Characterization of a K_{ATP} Channel–Independent Pathway Involved in Potentiation of Insulin Secretion by Efaroxan

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Efaroxan, like several other imidazoline reagents, elicits a glucose-dependent increase in insulin secretion from pancreatic β-cells. This response has been attributed to efaroxan-mediated blockade of K_{ATP} channels, with the subsequent gating of voltage-sensitive calcium channels. However, increasing evidence suggests that, at best, this mechanism can account for only part of the secretory response to the imidazoline. In support of this, we now show that efaroxan can induce functional changes in the secretory pathway of pancreatic β-cells that are independent of K_{ATP} channel blockade. In particular, efaroxan was found to promote a sustained sensitization of glucose-induced insulin release that persisted after removal of the drug and to potentiate Ca^{2+}-induced insulin secretion from electropermeabilized islets. To investigate the mechanisms involved, we studied the effects of the efaroxan antagonist KU14R. This agent is known to selectively inhibit insulin secretion induced by efaroxan, without altering the secretory response to glucose or KCl. Surprisingly, however, KU14R markedly impaired the potentiation of insulin secretion mediated by agents that raise cAMP, including the adenylate cyclase activator, forskolin, and the phosphodiesterase inhibitor isobutylmethyl xanthine (IBMX). These effects were not accompanied by any reduction in cAMP levels, suggesting an antagonistic action of KU14R at a more distal point in the pathway of potentiation. In accord with our previous work, islets that were exposed to efaroxan for 24 h became selectively desensitized to this agent, but they still responded normally to glucose. Unexpectedly, however, the ability of either forskolin or IBMX to potentiate glucose-induced insulin secretion was severely impaired in these islets. By contrast, the elevation of cAMP was unaffected by culture of islets with efaroxan. Taken together, the data suggest that, in addition to effects on the K_{ATP} channel, imidazolines also interact with a more distal component that is crucial to the potentiation of insulin secretion. This component is not required for Ca^{2+}-dependent secretion per se but is essential to the mechanism by which cAMP potentiates insulin release. Overall, the results indicate that the actions of efaroxan at this distal site may be more important for control of insulin secretion than its effects on the K_{ATP} channel. ***Diabetes* 50:340–347, 2001**

Imidazoline reagents, such as efaroxan (1–5), phentolamine (6–8), and RX871024 (9–14), can stimulate insulin secretion both in vivo and in vitro, and a number of structurally related compounds are currently under development as novel oral antihyperglycemic agents. Despite this, the mechanism of action of imidazoline insulin-secretagogues has not been fully established, and their principal molecular target has not been defined at the level of the β-cell. Convincing evidence is available that imidazolines can regulate the efflux of K^+ ions from the β-cell (14–18), and several studies have indicated that the pore-forming subunit of the K_{ATP} channel, Kir6.2, may be an imidazoline binding protein (18–20). This conclusion first emerged from electrophysiological studies performed in heterologous expression systems (18,19) but has also been confirmed more directly, using an affinity chromatographic approach in which endogenous Kir6.2 was selectively retained on an efaroxan-conjugated affinity matrix (20).

On this basis, it appears that one mechanism by which efaroxan and related reagents may stimulate insulin secretion involves blockade of K_{ATP} channels, with the subsequent gating of voltage-sensitive Ca^{2+} channels in response to membrane depolarization. In this respect, these reagents mirror the actions of sulfonylureas and can be considered to elicit the activation of a K_{ATP} channel–dependent pathway of insulin secretion.

However, the actions of efaroxan differ from those of many other depolarizing agents in one important respect, namely, that stimulation of insulin secretion by this compound is entirely glucose dependent (1,2,14). Thus, efaroxan is ineffective at subthreshold glucose concentrations (despite closing K_{ATP} channels under these conditions) but becomes progressively more effective as the glucose level rises between 4 and 12 mmol/l. This suggests that the stimulatory actions of efaroxan require an unidentified factor that acts in concert with a rise in β-cell Ca^{2+} and implies that blockade of K_{ATP} channels cannot account completely for the ability of the imidazoline to stimulate insulin secretion.

Additional support for a second, more distal site of action of imidazolines has come from β-cell desensitization studies performed with efaroxan. Initially, we demonstrated that culture of rat islets in the presence of efaroxan leads to selective loss of responsiveness (21,22) and, more recently, similar conclusions have been drawn from experiments with mouse islets (23) and clonal BRIN-BD11 cells (5,24). Significantly, it

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IBMX, isobutylmethyl xanthine or 3-isobutyl-1-methyl xanthine.

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has now become evident that this loss of responsiveness is not associated with any change in the ability of efaroxan to induce closure of β-cell K_{ATP} channels and that it does not result from inhibition of Ca^{2+} influx or a decrease in cytosolic Ca^{2+} levels (5,23). Hence, desensitization to efaroxan appears to involve the selective loss of a distal component of the insulin secretory pathway that is absolutely required for the functional activity of the imidazoline. This component cannot be associated with the K_{ATP}-dependent pathway of secretion per se, because there is no alteration in the insulin secretory response to glucose or KCl (2,21,22). In this respect, the desensitization caused by efaroxan differs markedly from the more generalized response that accompanies prolonged stimulation of islets with either glucose or sulfonylureas. In the case of both of these agents, desensitization results in attenuation of the secretory response to multiple stimuli and is associated with reduced Ca^{2+} influx (24–27).

More direct support for the concept that imidazolines can control insulin exocytosis independently of the membrane potential has emerged from studies with RX871024 (9). Like efaroxan, this agent has the ability to induce closure of β-cell K_{ATP} channels and to elicit a Ca^{2+}-dependent increase in insulin secretion. Surprisingly however, RX871024 also stimulates insulin secretion from electropermeabilized islets, in which the regulation of membrane K^+ permeability is lost (9). This provides strong evidence that RX871024 must be capable of influencing insulin secretion by interaction with components of the secretory pathway that lie distal to the K_{ATP} channel and are independent of the membrane potential.

