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Desensitization of insulin secretory response to imidazolines, tolbutamide, and quinine
II. Electrophysiological and fluorimetric studies

Ingo Rustenbeck[a,*], Corinna Dickel[b] Thomas Grimmsmann[1,b]

[a]*Institute of Clinical Biochemistry, Hannover Medical School, D-30623 Hannover, Germany*
[b]*Institute of Pharmacology, University of Göttingen, D-37075 Göttingen, Germany*

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Abstract

Prolonged *in vitro* exposure (18 h) of pancreatic islets to insulin secretagogues that block ATP-dependent K^+ channels (K_{ATP} channels), such as sulfonylureas, imidazolines, and quinine, induced a desensitization of insulin secretion (Rustenbeck *et al.*, pages 1685–1694, this issue). To elucidate the underlying mechanisms, K_{ATP} channel activity, plasma membrane potential and the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) were measured in mouse single B-cells. In B-cells desensitized by phentolamine or quinine (100 μM each) K_{ATP} channel activity was virtually absent and could not be elicited by diazoxide. Desensitization by alinidine (100 μM) induced a marked reduction of K_{ATP} channel activity, which could be reversed by diazoxide, whereas exposure to idazoxan (100 μM) or tolbutamide (500 μM) had no lasting effect on K_{ATP} channel activity. Correspondingly, phentolamine-, alinidine-, and quinine-desensitized B-cells were markedly depolarized, whereas B-cells that had been exposed to tolbutamide or idazoxan had an unchanged resting membrane potential. The increase in $[Ca^{2+}]_i$ normally elicited by phentolamine and alinidine was suppressed after desensitization by these compounds, whereas the $[Ca^{2+}]_i$ increase by re-exposure to quinine was markedly reduced and that by tolbutamide only minimally affected as compared with control-cultured B-cells. The increase in $[Ca^{2+}]_i$ elicited by a K^+ depolarization was diminished in secretagogue-pretreated B-cells, the extent depending on the secretagogue. This effect was closely correlated with the degree of depolarization after pretreatment with the respective secretagogue. In conclusion, the apparently uniform desensitization of secretion by K_{ATP} channel blockers is due to different effects at two stages located distally in the stimulus-secretion coupling: either at the stage of $[Ca^{2+}]_i$ regulation, where the increase is depressed as a consequence of a persistent depolarization (e.g. in the case of phentolamine or alinidine) and/or at the stage of exocytosis, which responds only weakly to substantial increases in $[Ca^{2+}]_i$ (in the case of tolbutamide).

Keywords: Pancreatic B-cells; Desensitization; K_{ATP} channels; Cytosolic calcium concentration; Imidazolines; Tolbutamide

1. Introduction

In the companion paper it was shown that an 18-h incubation of mouse pancreatic islets with the imidazoline compounds phentolamine and alinidine, the sulfonylurea tolbutamide and the cinchona alkaloid quinine, but not with the imidazoline idazoxan led to a reduced secretory responsiveness when the islets were exposed again to the same drug [1]. These observations are compatible with earlier reports, in particular that tolbutamide induces a desensitization that is not explained by a lack of insulin [2] and that exposure to idazoxan had virtually no effect on the secretory responsiveness [3]. In contrast to earlier reports we found that the desensitized state produced by exposure to imidazolines was not selective for these compounds, because the secretory response to tolbutamide and quinine was also strongly diminished. Furthermore, prolonged exposure to a high potassium concentration also induced a desensitization, albeit to a lesser degree than that produced by the secretagogues.

Thus, the secretion studies raised doubt that the imidazoline-induced desensitization of insulin secretion corre-

* Corresponding author: Dr. I. Rustenbeck, Institute of Pharmacology and Toxicology, Technical University of Braunschweig, Mendelssohnstr. 1, D-38106 Braunschweig, Germany. Fax: +49-531-391-8182.

E-mail address: i.rustenbeck@tu-bs.de (I. Rustenbeck).

[1] Present address: Lübeck Medical School, Dept. of Internal Medicine, Ratzeburger Allee 160, D-23538, Lübeck, Germany.

Abbreviations: $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; K_{ATP} channel, ATP-dependent K^+ channel.

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sponds to a homologous desensitization, which is regarded to consist in a dissociation between receptor occupancy and subsequent response-generating elements in the signal transduction pathway [4]. A homologous desensitization of secretion by imidazolines would be an important argument for the concept that a specific B-cell imidazoline receptor exists, which by a second messenger cascade indirectly affects the activity of K_{ATP} channels and/or other mechanisms contributing to the enhancement of insulin secretion [3,5]. Recent experiments by using a truncated variant of the inwardly rectifying K^+ channel Kir6.2 [6] have provided evidence that imidazolines directly interact with the pore-forming subunit of the K_{ATP} channel to inhibit channel activity [7]. This is an unlikely mechanism to induce a homologous desensitization, even though the existence of a specific islet imidazoline receptor is not disproved by this observation.

