Desensitization of Insulin Secretion by Depolarizing Insulin Secretagogues

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Prolonged stimulation of insulin secretion by depolarization and Ca^{2+} influx regularly leads to a reversible state of decreased secretory responsiveness to nutrient and nonnutrient stimuli. This state is termed "desensitization." The onset of desensitization may occur within 1 h of exposure to depolarizing stimuli. Desensitization by exposure to sulfonylureas, imidazolines, or quinine produces a marked cross-desensitization against other ATP-sensitive K^+ channel (K_{ATP} channel)-blocking secretagogues. However, desensitized β-cells do not necessarily show changes in K_{ATP} channel activity or Ca^{2+} handling. Care has to be taken to distinguish desensitization-induced changes in signaling from effects due to the persisting presence of secretagogues. The desensitization by depolarizing secretagogues is mostly accompanied by a reduced content of immunoreactive insulin and a marked reduction of secretory granules in the β-cells. In vitro recovery from a desensitization by the imidazoline efaroxan was nearly complete after 4 h. At this time point the depletion of the granule content was partially reversed. Apparently, recovery from desensitization affects the whole lifespan of a granule from biogenesis to exocytosis. There is, however, no direct relation between the β-cell granule content and the secretory responsiveness. Even though a prolonged exposure of isolated islets to depolarizing secretagogues is often associated with the occurrence of ultrastructural damage to β-cells, we could not find a cogent link between depolarization and Ca^{2+} influx and apoptotic or necrotic β-cell death. *Diabetes* 53 (Suppl. 3):S140–S150, 2004

DESENSITIZATION OF INSULIN SECRETION: DEFINITION AND POSSIBLE PATHOGENIC ROLE

A state of decreased secretory responsiveness to physiological or pharmacological stimuli of insulin secretion evoked by prior exposure to effective concentrations of the same stimuli is called "desensitization." The desensitized state can be induced by nutrient and nonnutrient stimuli and is readily reversible upon removal of the stimuli. The glucose-induced desensitization as defined above has to be distinguished from the more popular term "glucose toxicity." Glucose toxicity infers a damaging effect, leading not only to functional changes, but also to structural alterations in the β-cells (1,2), which may progress to the loss of β-cell mass occurring in advanced type 2 diabetes (3,4).

The concept of glucose desensitization of insulin secretion has attracted considerable interest in the last few years because it may be relevant for the natural history of type 2 diabetes, representing the link between functional abnormalities of insulin secretion and overt β-cell failure. The desensitization induced by non-nutrient stimuli is of relevance for the still unresolved problem of why the efficacy of treatment with oral antidiabetic agents decreases with time (5,6), but may also contribute to the understanding of glucose desensitization. In particular, it is of relevance for the question of whether a pharmacological stimulation of insulin secretion may actually accelerate the progression toward β-cell failure.

GLUCOSE DESENSITIZATION: CONCEPTS AND CONTROVERSIES

A more detailed review on the desensitization by nutrient secretagogues is beyond the scope of the present article (for a recent overview, see Rustenbeck [7]). However, the basic features are well worth presenting because the insights gained from investigations on glucose desensitization are also shaping the lines of thought in investigations of the desensisitization by nonnutrient, pharmacological insulin secretagogues. The main feature that distinguishes glucose desensitization from glucose toxicity is the reversibility of the diminished secretory responsiveness (8). This reversibility can be suprisingly rapid: it was described to occur in vitro within minutes after changing from a moderately elevated glucose concentration back to a nonstimulatory concentration (9). However, 7–14 days were necessary for cultured islets to recover from a desensitization by a maximally effective glucose concentration (10).

Together with observations that in vitro– and in vivo–induced glucose desensitization was specific or at least preferential for glucose and nutrient secretagogues (10–12), the fast reversibility suggests that the main alterations that cause the decrease in secretion are located proximally in stimulus-secretion coupling. Whether this is in the process of glucose recognition, creating a state of "glucose nonsense" of the β-cell (13) or in those steps of energy metabolism that are common for all nutrient secretagogues, is an open question.

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$[Ca^{2+}]_c$, cytosolic free calcium concentration; K_{ATP} channel, ATP-sensitive K^+ channel.

Opposed to the concept of desensitized glucose recognition is the view that during prolonged exposure to elevated glucose concentrations, β-cells secrete more insulin than they are able to deliver to the exocytotic machinery. The concept of the "exhausted" or "overworked" β-cell (14) is supported by the observations that the insulin content and/or granule number of in vitro– and in vivo–desensitized β-cells is often reduced and that the desensitization by glucose can also diminish the response to other stimuli. There are conflicting results as to whether the decreased content is due to an absolutely lowered insulin synthesis (15) or to an imbalance between an increased supply and an even more increased demand (16,17).

Thus, one could define desensitization as a state of decreased glucose responsiveness when there is no global reduction in insulin or granule content of the β-cells. This definition is practically identical with that of the "third phase of insulin secretion" as given by Grodsky (18). It could be possible that the exhausted β-cell is not a so much an alternative concept of desensitization but an advanced state of desensitization. The lack of releasable insulin implicit in this definition requires the presence of morphological alterations: at least a partial degranulation of the β-cell should be recognizable. These alterations have to be fully reversible in the normal course of stimulus-secretion coupling. Similarly, the "overstimulated" β-cell (19), for which the definition emphasizes an enhanced secretory activity as the relevant mechanism, may represent a more advanced stage of desensitization. The further progression to β-cell damage, possibly by prolonged endogenous production of reactive oxygen species derived from glucose metabolism (20), would constitute the glucose toxicity. At this stage there is no reversibility in a strict sense; only repair is possible.

In the following review of secretagogue-induced desensitization we will use "desensitization" as a general phenomenological description of a reduced secretory responsiveness, thereby excluding neither the occurrence of β-cell exhaustion nor β-cell damage as possible companions or sequels. As with glucose-induced desensitization, the characteristics of interest are *1*) the onset and reversibility of desensitization, *2*) the specificity, *3*) the relation between changes in signaling and exhaustion of releasable insulin, and finally *4*) the possible induction of β-cell damage.

