmol/l) suppresses IL-1 $\beta$ -induced apoptosis of pancreatic  $\beta$ -cells ( $n = 3$ ). *B:* NO production in isolated pancreatic islets measured as release of nitrite into culture media during 40 h with 2 ng/ml IL-1 $\beta$  in the presence and absence of 50  $\mu$ mol/l RX871024 (RX) ( $n = 3$ ). *C:* Experiments showing expression of iNOS by 2 ng/ml IL-1 $\beta$  (IL-1 $\beta$ ) and inhibition of this expression by 50  $\mu$ mol/l RX871024 (IL-1 $\beta$  + RX); control represents a control without IL-1 $\beta$  or RX871024. *D:* Data analysis of five separate experiments depicted in *C* (IL-1 $\beta$  alone is taken as 100%). Statistical significance between means was assessed by Student's *t* test for unpaired values. \*\* $P < 0.01$  relative to control; # $P < 0.05$  relative to 2 ng/ml IL-1 $\beta$ .

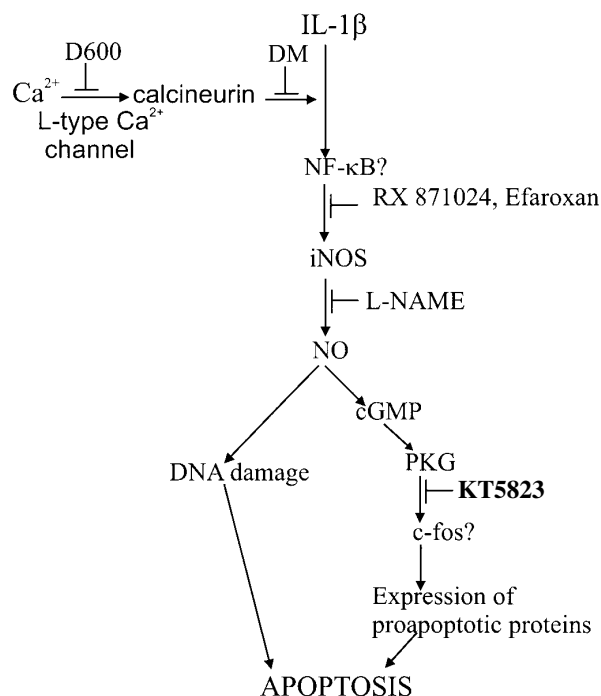
T-type Ca<sup>2+</sup> channels (24). Here we show that  $\beta$ -cell apoptosis in *ob/ob* mouse does not involve voltage-gated T-type Ca<sup>2+</sup> channels because they are not induced during IL-1 $\beta$  treatment. The results of this study point to an important role of voltage-gated L-type Ca<sup>2+</sup> channels in IL-1 $\beta$ -induced apoptosis because inhibition of Ca<sup>2+</sup> influx into the  $\beta$ -cell by D-600 suppresses this process. What molecular mechanism is then underlying Ca<sup>2+</sup>-mediated IL-1 $\beta$ -induced  $\beta$ -cell apoptosis? Our results show that IL-1 $\beta$  does not induce apoptosis in *ob/ob* mouse  $\beta$ -cells by directly activating voltage-gated L-type channels. In addition, the imidazoline compounds used in this study, known to increase [Ca<sup>2+</sup>]<sub>i</sub> in  $\beta$ -cells (3,8,38), do not stimulate but inhibit IL-1 $\beta$ -induced apoptosis. The inhibitory effect of 20 nmol/l deltamethrin on IL-1 $\beta$ -induced  $\beta$ -cell apoptosis points to the involvement of calcineurin in this process, which could be explained by participation of the protein phosphatase in the expression of iNOS. In Jurkat cells, it was demonstrated that calcineurin activates nuclear factor (NF)- $\kappa$ B