A final piece of evidence that is consistent with the possibility that imidazolines control insulin secretion independently of the membrane potential derives from studies with KU14R. This reagent is the imidazole analog of efaroxan (28,29) and, like the parent molecule, it retains the ability to close β-cell K_{ATP} channels in the absence of other agents (29). However, despite this, KU14R does not stimulate insulin secretion from rat islets under any condition studied. Rather, it acts as a potent antagonist of the secretory response to efaroxan (2,13,28–30). This suggests that KU14R may interact with two sites in the β-cell. The first of these is associated with the K_{ATP} channel, but the second must lie more distally and is likely to be the site at which the antagonistic actions of KU14R are primarily manifest.

When considered as a whole, these results suggest that efaroxan (and other imidazolines) probably activates both a K_{ATP} channel–dependent and a K_{ATP} channel–independent pathway to regulate insulin exocytosis. In the present work, we have attempted to identify the K_{ATP} channel–independent pathway in rat islets and provide evidence that it involves components that are also important for potentiation of insulin secretion by cAMP. In addition, we show that the imidazoline-regulated step lies distal to the point at which cAMP-dependent events are controlled.

RESEARCH DESIGN AND METHODS

Islet isolation and incubation. Islets were isolated from male Wistar rats (180–230 g body weight; Charles River, Wilmington, MA) by collagenase digestion. The animals were maintained on a 12-h light-dark cycle and were provided with free access to food and water. Immediately after the rats were killed, the pancreata were removed and distended with bicarbonate-buffered physiological salt solution (31) supplemented with 4 mmol/l glucose and 1 mmol/l $CaCl_2$. Each organ was chopped finely and then transferred to a 25-ml conical flask containing 7 mg collagenase (Type XI; Sigma, Poole, U.K.). The mixture was shaken vigorously for 5–7 min at 37°C until the islets were free of contaminating exocrine tissue. They were then selected by hand under a dissecting microscope. Islets were hand picked twice on all occasions to minimize the contamination with exocrine fragments.

For desensitization experiments, islets were isolated and then divided into groups of 400. Each separate group was transferred to a sterile microfuge tube and washed twice with culture medium RPMI-1640 (Flow Laboratories, Irvine, U.K.) containing 10% fetal calf serum (Flow Laboratories) 2 mmol/l glutamine, 400 U/ml penicillin, and 200 µU/ml streptomycin (Sigma). The islets were then incubated in 3 ml of this medium in 5-ml Petri dishes for 24 h under either control conditions (no further additions) or after addition of 100 µmol/l efaroxan or KU14R (synthesized by Prof. Chris A. Ramsden, School of Chemistry and Physics, Keele University, Keele, U.K.). After the culture period, each group of islets was washed with 20 ml of bicarbonate-buffered physiological salt solution and allowed to equilibrate in this solution for 30 min. After this time, they were used in insulin secretion or cAMP studies.

For experiments with freshly isolated islets, the islets were equilibrated for 30 min after isolation (in bicarbonate-buffered physiological salt solution) and then used immediately for secretion studies or cAMP measurements. For static incubation experiments, groups of three individual islets were incubated in 200 µl of bicarbonate-buffered physiological salt solution supplemented with 1 mg/ml bovine serum albumin (Wilfred Smith, Edgeware, U.K.) in a microtiter plate. The plate was incubated at 37°C for 1 h and the medium sampled for measurement of insulin by radioimmunoassay. Guinea-pig anti–bovine insulin serum for the radioimmunoassay was obtained from Sigma and ^{125}I-insulin from Biogenesis. Bound insulin and free insulin were separated using Sac-Cel antibody-coated beads (IDS, Tyne and Wear, U.K.).

For perifusion studies, groups of 100 islets were placed in male-female luer connectors plugged with glass wool. The chambers containing the islets were then connected to a peristaltic pump (Anachem, Luton, U.K.) and perifused with bicarbonate-buffered physiological salt solution supplemented with 1 mg/ml bovine serum albumin at a flow rate of 1 ml/min. The islets and medium reservoir were placed inside a heated chamber at 37°C and perifused for 30 min before the start of the experiment. Test reagents were introduced either directly into the bulk medium or were delivered via a second peristaltic pump set at a flow rate of 10 µl/min. Effluent medium was collected and sampled for measurement of insulin by radioimmunoassay.

Electropermeabilization of islets. For studies with electropermeabilized islets, the islets were transferred into ice-cold bicarbonate-buffered physiological salt solution immediately upon isolation and the subsequent permeabilization was carried out at 4°C. The islets were washed three times in ice-cold permeabilization buffer (140 mmol/l potassium glutamate, 2 mmol/l $MgSO_4$, 1 mmol/l EGTA, 15 mmol/l HEPES, pH 7.0) and collected by centrifugation at 1,000*g* for 30 s. They were transferred to precooled cuvettes containing 0.8 ml of ice-cold permeabilization buffer and exposed for 6×200 µs to an electric field of 3.5 kV/cm (Gene Pulser; BioRad, Hertfordshire, U.K.). The islets were gently resuspended between each pulse and finally washed twice in a large volume of permeabilization buffer before placing in incubation vials.

Groups of five individual islets were incubated at 37°C in 0.5 ml of permeabilization buffer containing ATP (0.2 or 2 mmol/l according to the experiment) and an ATP regenerating system (2 mmol/l creatine phosphate, 10 U/ml creatine phosphokinase) plus appropriate amounts of $CaCl_2$ to achieve free Ca^{2+} concentrations of 50 nmol/l or 10 µmol/l. These were calculated according to the method of Fohr et al. (32). After incubation for 15 min, samples of medium were withdrawn for assay of insulin by radioimmunoassay.

Measurement of islet cAMP levels. For estimation of islet cAMP levels, groups of 10 islets were incubated with test substances for 30 min at 37°C. Samples of the medium were removed for measurement of insulin by radioimmunoassay and the islets were centrifuged at 3,000*g* for 2 min. The pellet was disrupted ultrasonically in 100 µl of ice-cold 6% trichloroacetic acid and centrifuged briefly. The supernatant was extracted four times with five volumes of water-saturated diethyl ether. The aqueous phase was then lyophilized before resuspension in 250 µl of assay buffer provided with the cAMP Biotrak Enzyme immunoassay kit (Amersham International, Buckinghamshire, U.K.). The assay was performed using the nonacetylated procedure according to the manufacturer's instructions.