DESENSITIZATION BY DEPOLARIZING INSULIN SECRETAGOGUES

Modes of action of depolarizing insulin secretagogues. Currently, clinically used stimulators of insulin secretion are blockers of ATP-sensitive K^+ channels (K_{ATP} channels). When K_{ATP} channels are closed, an inward leak current depolarizes the β-cells and elicits a Ca^{2+} influx via voltage-dependent Ca^{2+} channels (21). Sulfonylureas, the prototypical K_{ATP} channel blockers, have at least one additional site of action that contributes to the insulinotropic effect. They enhance the acidification of the secretory granules of the β-cells by binding to a granular 65-kDa protein, thus activating chloride influx into the granules (22). The relevance of the latter mechanism for the clinical effect of sulfonylureas is an open question, but it has to be taken into account when analyzing in vitro observations. Benzoic acid derivatives and phenylalanine derivatives block K_{ATP} channels via binding to SUR1 and can thus be termed as sulfonylurea analogs. The benzoic acid derivative repaglinide is probably devoid of direct effects on secretory granules in β-cells (23); it is unclear whether this is true for benzoic acid derivatives in general or the phenylalanine derivative nateglinide.

In previous years, imidazolines have gained considerable interest as potential oral antidiabetic drugs, because some of these compounds, which were originally synthesized to act as α-adrenoceptor ligands, enhance insulin secretion only in the presence of a stimulatory glucose concentration (24–26). Like sulfonylureas, imidazolines inhibit K_{ATP} channels (27), but in contrast to sulfonylureas, the actions of imidazolines are probably due to a direct interaction with the pore-forming subunit Kir6.2 (28). Some imidazolines exert additional effects, such as release of Ca^{2+} from internal stores or activation of protein kinases, that may or may not contribute to the enhancement of insulin secretion (29–31). Recently, insulinotropic imidazoline compounds, which do not block K_{ATP} channels, have been described (32). These imidazolines are thought to act solely by mechanisms affecting the transduction of Ca^{2+} signals into exocytotic events (33). One such mechanism appears to be similar to that of sulfonylureas in that it involves the acidification of secretory granules (34). However, no data on desensitization by these compounds have been published thus far.

When insulin secretion is measured in vitro, a typical maneuver to elicit secretion by Ca^{2+} influx is to depolarize the β-cell plasma membrane by a high extracellular K^+ concentration (20–40 mmol/l). Ca^{2+} influx by itself, in addition to the stimulation of exocytosis, exerts a number of additional effects (feed-forward and feed-back), which may render the interpretation of long-term experiments less straightforward than expected. When the use of a high K^+ concentration is not feasible (typically in in vivo experimentation), 5–20 mmol/l arginine can be used to depolarize the β-cell membrane. In contrast to other insulinotropic amino acids, arginine is believed to stimulate insulin secretion not by acting as a fuel, but by depolarization, apparently because arginine is taken up by an electrogenic transporter (35).

Desensitization by sulfonylureas. Approximately 30 years ago a reversible impairment of insulin secretion by sulfonylureas, such as tolbutamide or glibenclamide, was noted (36). The sulfonylurea-induced desensitization was described to be selective for sulfonylureas (37,38), but in vitro experimentation showed that exposure of islets to sulfonylureas also markedly reduced glucose-induced insulin secretion (39–41). In vitro, the onset of an inhibitory effect of tolbutamide on insulin secretion became visible after 30 min (42). The higher the tolbutamide concentration, the more effective it was at diminishing the subsequent secretory response to glucose, even though the amount of insulin released during the tolbutamide exposure was virtually the same. In particular, the first-phase increase by glucose was blunted by the prior exposure to tolbutamide, whereas the second phase was reduced by ~50% (42).

When isolated mouse islets were cultured for a prolonged (18-h) period of time in the presence of 500 μmol/l

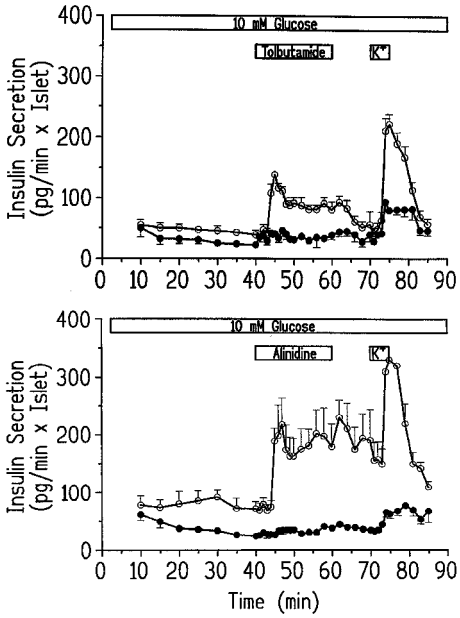

FIG. 1. Desensitization of insulin secretion by maximally effective concentrations of K_{ATP} channel–blocking secretagogues. Isolated mouse pancreatic islets were cultured for 18 h in RPMI 1640 with 5 mmol/l glucose containing 500 μmol/l tolbutamide, 100 μmol/l alinidine, or no secretagogue (control culture). Cultured islets were collected and perifused with a Krebs-Ringer medium containing 10 mmol/l glucose and the same secretagogue to which they had been exposed previously (●). This was followed by a K^+ depolarization. The secretory responses were compared with those of control-cultured islets (○). Adapted from Rustenbeck et al. (50).

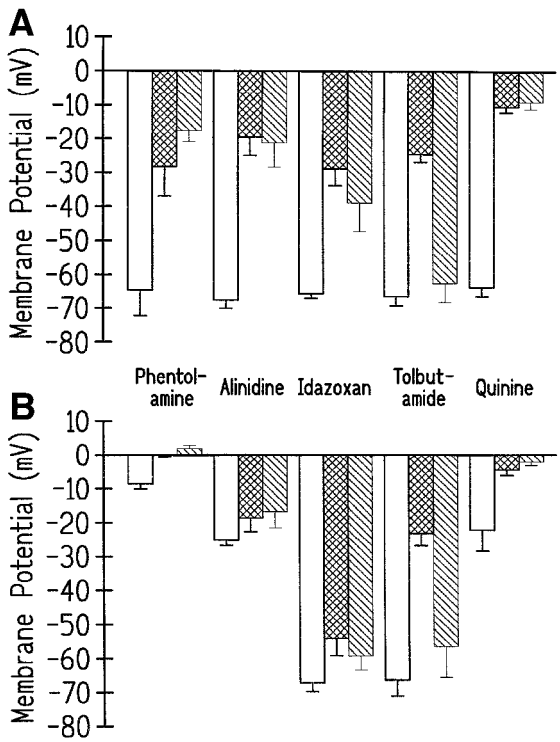

FIG. 2. Comparison of the effects of depolarizing insulin secretagogues on membrane potential of control-cultured (*A*) and secretagogue-desensitized β-cells (*B*). Isolated β-cells were cultured for 18 h in RPMI 1640 containing 5 mmol/l glucose and, additionally, 100 μmol/l of the named secretagogues (tolbutamide 500 μmol/l) or no secretagogue (control culture). After cell culture the membrane potential was measured by patch clamping in the conventional whole-cell configuration under current clamp condition. □, values measured immediately before reexposure to the secretagogue to which the cells had been exposed previously; ▨, values during exposure; ▧, values after return to basal extracellular medium. Data are means ± SE of four to six experiments. Adapted from Rustenbeck et al. (44).