by enhancing inactivation of I $\kappa$ B $\alpha$  (inhibitor of NF- $\kappa$ B), thereby increasing NF- $\kappa$ B DNA binding activity (25). In rodent  $\beta$ -cells, IL-1 $\beta$  has been shown to induce rapid translocation of NF- $\kappa$ B from cytosol to the nucleus, and it has been suggested that NF- $\kappa$ B activation is necessary for IL-1 $\beta$ -induced NO production (18,39,40). Taking all our data into consideration, we propose the following mechanism for IL-1 $\beta$ -induced apoptosis in *ob/ob* mouse  $\beta$ -cells. IL-1 $\beta$  induces expression of iNOS, which involves activation of NF- $\kappa$ B. The expression of NF- $\kappa$ B is controlled by [Ca<sup>2+</sup>]<sub>i</sub> and involves activation of calcineurin. NO generated by the expressed iNOS may stimulate  $\beta$ -cell apoptosis by a mechanism involving PKG, probably through expression of *c-fos* (35) and leading to expression of proapoptotic proteins, and by a mechanism involving direct DNA damage (Fig. 7) (41).

One of the major findings in this study is that imidazoline compounds RX871024 and efaroxan, discussed as prototypes of a new generation of drugs against type 2 diabetes (3,4,7),



**FIG. 6.** The role of cGMP-dependent protein kinase in IL-1 $\beta$ -induced apoptosis. **A:** PKG inhibitor KT5823 (1  $\mu$ mol/l,  $n = 3$ ) suppresses IL-1 $\beta$ -induced apoptosis in  $\beta$ -cells. **B:** Effect of dibutyryl-cGMP on voltage-gated Ca<sup>2+</sup> currents in the  $\beta$ -cell. The cell, before and during exposure to dibutyryl-cGMP, displays similar whole-cell Ca<sup>2+</sup> current traces evoked by step depolarizing voltage pulses from a holding potential of -70 mV (upper panel). Compiled data show that the exposure to dibutyryl-cGMP did not alter whole-cell Ca<sup>2+</sup> currents ( $n = 7$ ) (lower panel). **C:** Effect of KT5823 on voltage-gated Ca<sup>2+</sup> currents in the pancreatic  $\beta$ -cell. The cell, subsequent to treatment with KT5823, exhibits no appreciable changes in whole-cell Ca<sup>2+</sup> current traces evoked by step depolarizing voltage pulses from a holding potential of -70 mV. Compiled data illustrate that the treatment with KT5823 did not cause significant changes in whole-cell Ca<sup>2+</sup> currents ( $n = 7$ ) (lower panel). Statistical significance between means was assessed by Student's *t* test for paired values. \* $P < 0.05$  relative to control; # $P < 0.05$  relative to IL-1 $\beta$ .



**FIG. 7.** Hypothetical mechanism involved in IL-1 $\beta$ -induced apoptosis in pancreatic  $\beta$ -cells. IL-1 $\beta$  induces expression of iNOS, which involves activation of the transcription factor NF- $\kappa$ B, controlled by the increase in [Ca<sup>2+</sup>], resulting from Ca<sup>2+</sup> influx through voltage-gated L-type Ca<sup>2+</sup> channels and involves activation of calcineurin. NO generated by the expressed iNOS may stimulate  $\beta$ -cell apoptosis by a mechanism involving PKG, probably through expression of *c-fos* and leading to expression of proapoptotic proteins and by a mechanism involving direct DNA damage. Imidazoline compounds RX871024 and efaroxan inhibit the expression of iNOS and thereby protect against IL-1 $\beta$ -induced apoptosis in pancreatic  $\beta$ -cells. Inhibition of apoptosis is achieved when the described pathways are blocked by an inhibitor of voltage-gated L-type Ca<sup>2+</sup> channels (D600), an inhibitor of calcineurin (deltamethrin [DM]), a blocker of NO synthase (L-NAME), and an inhibitor of PKG (KT5823).

can protect against IL-1 $\beta$ -induced apoptosis in pancreatic  $\beta$ -cells by inhibiting the expression of iNOS—a key element in IL-1 $\beta$ -induced apoptosis in  $\beta$ -cells. In addition, we have demonstrated that long-term exposure to the imidazoline compound RX871024 is not toxic to  $\beta$ -cells. These data, together with the observations published previously (3–7,42), suggest that imidazoline compounds should be explored as potential therapeutic agents for the treatment of both type 1 and type 2 diabetes.

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