Statistical analysis. The statistical significance of test results was determined either by analysis of variance or by Student's *t* test. Results were considered to be statistically different when $P < 0.05$.

RESULTS

Effects of efaroxan on insulin secretory responses from intact and permeabilized rat islets. One important feature of the blockade of K_{ATP} channels in the β-cell by

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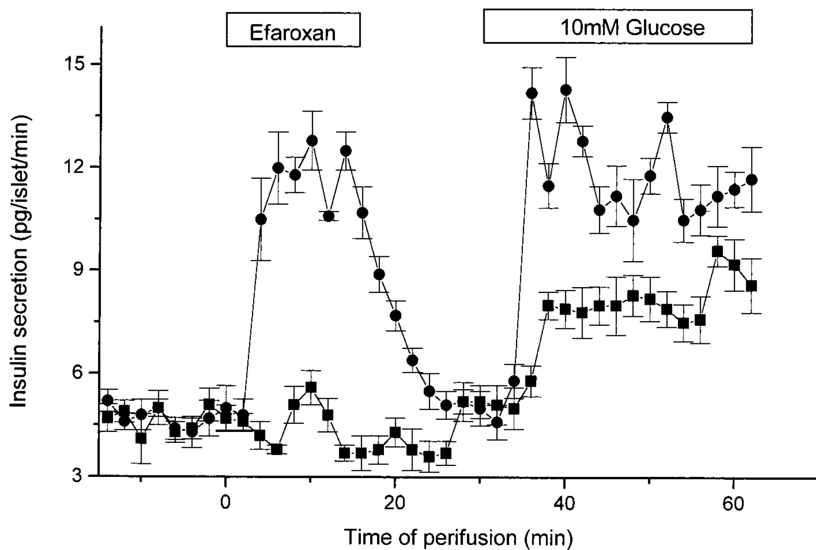

FIG. 1. Sensitization of glucose-induced insulin secretion in islets exposed to efaroxan. Groups of 100 islets were perifused in medium containing 5 mmol/l glucose for 30 min before t = 0. Control islets (■) were then perifused with 5 mmol/l glucose for a further 30 min, whereas test groups (●) were exposed to efaroxan for 15 min from t = 0. After this time, the efaroxan was removed. From t = 30 min, 10 mmol/l glucose was introduced into all channels of the perifusion system for the duration of the experiment. Samples of effluent medium were collected at 1-min intervals and assayed for insulin by radioimmunoassay. Each point represents the mean value ± SE from four separate islet preparations.

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efaroxan is that removal of the reagent is associated with rapid recovery of channel activity (5,14,29). This has been confirmed in a number of studies and would be expected to result in an equally rapid decline in insulin secretion when efaroxan is removed from the incubation medium. This prediction was confirmed by the experiments shown in Fig. 1 that reveal that, in perifused islets, efaroxan-induced insulin release was readily—and completely—reversible upon removal of the imidazoline. However, a surprising observation was made when the islets were subsequently stimulated with a submaximal (10 mmol/l) concentration of glucose. Islets that had been previously treated with efaroxan exhibited a much-enhanced response to 10 mmol/l glucose (Fig. 1). This response was manifest as a more rapid rise in insulin secretion, which then achieved a higher rate than that measured in control islets. Insulin secretion was sustained at this elevated level throughout the remainder of the perifusion period (Fig. 1). Thus, even though efaroxan was no longer present in the perifusion medium during the period of glucose challenge, islets that had been exposed to the drug earlier in the experiment exhibited a markedly enhanced secretory response to 10 mmol/l glucose.

By contrast with these findings, preexposure of islets to efaroxan for 20 min did not lead to any increase in the subsequent secretory response to forskolin, suggesting that the sensitization mechanism may be associated principally with glucose-sensitive components of the secretory pathway (Control islets: 5 mmol/l glucose alone, 1.50 ± 0.10 ng · islet^{-1} · h^{-1}; 5 mmol/l glucose plus 5 µmol/l forskolin, 2.58 ± 0.25 [P < 0.001]; Islets preexposed to 100 µmol/l efaroxan: 5 mmol/l glucose alone, 1.46 ± 0.12; 5 mmol/l glucose plus 5 µmol/l forskolin, 2.40 ± 0.22).

These results imply that treatment of islets with efaroxan had induced a change or changes in the glucose-sensitive secretory pathway that was independent of K_{ATP} channel blockade. This, in turn, suggests that efaroxan must exert some control over insulin secretion by virtue of an action that is independent of the membrane potential. To confirm the validity of this conclusion, we studied the effects of efaroxan in islets rendered permeable to ions and small molecules by exposure to high-voltage electric discharge. The extent of islet cell permeabilization was assessed by trypan blue staining and it was established that exposure of the isolated islets to 6 × 200 µs pulses, at a field strength of 3.5 kV/cm, was sufficient to induce permeabilization of >90% of the cells. Incubation of these permeabilized islets in a buffer containing 10 µmol/l Ca^{2+} then resulted in a marked increase in insulin secretion above that measured when the medium contained 50 nmol/l Ca^{2+} (Fig. 2). This response was still apparent when the ATP concentration was reduced from 2 to 0.2 mmol/l (Fig. 2) but was absent when ATP was omitted entirely (not shown).