tolbutamide (in cell culture medium RPMI with 5 mmol/l glucose) and then used in perifusion experiments, the secretory rate in the presence of 10 mmol/l glucose was about half of the control value (Fig. 1). When the islets were re-exposed to 500 μmol/l tolbutamide, the amount of insulin released corresponded to 39% of the amount of control-cultured islets. A subsequent K^+ depolarization also yielded a clearly diminished response, as the amount of insulin secreted during this phase corresponded to 56% of the value of control-cultured islets (Fig. 1). In conclusion, a desensitization by sulfonylureas is of low specificity in that it does not only affect tolbutamide-induced secretion, but also affects—with considerable efficiency—the secretion induced by nutrient and other nonnutrient stimuli. The reported ability of nateglinide to be fully effective on tolbutamide- and glibenclamide-desenzitized islets (43) is thus unsuspected and not easily explained in view of the mechanisms discussed below.

β-Cells that had been cultured in the presence of tolbutamide under the same conditions as those for the secretion measurements had a K_{ATP} channel activity that was not significantly different from controls when there was a 30-min wash-out phase before the experiments (44). Accordingly, tolbutamide-desensitized β-cells had a normal resting membrane potential and reacted with a marked depolarization to a renewed tolbutamide exposure (Fig. 2). Also, tolbutamide or a high K^+ concentration increased the cytosolic free calcium concentration ($[Ca^{2+}]_c$) only slightly less in tolbutamide-desensitized β-cells than in controls (44). From these data, it seems that there are only minor changes in the K_{ATP} channel–dependent signal pathway in tolbutamide-desensitized β-cells, which cannot explain the strongly diminished secretion.

This conclusion is at variance with that of an investigation in which a high glibenclamide concentration (10 μmol/l) was used to desensitze MIN6 cells (45). Here, a diminished presence of sulfonylurea receptor (SUR)-1 and, hence, K_{ATP} channel activity in the plasma membrane was proposed to be the underlying cause, via partial depolarization, of an increased basal $[Ca^{2+}]_c$ and an impaired $[Ca^{2+}]_c$ increase upon reexposure to glibenclamide. This discrepancy may be explained by taking into account that glibenclamide is a compound of very slow reversibility, which is most likely due to its intracellular accumulation (46). Similar effects (e.g., an increased basal $[Ca^{2+}]_c$ and an increased basal rate of secretion) were seen in a study in which mouse islets had been exposed overnight to a therapeutic concentration (10 nmol/l) of glibenclamide (47). However, such effects were not produced by exposure to a comparably effective tolbutamide concentration (50 μmol/l). Only when tolbutamide was also present during the functional tests did such effects on signal transduction occur (47). In conclusion, when the objective is to characterize the lasting changes induced by sulfonylurea desensitization with as little interference as possible from the desensitizing agent, tolbutamide appears to be a more convenient experimental tool than glibenclamide.

Both sulfonylureas, however, led to a similar reduction in insulin content and glucose-induced insulin secretion (47). This confirms earlier in vitro observations that

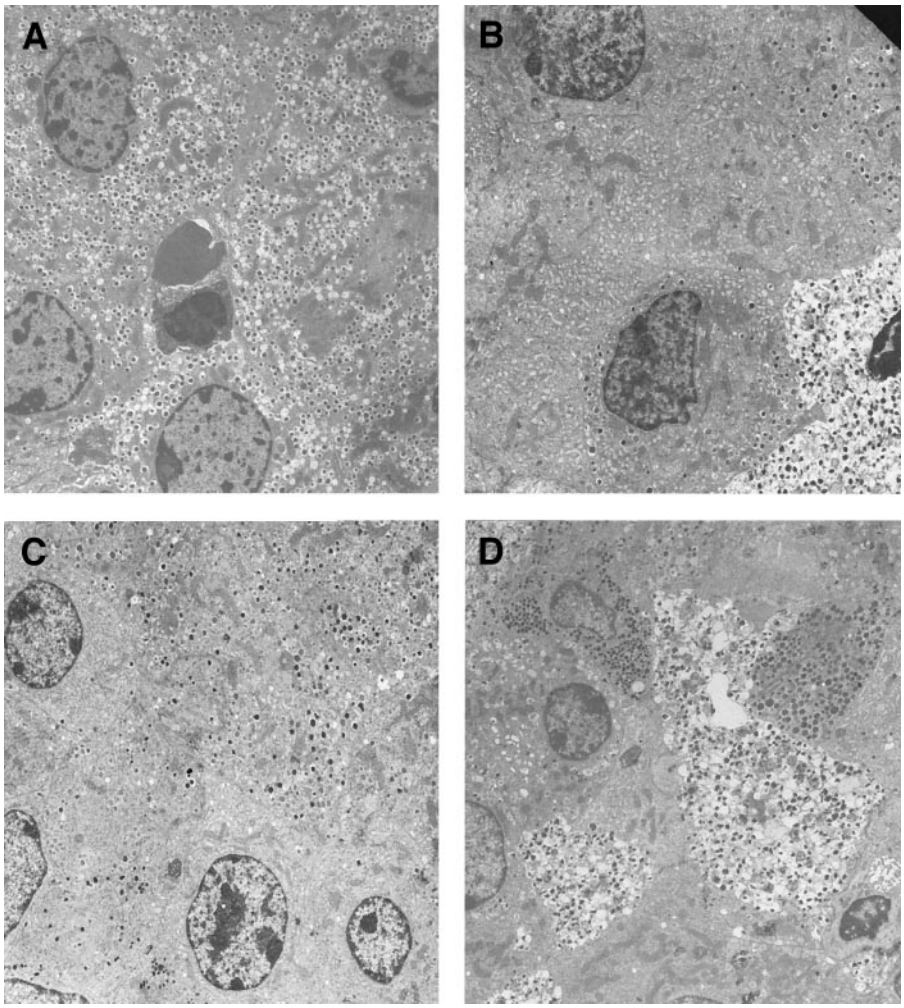

FIG. 3. Degranulation of pancreatic β-cells by induction of desensitization. Isolated pancreatic islets were cultured for 18 h in RPMI 1640 with 5 mmol/l glucose (control; *A*) or in the presence of 500 μmol/l tolbutamide (*B*), 100 μmol/l alinidine (*C*), or 100 μmol/l quinine (*D*). In contrast to the strongly degranulated β-cells with enlarged endoplasmic reticulum after tolbutamide treatment (*B*), alinidine-desensitized β-cells showed a partial degranulation but no other characteristic features (*C*). Nine percent of the β-cells were damaged or necrotic after quinine exposure (*D*). The percentage (mean ± SE) of degranulated β-cells was determined in five islets of medium size and was 86 ± 3 for tolbutamide, 66 ± 4 for alinidine, and 65 ± 7 for quinine. Under control conditions, the percentage of degranulated β-cells was 14 ± 2. Magnification 5,500×. *A* and *B* adapted from Rustenbeck et al. (50).