Treatment of permeabilized islets with efaroxan (100 µmol/l) resulted in a further significant (P < 0.01) rise in insulin release, beyond that elicited by Ca^{2+} alone, when the incubation buffer contained 2 mmol/l ATP (Fig. 2). However, this

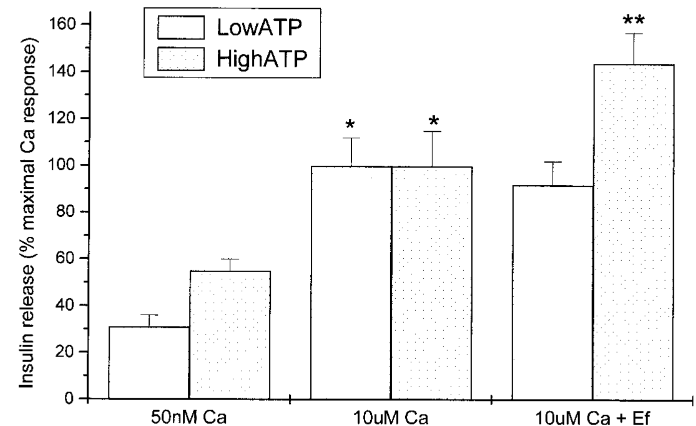

FIG. 2. Stimulation of insulin secretion from electropermeabilized islets by efaroxan. Groups of isolated rat islets were permeabilized by exposure to a high-voltage electric field as described in RESEARCH DESIGN AND METHODS. They were then incubated in the presence of either 50 nmol/l or 10 µmol/l Ca^{2+} in the presence or absence of efaroxan (Ef) (100 µmol/l). ATP was included at either 0.2 mmol/l ("low ATP"; ▨) or 2 mmol/l ("high ATP"; ▩). Insulin secretion was measured after incubation for 15 min at 37°C. The rate of insulin release induced by 10 µmol/l Ca^{2+} was set at 100%. The mean value under these conditions averaged 1.42 ± 0.11 ng · islet^{-1} · h^{-1} in the presence of 0.2 mmol/l ATP and 1.95 ± 0.18 ng · islet^{-1} · h^{-1} in the presence of 2 mmol/l ATP. Results show mean values ± SE from four separate islet preparations. *P < 0.01 relative to 50 nmol/l Ca^{2+}; **P < 0.01 relative to 10 µmol/l Ca^{2+} in the absence of efaroxan.

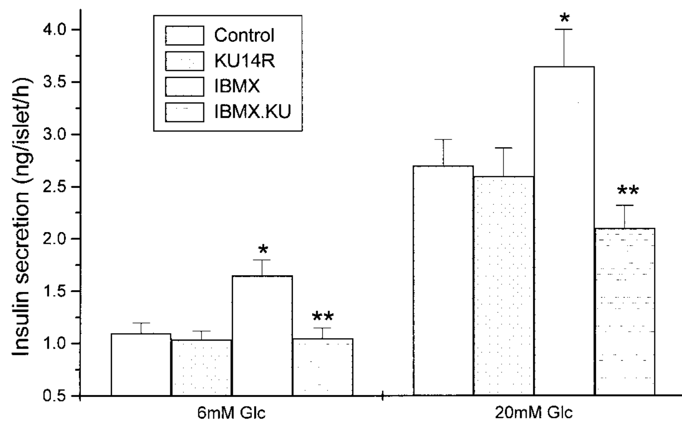

FIG. 3. Blockade of IBMX-induced insulin secretion by KU14R. Groups of three freshly isolated islets were incubated in the presence of either 6 or 20 mmol/l glucose. KU14R (100 μmol/l) and IBMX (50 μmol/l) were included as shown. Insulin secretion was measured after incubation of the islets for 1 h. Results are shown as mean values ± SE ($n = 6$) from a representative experiment that was repeated three times with similar results. *$P < 0.01$ relative to glucose alone; **$P < 0.01$ relative to IBMX in the absence of KU14R.

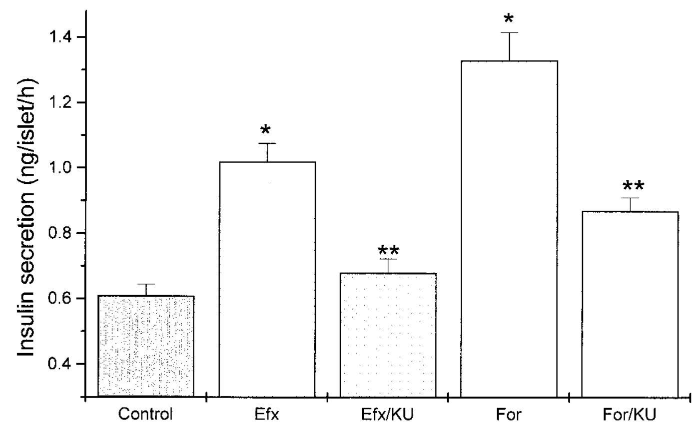

FIG. 4. Effects of KU14R on insulin secretion induced by efaroxan or forskolin. Groups of freshly isolated islets were incubated in the presence of 6 mmol/l glucose (■) or 6 mmol/l glucose plus test reagents. Efaroxan (Efx) and KU14R (KU) were present at 100 μmol/l, and forskolin (For) was used at 5 μmol/l. Insulin secretion was measured after incubation of the islets for 1 h. Results are shown as mean values ± SE ($n = 6$) from a representative experiment that was repeated twice with similar results. *$P < 0.001$ relative to glucose alone; **$P < 0.001$ relative to equivalent condition in the absence of KU14R.

response was not seen when the ATP concentration was reduced to 0.2 mmol/l, even though the islets responded to the Ca^{2+} stimulus under these conditions (Fig. 2). Efaroxan failed to increase insulin secretion from islets incubated with 2 mmol/l ATP in the presence of 50 nmol/l Ca^{2+} (not shown).

When intact islets were substituted for electropermeabilized islets under identical incubation conditions, neither 10 μmol/l Ca^{2+} nor efaroxan elicited any increase in insulin release irrespective of the ATP concentration (not presented). This result confirms that the ability of efaroxan to potentiate Ca^{2+}-induced insulin secretion requires cell permeabilization and is independent of the β-cell membrane potential.

Effects of imidazoline reagents on potentiation of insulin secretion by cAMP-dependent mechanisms. Because efaroxan can potentiate Ca^{2+}-induced insulin secretion by a mechanism that is independent of membrane potential, we investigated whether the actions of the imidazoline are mediated via a pathway that is also used by other potentiators of secretion. Previous work has shown that protein kinase C is not required for the secretory actions of imidazolines (7,12), and thus we explored the possible involvement of a protein kinase A–dependent mechanism.

For this work, we took advantage of the recent observation that a close structural analog of efaroxan, KU14R, acts as a functional antagonist of imidazoline-mediated responses in the pancreatic β-cell (2,13,28–30). Previous studies have shown that, despite blocking K_{ATP} channels, KU14R does not modify the rate of glucose-induced insulin secretion from rat islets (29,30). In accord with this finding, KU14R failed to alter the response to either 6 or 20 mmol/l glucose in the current work (Fig. 3). By contrast, KU14R significantly impaired the ability of 100 μmol/l efaroxan to potentiate glucose-induced insulin secretion (Fig. 4), confirming its antagonist activity. More strikingly, it also inhibited the potentiation mediated by either the phosphodiesterase inhibitor isobutylmethyl xanthine (IBMX) (Fig. 3) or the adenylate cyclase activator forskolin (Fig. 4) in islets not exposed to efaroxan. KU14R did not, however, reduce insulin secretion stimulated by depolarization of the β-cells with 50 mmol/l KCl (not shown; 29,30). These effects of KU14R were not secondary to any change in islet cAMP levels since the ability of forskolin or IBMX to increase islet cAMP was not influenced by KU14R (Fig. 5). Moreover, like efaroxan (14), KU14R failed to alter islet cAMP levels directly (Fig. 5).

These data suggest that efaroxan and KU14R can both interact with a component of the exocytotic pathway that is involved in potentiation of insulin secretion. Binding of efaroxan results in activation of this pathway, whereas KU14R antagonizes the response. This imidazoline-sensitive component is clearly not required for Ca^{2+}-induced secretion but does contribute to the effects of cAMP.

Effects of desensitization with efaroxan on potentiation of insulin secretion by agents raising cAMP. Because the aforementioned studies have implicated an imidazoline-sensitive component in the pathway by which cAMP potentiates insulin secretion, we investigated whether desensitization of islets to imidazoline agonists leads to any impairment of their response to agents acting via cAMP-dependent mechanisms. In these experiments, rat islets were cultured with efaroxan for 24 h to induce a functional desensitization. The success of this procedure is illustrated in Fig. 6. Efaroxan induced a marked potentiation of glucose-induced insulin secretion from islets cultured under control conditions but, as expected (2,5,21,22,24), it failed to enhance the response from islets cultured in the presence of the drug. Such islets were, therefore, desensitized to efaroxan. The secretory response to 20 mmol/l glucose was unaffected in efaroxan-desensitized islets (Fig. 6), consistent with the view that Ca^{2+}-dependent exocytosis does not require the imidazoline-sensitive component. By contrast, the ability of the adenylate cyclase activator forskolin to enhance insulin release was markedly impaired in islets cultured with efaroxan (Fig. 6).

This phenomenon was further investigated in a set of experiments in which islet cAMP levels were elevated by different means (Fig. 7). As expected, addition of the phospho-

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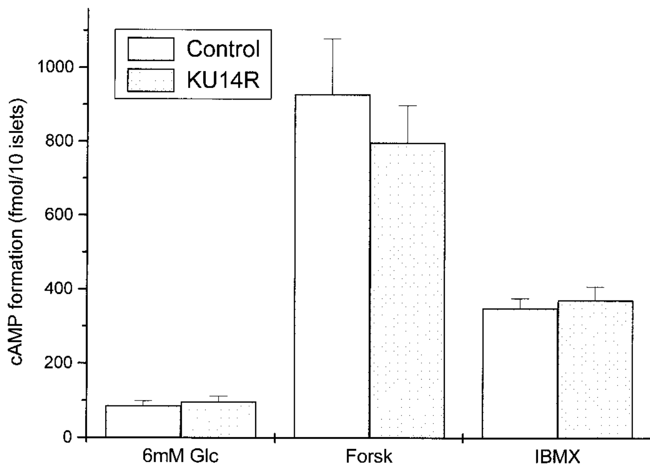

FIG. 5. Effects of KU14R on elevation of islet cAMP levels by IBMX and forskolin. Groups of freshly isolated islets were incubated in the presence of 6 mmol/l glucose alone or 6 mmol/l glucose plus either forskolin (5 µmol/l) or IBMX (50 µmol/l) in the absence (▨) or presence (☒) of 100 µmol/l KU14R, as shown. cAMP levels were measured as described in RESEARCH DESIGN AND METHODS after incubation of the islets for 30 min. Results represent mean values ± SE from three separate islet preparations.

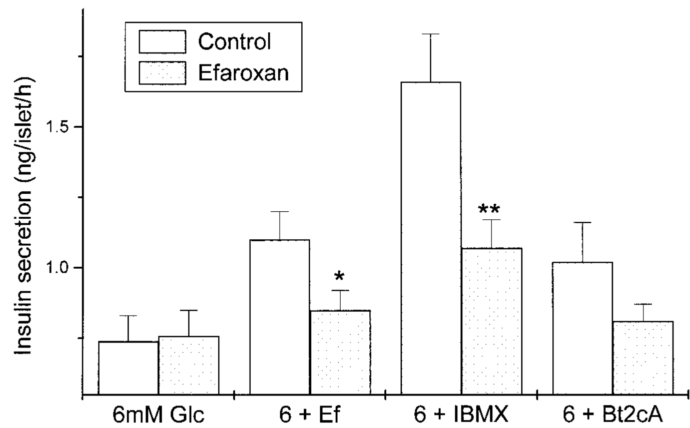

FIG. 7. Effect of culture in the presence of efaroxan on stimulation of insulin secretion by IBMX and dibutyryl-cAMP. Groups of islets were cultured for 24 h in either the absence (▨) or presence (☒) of efaroxan as described in RESEARCH DESIGN AND METHODS. After the culture period, the islets were washed and incubated for 1 h with 6 mmol/l glucose, 6 mmol/l glucose plus efaroxan (Ef) (100 µmol/l), 6 mmol/l glucose plus IBMX (100 µmol/l), or 6 mmol/l glucose plus dibutyryl-cAMP (Bt2cA) (2 mmol/l). Results represent mean values ± SE (n = 8) from an experiment that was repeated twice with similar results. *P < 0.05 relative to islets cultured under control conditions; **P < 0.001 relative to islets cultured under control conditions.