chronic exposure to not only a high but also a low therapeutically relevant tolbutamide concentration decreases the response to a glucose stimulus (48). This cross-desensitization could be due to the functional changes in signal transduction and/or the depletion of insulin stores, reflecting the controversy of β-cell desensitization versus β-cell exhaustion. Measurements of immunoreactive insulin have mostly shown moderate reductions (mostly by 10–30%) of insulin content, even after extended periods of time (41,45,48–50).

Electron microscopy of islets after exposure to sulfonylureas showed more impressive changes (Fig. 3). Islets that had been cultured for 18 h in the presence of 500 μmol/l tolbutamide (using cell culture medium RPMI 1640 with 5 mmol/l glucose, the same conditions as for the secretion measurements) showed a strong degranulation of the β-cells and at the same time a cystic enlargement of the rough endoplasmic reticulum and a well-developed Golgi complex (Fig. 3*B*). A number of small clear vesicles were present in the cytoplasm, often situated in the vicinity of the dilated cisternae. Quantitatively, 86% of the β-cells in tolbutamide-desensitized islets were degranulated. Under control conditions all endocrine cell types in the isolated islets were ultrastructurally well preserved. Only a minority of the β-cells were degranulated (~15%), and the cell organelles involved in the synthesis of insulin, such as the cisternae of the rough endoplasmic reticulum and the Golgi complex, were developed to the same extent as in mouse β-cells under in vivo conditions, excluding unspecific effects of the cell culture conditions (Fig. 3*A*).

The observation of a strong degranulation by tolbutamide concurs with earlier light and electron microscopic measurements in which massive degranulations were found after in vitro and in vivo exposure to tolbutamide or other sulfonylureas (51–54). In those studies, at least 2–3 h were necessary for a degranulation to become significant, and the minimal content of granules was reached after 18–24 h. It is remarkable that a complete regranulation after a single high dose of tolbutamide in vivo required 48 h, and even a partial regranulation required 24 h after the nadir of granule content (55). A similarly strong degranulation was observed after in vivo (55) and in vitro (56) exposure to a high dose of glibenclamide and again reversibility required several days (53,55,56). As described above, a cystic enlargement of the endoplasmic reticulum was seen in the degranulated β-cells, suggestive of an increased biosynthetic activity. In fact, prolonged exposure to high concentrations of sulfonylureas induces an islet hypertrophy, which was originally believed to be therapeutically relevant (57). Paradoxically, both insulin content and insulin synthesis are decreased under this condition (48,56,58), which may contribute to the slow reversibility of the sulfonylurea-induced degranulation.

It is conceivable that the early phase of desensitization

by tolbutamide, and after prolonged exposure, may involve different mechanisms. After all, an ultrastructurally visible degranulation cannot account for the early events. In this context, it is interesting that a long-term low-dose application of tolbutamide in vivo induced a desensitization without reducing the insulin content, while a high dose clearly did so. Unfortunately, no ultrastructural examinations were performed in this study (48). A mechanism that could contribute to both the acute and chronic effect is an interference with energy metabolism. It has long been known that tolbutamide decreases ATP levels of islets (59,60). This effect is already measurable after 15 min in the presence of 100 μmol/l and even of 30 μmol/l, which is close to therapeutic concentrations (61). At this early time point, the ATP loss cannot be explained by degranulation. Because there is also an increase in oxygen consumption under this condition (61), tolbutamide may have a partial uncoupling effect on oxidative phosphorylation.

There are two obvious questions remaining: *1*) is the degranulation sufficiently explained by the depolarizing effect of the secretagogues, and *2*) is regranulation a prerequisite for a functional recovery of secretion? These aspects will be discussed below after presenting data on the imidazoline-induced desensitization.

Desensitization by imidazolines and other depolarizing secretagogues. The desensitization of insulin secretion by imidazolines was explored initially to substantiate the hypothesis that imidazolines stimulate insulin secretion by binding to a receptor that activates a second messenger cascade (62). The desensitization by imidazolines was described to be specific, since the pretreated islets still responded to high glucose and to diazoxide, whereas the typical effect of imidazolines to overcome the diazoxide block of secretion was lost. Only imidazolines that stimulated insulin secretion, such as phentolamine and efaroxan, but not an apparently inactive imidazoline, idazoxan, induced a desensitization (63).

When isolated islets were cultured in the presence of 100 μmol/l of the imidazoline alinidine, under the same condition as described above for tolbutamide desensitization, a strongly reduced secretory responsiveness resulted (Fig. 1). The secretory response was not only diminished upon reexposure to the imidazoline (19% of control-cultured islets), but also upon K^+ depolarization (36% of control). On the whole, the desensitization by alinidine resembled the desensitization induced by tolbutamide (Fig. 1). The exposure to alinidine and the prototypical imidazoline phentolamine desensitized isolated islets not only against reexposure to the same compound but also against a stimulation by tolbutamide and quinine (50). The cross-desensitization does not come as a surprise when bearing in mind that the imidazolines share essential parts of the β-cell signaling cascade with the sulfonylureas and quinine. Likewise, overnight culture in a medium with a high K^+ concentration moderately but significantly reduced the secretory response to sulfonylureas, imidazolines, and quinine (50). The conclusion, that a prolonged depolarization is sufficient to elicit a desensitization, is supported by the observation that arginine also decreased the response to a subsequent glucose or tolbutamide stimulus (11).