diesterase inhibitor IBMX (100 µmol/l) to control islets caused a marked potentiation of glucose-induced insulin secretion. However, in islets previously cultured for 24 h with efaroxan, this response was virtually absent (Fig. 7). Moreover, the small increase in secretion induced by exogenous dibutyryl cAMP was also inhibited by preculture in the presence of efaroxan, suggesting that the inhibitory effect is not due to any change in the concentration of cAMP after efaroxan treatment. This was confirmed by direct measurements of cAMP (Fig. 8), which revealed that both forskolin and IBMX were able to promote an equivalent rise in cAMP in control and efaroxan-cultured islets. These studies also confirm previous data (14) showing that acute exposure of islets to efaroxan does not cause an increase in islet cAMP levels. In addition, they reveal that longer-term treatment with the drug fails to alter islet cAMP (Fig. 8).

In a third set of desensitization experiments, the effect of long-term treatment with KU14R on imidazoline-mediated

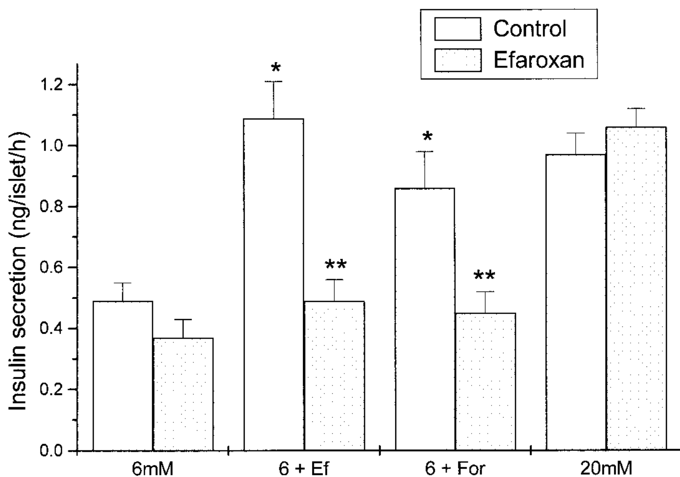

FIG. 6. Effect of culture in the presence of efaroxan on stimulation of insulin secretion from rat islets. Groups of islets were cultured for 24 h in either the absence (▨) or presence (☒) of efaroxan as described in RESEARCH DESIGN AND METHODS. After the culture period, the islets were washed and incubated for 1 h with 6 mmol/l glucose, 6 mmol/l glucose plus efaroxan (Ef) (100 µmol/l), 6 mmol/l glucose plus forskolin (For) (5 µmol/l), or 20 mmol/l glucose. Results represent mean values ± SE (n = 8) from an experiment that was repeated four times with similar results. *P < 0.001 relative to 6 mmol/l glucose alone; **P < 0.001 relative to islets cultured under control conditions.

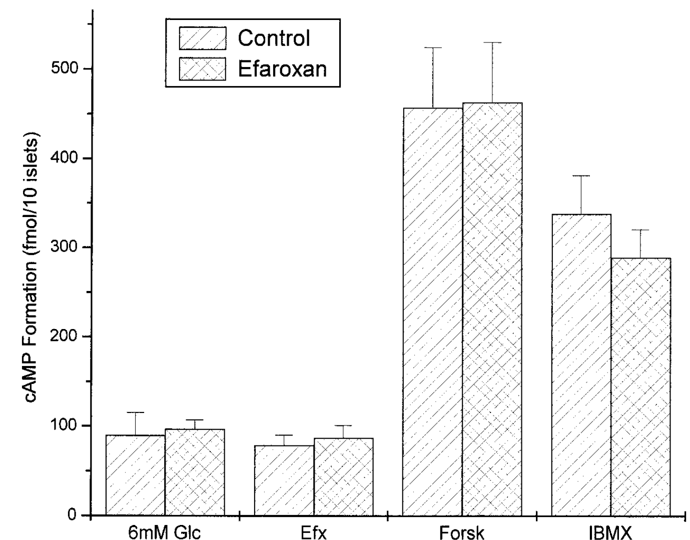

FIG. 8. Effect of culture in the presence of efaroxan on islet cAMP levels. Groups of islets were cultured for 24 h in either the absence (▨) or presence (☒) of efaroxan as described in RESEARCH DESIGN AND METHODS. After the culture period, the islets were washed and incubated with 6 mmol/l glucose alone, 6 mmol/l glucose plus 100 µmol/l efaroxan (Ef), 6 mmol/l glucose plus 5 µmol/l forskolin (Forsk), or 6 mmol/l glucose plus IBMX (100 µmol/l) for 30 min. They were then centrifuged and their cAMP content measured. Results represent mean values ± SE from four separate islet preparations.

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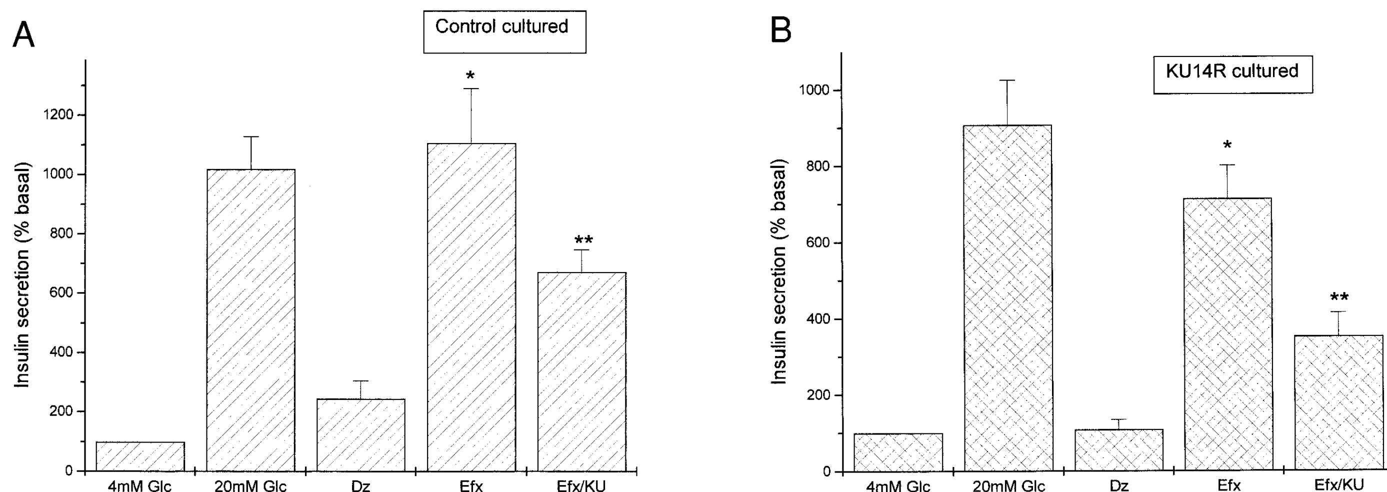

FIG. 9. Effect of culture in the presence of KU14R on stimulation of insulin secretion from rat islets. Groups of islets were cultured for 24 h in either the absence (i.e., control cultured) (*A*) or presence (i.e., KU14R cultured) (*B*) of 100 μmol/l KU14R as described in RESEARCH DESIGN AND METHODS. After the culture period, the islets were washed and incubated for 1 h with 4 mmol/l glucose, 20 mmol/l glucose, or 20 mmol/l glucose plus either diazoxide alone (Dz) (250 μmol/l), diazoxide plus efaroxan (Efx) (100 μmol/l), or diazoxide plus efaroxan plus KU14R (KU) (100 μmol/l). Results represent mean values for insulin secretion relative to 4 mmol/l glucose (100%) ± SE from three separate islet preparations. **P* < 0.001 relative to diazoxide in the absence of efaroxan; ***P* < 0.001 relative to efaroxan in the absence of KU14R.

responses was studied. As indicated above, KU14R blocks K_{ATP} channels when administered to islets in the absence of efaroxan. Thus, if long-term K_{ATP} channel blockade underlies the loss of sensitivity to efaroxan, then it follows that KU14R should be capable of causing β-cell desensitization. However, after exposure of rat islets to KU14R for 24 h, they still responded normally to glucose and diazoxide (Fig. 9). In these islets, efaroxan was still capable of preventing the inhibition of insulin secretion caused by diazoxide (a response that is lost in islets desensitized to the imidazoline [21,22]) and KU14R antagonized the efaroxan response just as effectively as in islets cultured under control conditions (Fig. 9). Thus, there was no evidence for loss of sensitivity to imidazolines after long-term KU14R treatment.

DISCUSSION

Until recently, the effects of efaroxan on insulin secretion were attributed solely to its well-described ability to close K_{ATP} channels, a response observed both in whole-cell recordings and in isolated patches of membrane (1,2,5,14,23,28,29). The finding that efaroxan can block K_{ATP} channels in membrane patches provides strong evidence that the binding site responsible for mediating this action must reside within the membrane domain. In support of this, electrophysiological and biochemical evidence has confirmed that the pore-forming subunit of the K_{ATP} channel, Kir6.2, contains an imidazoline binding site (18–20). It seems probable, therefore, that the interaction of efaroxan with Kir6.2 leads to a change in the conductance properties of the channel, resulting in a reduction in K^+ efflux from the β-cell. This, in turn, promotes membrane depolarization, Ca^{2+} influx, and a rise in insulin secretion.

However, this relatively simple model is not easily reconciled with a number of features of the secretory response to efaroxan. For example, patch-clamp studies show that concentrations of efaroxan in the low micromole-per-liter range can elicit complete blockade of β-cell K_{ATP} channels (14), whereas studies of insulin secretion have revealed that, even at much higher concentrations, its effects are strictly glucose dependent (2,14,28). Thus, there is no direct correlation between K_{ATP} channel closure and the insulin secretory response in islets treated with efaroxan. More strikingly, recent work has revealed that culture of islets with efaroxan for 18–24 h leads to functional desensitization (2,5,21,22,24), but that this is not associated with any change in the ability of imidazolines to close β-cell K_{ATP} channels (5). Additionally, there is no reduction in the level of intracellular Ca^{2+} after membrane depolarization in these islets (5,23). Thus, islets that are desensitized to efaroxan exhibit normal ionic responses to the drug, but the ionic changes become uncoupled from regulation of insulin secretion. These results imply that the ability of efaroxan to regulate K^+ and Ca^{2+} fluxes may be secondary to a more important action involving other components of the exocytotic pathway.

The above-listed considerations are supported and extended by the results obtained in the present work, in which it has been established that efaroxan can elicit a Ca^{2+}-dependent increase in insulin secretion from islet cells rendered permeable to small molecules, by exposure to high-voltage electric discharge (Fig. 2). In such islets, control of plasma membrane ion fluxes is lost and Ca^{2+} can enter the cells freely from the incubation medium. If the primary action of efaroxan was to block K_{ATP} channels in the β-cell, then it would be anticipated that the molecule would be ineffective under conditions in which these channels are unable to exert an influence on insulin secretion. This, however, was not the case (Fig. 2), because in electropermeabilized islets, efaroxan enhanced Ca^{2+}-dependent secretion. Similar results have been obtained previously with the imidazoline RX871024 (9).