Measurements of the β-cell membrane potential showed clear differences between the imidazolines and tolbutamide (Fig. 2). Under control conditions, the depolarization by all imidazoline compounds tested was much less reversible than the depolarization by tolbutamide. Most conspicuously, the depolarization by phentolamine and also by quinine was still increasing during wash out of the compounds. Thus, it is not surprising that after overnight exposure to phentolamine, the desensitized β-cells were strongly depolarized even before re-exposure to this compound (Fig. 2). Actually, it took 1 day for phentolamine-desensitized β-cells to regain a modest spontaneous K_{ATP} channel activity (44). This is similar to the use of glibenclamide, which is apparently retained in β-cells for several days, thus rendering difficult the distinction between mechanisms of desensitization and effects caused by the persisting presence of the secretagogue.

The immunoreactive insulin content of the imidazoline-desensitized islets was differently affected: after an 18-h exposure to phentolamine, there was no decrease in insulin content, whereas alinidine desensitization produced a reduction of ~25% (50). This was confirmed by ultrastructural examination in which the β-cell degranulation in phentolamine-exposed islets was not significantly different from that of control-incubated islets, whereas 100 μmol/l alinidine led to a degranulation in ~40% of the β-cells (Fig. 3*C*). This degree of degranulation was comparable to that induced by an 18-h exposure to 100 μmol/l quinine or 40 mmol/l K^+ (50). The ultrastructure of imidazoline- and quinine-incubated islets differed from that of sulfonylurea-incubated islets in that the β-cells showed no cystic enlargement of the endoplasmic reticulum (Fig. 3*C* and *D*). Apparently, the above-discussed biosynthetic activation is specific for sulfonylureas and not a general property of K_{ATP} channel–blocking insulin secretagogues. The degranulation, even though significantly less extensive than after tolbutamide exposure, was still of a magnitude that an exhaustion of insulin stores could be involved in the decreased secretory response. Thus, the same challenge arises with imidazolines as with nutrients and sulfonylureas: how to distinguish β-cell exhaustion from β-cell desensitization.

This question was addressed using the imidazoline efaroxan (64) because its acute effects on stimulus-secretion coupling in the β-cell, like those of tolbutamide, were quickly reversible (65). As with the previous experiments, the desensitization was brought about by culturing overnight (18–20 h) in RPMI 1640 medium containing 5 mmol/l glucose and 100 μmol/l of the secretagogue. Compared with control-cultured islets, the efaroxan-induced secretion in the presence of 10 mmol/l glucose was reduced to ~20%, whereas the basal rates in the presence of 5 mmol/l glucose alone were similar (Fig. 4). Interestingly, efaroxan had a slight stimulatory effect on control-cultured islets in the presence of 5 mmol/l glucose, whereas it had no or even a slight inhibitory effect on freshly isolated islets under this condition (65). The extent of desensitization by efaroxan was similar when the cell culture medium contained 10 mmol/l glucose (data not shown).

The efaroxan-induced desensitization also affected the stimulation by 500 μmol/l tolbutamide, which was reduced by ~50% but left intact the response to a subsequent K^+

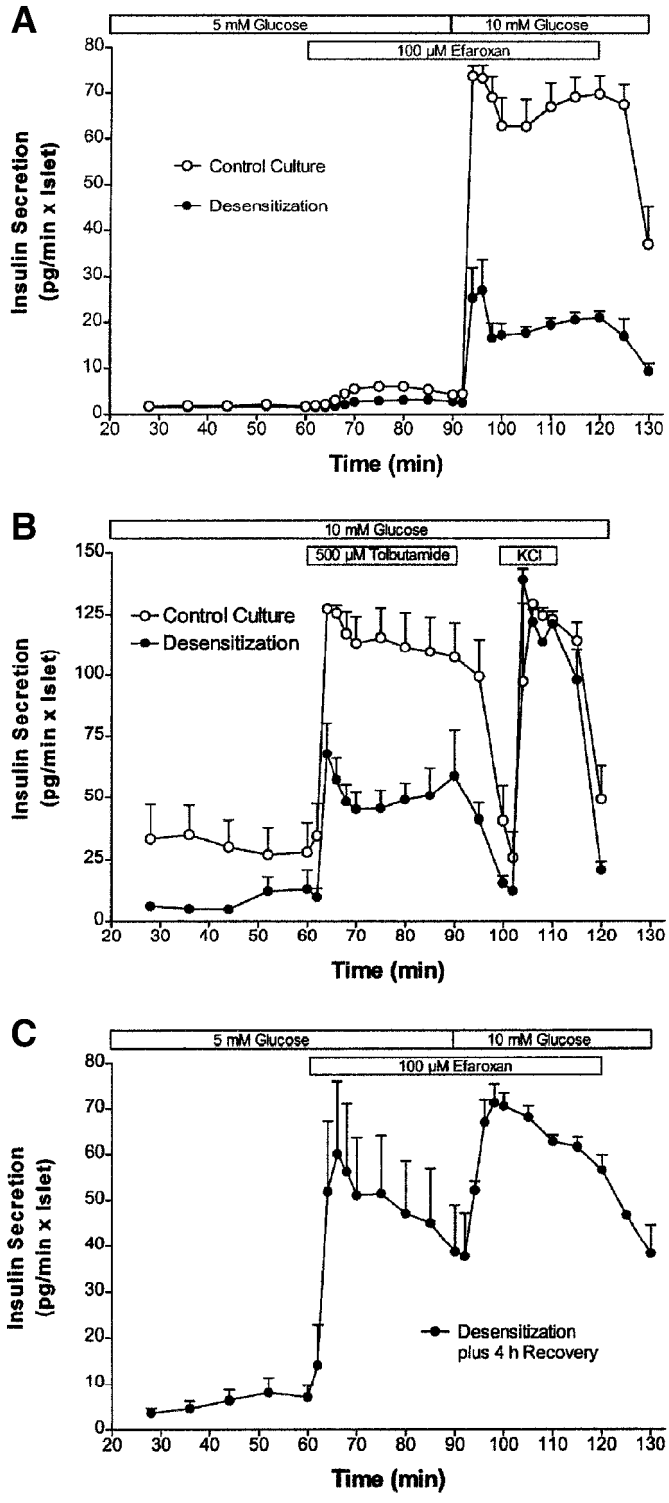

FIG. 4. Desensitization of insulin secretion by the imidazoline efaroxan. Isolated mouse pancreatic islets were cultured for 18 h in RPMI 1640 with 5 mmol/l glucose containing either 100 µmol/l efaroxan or no secretagogue (control culture). Cultured islets were collected and perifused with a Krebs-Ringer medium containing glucose and secretagogues, as indicated. In comparison with control-cultured islets (○), efaroxan-exposed islets have a much reduced response in the presence of 10 mmol/l glucose (*A*). The response of efaroxan-exposed islets to 500 µmol/l tolbutamide is reduced to ~50% as compared with control-cultured islets, but the response to a K^+ depolarization is virtually unchanged (*B*). After an additional culture period of 4 h without a secretagogue, efaroxan-exposed islets show a largely restored response to efaroxan in the presence of 10 mmol/l glucose and even have an overshoot response in the presence of 5 mmol/l glucose (*C*).

depolarization (Fig. 4). This observation concurs with the above conclusion that desensitization by imidazolines also reduces the response to other K_{ATP} channel–blocking agents, but is in contrast to other investigations that found no such cross-desensitization between efaroxan and tolbutamide (66,67). Perhaps the discrepancy is due to specific properties of the insulin-secreting BRIN BD11 cell line used in these investigations. This would also explain the observation that the depolarizing secretagogue BTS 67 582 desensitized against a tolbutamide stimulus but not against an efaroxan stimulus (49).

The virtually unimpaired response to K^+ depolarization after efaroxan desensitization is remarkably different from the modest response to a K^+ depolarization after desensitization by the imidazoline alinidine and by tolbutamide (Fig. 1). Presuming that here the desensitization was due to changes in signaling rather than a shortage of secretion-ready granules, the reversibility of efaroxan-induced desensitization was assessed. The usual desensitization procedure was followed by a 4-h recovery period in cell culture medium without efaroxan. When these islets were perifused with 10 mmol/l glucose, the efaroxan-induced secretion was nearly as high as that of control-cultured islets (Fig. 4). The response to efaroxan in the presence of 5 mmol/l glucose was even much stronger than the slight increase produced by control-cultured islets, which may represent a sort of rebound phenomenon. Of note, in freshly isolated mouse islets, efaroxan has no stimulatory effect in the presence of 5 mmol/l glucose.

The obvious question was whether the fast recovery of secretory responsiveness was accompanied by a change in the granulation status of the β-cells. Electron microscopy of efaroxan-desensitized islets showed that ~90% the β-cells were degranulated (Fig. 5*A*), similar to the tolbutamide desensitization. There were even fewer secretory granules in the vicinity of the plasma membrane than after tolbutamide exposure. In contrast to tolbutamide-desensitization (Fig. 3*B*), but similar to islets cultured in the presence of a high K^+ concentration, alinidine, or quinine, there were no signs of stimulated biosynthetic activity (Fig. 5*A*). After the 4-h recovery period, the granule content had already increased but was still significantly below that of normal islets. Thus, there is a clear dissociation between secretory responsiveness and granulation state of the β-cells under this condition. Another remarkable feature appearing during the recovery period was the enlargement of the Golgi apparatus in the majority of the β-cells (Fig. 5*B*). The prominent Golgi apparatus would fit to a rebounding insulin synthesis and granule biogenesis. A restored secretory responsiveness in the presence of a strongly diminished granule pool necessitates a large increase in turnover. A moderate increase of turnover in the granule pool may also occur during sulfonylurea-induced desensitization (68), but more often the decrease in insulin content was matched by the decrease in secretion (47,69).

The mechanisms responsible for the decreased secretory response after efaroxan desensitization do not seem to involve the K_{ATP} channel. There was an unchanged resting plasma membrane potential (-75.8 ± 0.4 mV vs. -73.3 ± 1.7 mV in controls) and a marked depolarizing effect (-32.9 ± 5.4 mV vs. -28.3 ± 3.4 mV in controls) by

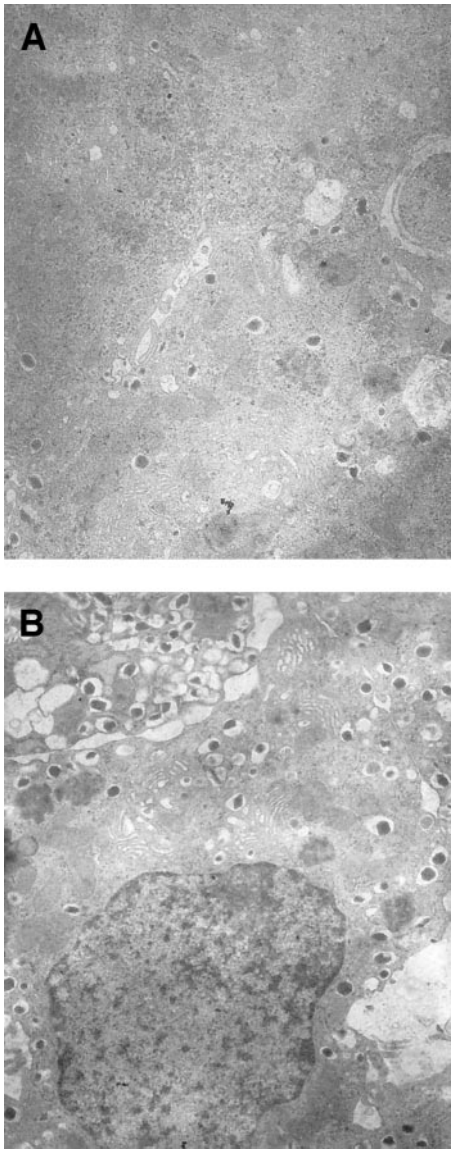

FIG. 5. Effect of exposure to efaroxan (*A*) and recovery from efaroxan exposure (*B*) on the ultrastructure of pancreatic β-cells. Isolated pancreatic islets were cultured for 18 h in RPMI 1640 with 5 mmol/l glucose in the presence of 100 μmol/l efaroxan (*A*) or were subsequently cultured for additional 4 h in RPMI 1640 with 5 mmol/l glucose without efaroxan (*B*). Note the prominent Golgi apparatus and the occurrence of vesicles at different stages of maturation during recovery. Magnification 11,400×.

reexposure to 100 μmol/l efaroxan. When the membrane potential was measured using the perforated patch mode, efaroxan induced oscillatory patterns of depolarization in desensitized as well as in normal cultured β-cells (65). Measurements of $[Ca^{2+}]_c$ in single β-cells showed that at all three efaroxan concentrations tested (10, 30, and 100 μmol/l), the increase in efaroxan-desensitized β-cells was the same as in control-cultured β-cells (Fig. 6*A*). Thus, the situation in efaroxan-desensitized β-cells is principally the same as in tolbutamide-desensitized β-cells (Fig. 6*A*): an intact Ca^{2+} signal meeting with a largely reduced insulin store. On the level of the intact islet, however, a difference between efaroxan-desensitized islets and controls could be found: While efaroxan normally elicited an oscillatory pattern of $[Ca^{2+}]_c$ increase, which was synchronized in the whole islet (65), efaroxan-desensitized islets showed smaller and desynchronized increases in $[Ca^{2+}]_c$ (Fig. 6*B*). Thus, changes in the β-cell–β-cell coupling within an islet may contribute to the secretagogue-induced desensitization.