More detailed analysis revealed that the secretory response to efaroxan in electropermeabilized islets was observed only when the ATP concentration was in the millimole-per-liter range. This finding cannot reflect a deficiency in the energy available from exogenous ATP since, although exocytosis is an ATP-dependent process, an ATP-regenerating system was included in the incubation buffer and Ca^{2+} was able to elicit a significant increase in insulin release when 0.2 mmol/l ATP was

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available (Fig. 2). This, in turn, implies that efaroxan may require ATP for an alternative reason other than as an energy source. One possibility is that ATP is required for "priming" of secretory granules, which are then competent for release (33). However, this explanation appears unlikely, because a rise in Ca^{2+} elicited an increase in secretion at low (0.2 mmol/l) ATP, suggesting that this concentration of the nucleotide was sufficient to support granule priming.

An alternative explanation is suggested by the recent report of Takahashi et al. (34), who argue that protein phosphorylation may be a necessary prerequisite to exocytosis in the β-cell and that donation of a phosphate group by ATP could play a role in the most distal steps of the process. However, in their study, ATP-dependent phosphorylation was reported to be necessary for Ca^{2+}-dependent exocytosis, whereas in the present work the higher ATP threshold was required only to support efaroxan-induced secretion. Moreover, Takahashi et al. (34) have proposed that the ATP-dependence of exocytosis reflects a requirement for generation of cAMP, and it is argued that the change in protein phosphorylation is mediated by protein kinase A (34,35). This mechanism cannot account for the effects of efaroxan on insulin secretion, since the imidazoline does not alter islet cAMP levels (Fig. 8; [14]) and its secretory effects are not impaired by inhibitors of protein kinase A (12). Furthermore, in permeabilized islets, any endogenous cAMP would be lost from the cells very rapidly by dilution into the incubation medium.

Although these considerations suggest that the effects of efaroxan on insulin secretion do not directly involve cAMP-dependent phosphorylation, there are certain features of the response that are consistent with the possibility that efaroxan and cAMP potentiate insulin release via a common pathway. The most persuasive evidence derives from the efaroxan-desensitization studies. As expected from earlier work, islets that had been exposed to efaroxan for 24 h displayed a normal secretory response to glucose but a reduced response to efaroxan. Because this response cannot be attributed to any change in the ability of efaroxan to block K_{ATP} channels in desensitized islets, it must reflect a functional modification to a more distal component of the potentiation pathway. Importantly, we have now observed that islets desensitized to efaroxan also exhibited a markedly reduced secretory response to agents that raise cAMP. This finding was entirely unexpected, because there has been no previous indication of any interaction between efaroxan and the cAMP system in β-cells. The response was not secondary to an alteration in cAMP levels in efaroxan-desensitized islets (Fig. 8), suggesting that the stimulatory mechanisms mediating the effects of efaroxan and cAMP must converge downstream of cAMP generation (and protein kinase A activation).

The validity of this conclusion is supported by data obtained with the antagonist KU14R. This reagent is a close structural analog of efaroxan (it differs only in the possession of an imidazole rather than an imidazoline ring [28,29]), which can induce the closure of K_{ATP} channels (29). Despite this, KU14R did not potentiate glucose-induced insulin release (Fig. 3), but rather it antagonized the secretory effects of efaroxan (Fig. 4) and closely related imidazolines (29). Surprisingly, however, KU14R also attenuated the potentiation of insulin secretion arising when freshly isolated rat islets were incubated with either IBMX (Fig. 3) or forskolin (Fig. 4). This effect was not secondary to a reduction in cAMP (Fig. 5) and it cannot readily be attributed to binding of KU14R to components of the K_{ATP} channel.

Taken together, these observations are consistent with the possibility that β-cells express a distally located imidazoline binding site that is involved in the regulation of exocytosis and is sensitive to both efaroxan (agonistic) and KU14R (antagonistic). This site lies downstream of the K_{ATP} channel but occupies a critical position in a pathway by which exocytosis is potentiated. The results further suggest that cAMP-dependent mechanisms involved in potentiation converge at or before the imidazoline binding site. Chronic activation of the site by efaroxan leads to loss of function, perhaps by promoting degradation of a key target protein (by analogy with the effects of phorbol esters during long-term activation of protein kinase C [36]). KU14R acts as a functional antagonist at this site and, as a consequence, it impairs the ability of imidazoline agonists and agents that raise cAMP to potentiate Ca^{2+}-dependent insulin secretion.

It is clear that the putative imidazoline binding site responsible for control of exocytosis remains to be identified, but the present results indicate that this site is also subject to a further level of regulation. Thus, in marked contrast to the loss of response that accompanies long-term treatment of islets with efaroxan, a shorter period of exposure resulted in sustained sensitization of the islets to 10 mmol/l glucose (Fig. 1). This effect was not due to any significant delay in the rate of dissociation of the imidazoline from its binding site, since withdrawal of efaroxan was followed by an immediate and rapid decline in insulin secretion. It seems likely, therefore, that binding of efaroxan had induced a stable modification to the target protein that persisted after drug withdrawal and that resulted in sensitization of the β-cells to glucose. Preexposure of islets to efaroxan did not, however, lead to any sensitization of the insulin secretory response to forskolin.

The molecular basis of this sensitizing action of efaroxan cannot be deduced with certainty; however, recent results suggest that certain other imidazolines, including S22068 (37) and moxonidine (38), can also promote a similar response. Sensitization of islets to glucose has also been reported after short-term exposure to sulfonylureas (39) and, in this case, was attributed to an increased association of hexokinase with mitochondria. Whether this mechanism could account for the sensitization observed with efaroxan remains to be determined, but it is noteworthy that there is controversy about the levels of expression of hexokinase in β-cells (40), and, in some studies, sulfonylureas have been reported to decrease mitochondrial hexokinase binding (41).

In summary, the results presented here reveal that efaroxan can regulate insulin secretion by mechanisms that are independent of the control of β-cell membrane potential. This finding suggests that closure of the K_{ATP} channel may not be the principal mechanism of action of imidazoline compounds in the β-cell and indicates that their functional effects could be mediated by an additional imidazoline binding protein (or proteins) located more distally within the exocytotic pathway. Characterization of this site should facilitate the development of a new generation of insulin secretagogues targeted to the direct control of exocytosis.

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