β-Cell toxicity of depolarizing insulin secretagogues. Finally, the question has to be addressed whether prolonged exposure to depolarizing insulin secretagogues is principally toxic for β-cells, similar to the progression from glucose desensitization to glucotoxicity. In fact, it was shown earlier that tolbutamide as well as high glucose concentrations induced apoptosis in isolated pancreatic β-cells and islets. This effect appeared to be dependent on Ca^{2+} influx through voltage-dependent Ca^{2+} channels (70). The authors concluded that a prolonged influx of Ca^{2+} into the β-cell, elicited either by glucose or tolbutamide, was the critical event triggering β-cell apoptosis. On the other hand, experiments with insulin-secreting cell lines showed that there were large differences in toxicity between imidazoline insulin secretagogues and that one imidazoline, efaroxan, was virtually nontoxic, despite being a known blocker of K_{ATP} channels (71).

Experiments with HIT cells and isolated islets showed that depolarization-induced Ca^{2+} influx was not necessarily leading to apoptosis (72). It was confirmed that two imidazolines, idazoxan and phentolamine, affected β-cell viability by inducing apoptosis. Quinine at high concentrations (1 mmol/l) was also markedly toxic, but in contrast to idazoxan and phentolamine, no signs of apoptosis could be detected. Tolbutamide and the imidazoline alinidine were both moderately toxic, but again this did not seem to involve apoptosis. Interestingly, efaroxan did not affect cell viability even at the highest concentration tested (1 mmol/l).

Ultrastructurally, there was a higher number of damaged β-cells (4–18%) in secretagogue-cultured islets than in control-cultured islets (2%). In control-cultured islets, damaged β-cells were only visible in the islet periphery, whereas in secretagogue-exposed islets, centrally located β-cells were also affected (Fig. 3*B* and *D*). In some β-cells, swollen mitochondria and intracytoplasmic vacuolization were seen as signs of a principally reversible damage. In the majority of the affected β-cells, ruptures of organelle membranes and of the plasma membrane, signs of irreversible damage, were found. Such alterations in the β-cell morphology were produced by the imidazolines idazoxan (18% of the β-cells affected), phentolamine (6%), and alinidine (5%). Quinine induced changes in 9% of the β-cells, and tolbutamide only in 4%. In islets exposed to efaroxan, no centrally located β-cells were affected, confirming the lack of toxicity found with β-cell lines (71,72).

The role of depolarization-induced Ca^{2+} influx was checked by ultrastructural examination of isolated mouse islets cultured for 18 h in RPMI medium containing either 40 mmol/l K^+ or one of the above secretagogues and, additionally, 50 μmol/l D600 (methoxyverapamil). Electron microscopy revealed that the number of damaged or necrotic β-cells (1.7 ± 0.5% in control-cultured islets) increased slightly to 3.5 ± 0.8% after 18 h of exposure to 40 mmol/l KCl. The percentage of damaged cells was unchanged by the concomitant presence of 50 μmol/l D600 in the incubation medium. Likewise, D600 did not reduce the occurrence of β-cell damage by exposure to 100 μmol/l idazoxan, 100 μmol/l phentolamine, or 500 μmol/l tolbut-

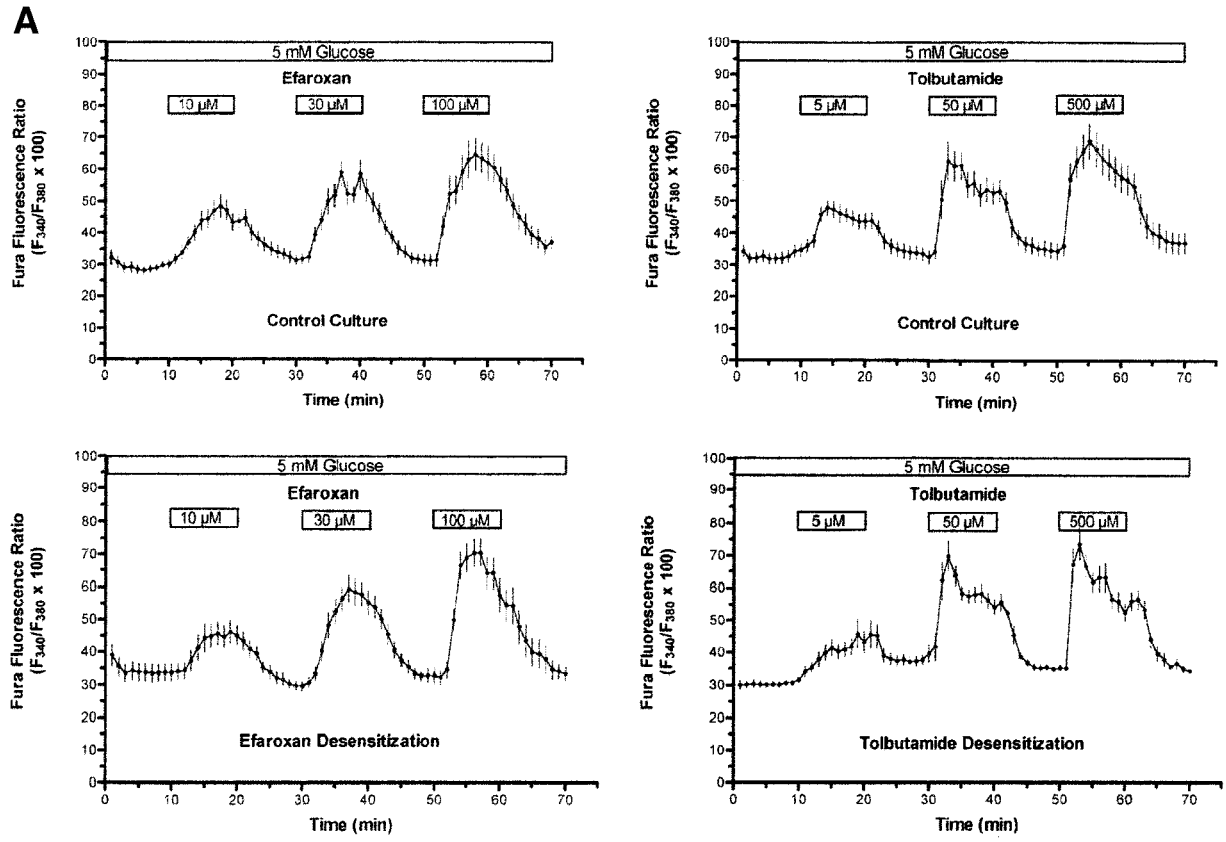

FIG. 6. Effect of desensitization by efaroxan on $[Ca^{2+}]_c$ in single β-cells (*A*) or isolated islets (*B*). Isolated pancreatic islets or single islet cells were cultured for 18 h in RPMI 1640 with 5 mmol/l glucose in the presence of 100 µmol/l efaroxan and were thereafter loaded with Fura 2/AM. *A*: Efaroxan-exposed cells responded in the same way to increasing concentrations of efaroxan as control-cultured cells (*left panels*). The same situation was found in tolbutamide-desensitized β-cells, which responded to tolbutamide like control-cultured cells (*right panels*). Data are means ± SE of three experiments. *B*: Whereas control-cultured islets responded to efaroxan with an oscillatory increase of $[Ca^{2+}]_c$ (*upper panel*), the response of efaroxan-exposed islets was a moderate increase with only few, desynchronized oscillations (*lower panel*). The registrations are typical for five experiments each.

amide. It is noteworthy that in none of the sections damaged, non–β-cells could be found underlining the much more pronounced vulnerability of β-cells (72). In contrast to glucose toxicity, there appears to be no common pathway for K_{ATP} channel–blocking secretagogues leading from a desensitization straight to β-cell damage.

CONCLUSIONS

Desensitization versus exhaustion. A desensitization produced by prolonged exposure to depolarizing insulin secretagogues is regularly accompanied by a marked reduction in the number of β-cell granules. This observation seems to support the concept of the exhausted β-cell. However, the amount of insulin released during the desensitizing exposure to the secretagogues is not increased. Thus, it could be possible that in vitro the reduced granule content may be due to a downregulation of granule formation rather than to an imbalance between a stimulated granule formation and an even more stimulated granule discharge.

Onset of desensitization. The onset of the secretagogue-induced desensitization within <1 h of exposure underlines the relevance of functional changes as opposed to a global lack of insulin. However, it is conceivable that a subpool of release-ready granules is not sufficiently refilled because signals for exocytosis and for refilling may diverge. Compared with the regulation of exocytosis (22), much less is known about the regulation of granule maturation and trafficking in the β-cell (73).

Recovery from desensitization. After removal of the desensitizing secretagogue, functional recovery was nearly complete when the granule content was not yet replenished. The granule biogenesis appeared stimulated even though only a basal (5 mmol/l) glucose concentration was present during the recovery period. This raises the question as to which signals regulate granule biogenesis. Likewise, stimulated secretion in the presence of a low-granule content requires an increased turnover in the granule pool. Whether this turnover is strictly sequential or involves the bypassing of aged granules by newly synthesized ones (68,74) is an intriguing question. New techniques (75) may provide an answer. Finally, the mechanism underlying the increased efficacy of the secretagogue in the presence of basal glucose is worth investigating.

Specificity. The desensitization by depolarizing insulin secretagogues appears to be of a low specificity because the glucose-induced secretion (basal and stimulated) was also affected. Also, a cross-desensitization between imidazolines and sulfonylureas was obvious. However, the characteristics of desensitization are not uniform for all secretagogues: some compounds (tolbutamide and alinidine) also decreased the secretion in response to a K^+

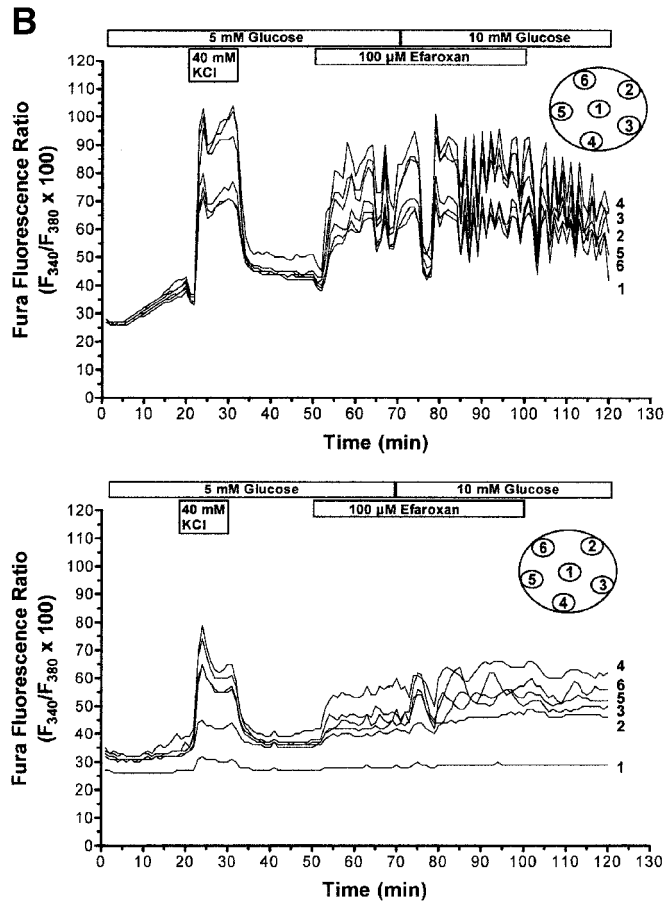

FIG. 6—*Continued*

depolarization while others (idazoxan and efaroxan) left this response unchanged.

Signaling. K_{ATP} channel–dependent signaling (channel closure, depolarization, increase of $[Ca^{2+}]_c$) is largely unimpaired when a prolonged exposure to a quickly reversible drug like tolbutamide or efaroxan has resulted in a desensitization of secretion. Lasting changes in these parameters, which are occasionally reported, are most likely due to the use of secretagogues, which accumulate in the β-cell (e.g., glibenclamide and phentolamine). An interference with K_{ATP} channel–independent metabolic signaling is conceivable, but the mechanisms of this "amplifying pathway" (76) still await clarification. Conceptually, the K_{ATP} channel–independent coupling of energy metabolism to secretion could be a relevant site for secretagogue-induced desensitization, since the energy metabolism may control insulin secretion at a step distal to Ca^{2+} influx (77–79).

Toxicity. The small but undeniable toxicity of high concentrations of the secretagogues is apparently not explained by depolarization and Ca^{2+} influx. It is nevertheless remarkable that efaroxan is devoid of such a toxicity. The ultrastructural changes produced by tolbutamide are typical for sulfonylurea exposure but not for a secretagogue-induced desensitization as such.

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