By characterizing the stimulus-secretion coupling in B-cells desensitized by various K_{ATP} channel blockers, the present study pursued a dual objective: the first aim was to clarify whether the imidazoline-induced desensitization involves a homologous desensitization, the second aim was to identify a possible common site of action where stimuli acting by closure of K_{ATP} channels might induce the desensitization of secretion. Such a common site might also be of interest for the mechanism of glucose-induced desensitization [8], because glucose signaling is critically dependent on K_{ATP} channel closure. Specifically, the following questions were to be answered to explain the loss of secretory responsiveness: (i) What is the activity state of the K_{ATP} channel in desensitized B-cells? (ii) Is the B-cell membrane potential still governed by K_{ATP} channel activity? (iii) Do the secretagogues still induce an increase of the cytosolic Ca^{2+} concentration? To this end, the following parameters were measured: the activity of K_{ATP} channels in intact B-cells and in inside-out patches from B-cells, the whole-cell currents and the plasma membrane potential of B-cells under current-clamp condition and the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) by using Fura-loaded single B-cells.

2. Materials and methods

2.1. Materials

Phentolamine was kindly donated by Ciba-Geigy (Tarrytown, NY, USA), alinidine by Boehringer Ingelheim (Ridgefield, CT, USA), and idazoxan by Research Biochemicals (Natick, MA, USA). Quinine and diazoxide were from Sigma (St. Louis, MO, USA), tolbutamide from Serva (Bay City, MI, USA), and nifedipine from Bayer (Wuppertal, Germany). Fura-2/AM was supplied by Molecular Probes (Eugene, OR, USA), and Collagenase P from Boehringer Mannheim (Indianapolis, IN, USA). Cell-culture medium RPMI 1640 (without glucose) was purchased from Life Technologies (Rockville, MD, USA) and fetal calf serum from Biochrom (Berlin, Germany). All other reagents of analytical grade were from Merck (West Point, PA, USA). Idazoxan was kept tightly sealed and in the dark because of the chemical instability of the dioxane ring structure. Diazoxide was dissolved in dry dimethylsulfoxide to prepare stock solutions of various concentrations. Dimethylsulfoxide concentrations during the experiments were less than 0.5% and had no effects of their own. Tolbutamide stock solutions were prepared in 1 N NaOH.

2.2. Tissues

Islets were isolated from the pancreas of ob/ob mice and albino mice by a conventional collagenase digestion technique. Islets were hand picked under a stereomicroscope. Single cells were obtained by incubation of the islets for 10 min in a Ca^{2+}-free medium and subsequent vortex-mixing for 2 min. Single islet cells were cultured in cell-culture medium RPMI 1640 with 10% fetal calf serum in a humidified atmosphere of 95% air and 5% CO_2 at 37°. The glucose concentration was 5 mM to avoid continuous stimulation of insulin secretion. Incubation of the single islet cells with the insulin secretagogues was performed using the same conditions as to induce the desensitization of insulin secretion of isolated islets (see companion paper). The concentration of the test agents was thus 100 μM (tolbutamide 500 μM) and the cell-culture period of 18 h.

2.3. Electrophysiological recordings

K_{ATP} channel activity was measured by a standard patch-clamp technique using the cell-attached, inside-out, and whole-cell configurations [9]. Pipettes were pulled from borosilicate glass (2-mm outer diameter, 1.4-mm inner diameter, supplied by Hilgenberg, Malsfield, Germany) by a two-stage vertical puller (List Electronic), and had resistances between 3 and 8 MOhm when filled with solution. Currents were recorded by an EPC 7 patch-clamp amplifier (List Electronic, Darmstadt, Germany), low pass-filtered by a 4-pole Bessel filter at 2 kHz and stored on a videotape. The pipette holding potential was 0 mV in cell-attached experiments and +50 mV in inside-out experiments. The membrane potential of B-cells that had been preincubated according to the desensitization protocol was determined by using the whole-cell mode of the patch clamp technique under current clamp conditions. At three time points (before, during, and after exposure to the test agents) the whole-cell current was measured by switching the amplifier from current clamp to voltage clamp at a holding potential of −50 mV. The composition of the bath solution (extracellular solution) was: 140 mM NaCl, 5.6 mM KCl, 1.2 mM $MgCl_2$, 2.6 mM $CaCl_2$, and 10 mM HEPES, pH 7.40. The pipette solution in the cell-attached and whole-cell experiments consisted of 146 mM KCl, 1.2 mM $MgCl_2$, 2.6 mM $CaCl_2$, and 10 mM HEPES, pH 7.4, and of 140 mM KCl, 1.0 mM $MgCl_2$, 2.0 mM $CaCl_2$, 10 mM EGTA, and 5.0 mM

HEPES, pH 7.15, respectively [10]. There was neither glucose nor any other metabolic substrate in the bath solution. All experiments were performed at room temperature (21–23°).

Data were analyzed off-line by using pClamp software (Axon Instruments, Foster City, CA, USA). In inside-out and whole-cell experiments the current amplitudes were determined as a measure of K_{ATP} channel activity. In cell-attached experiments the channel activity ($N * P_O$) was calculated as $1/T * \Sigma n_i * t_i$, where N is the number of channels, P_O the open probability of a single channel, t_i the time spent at each current level n_i, and T the total time for which quantitation was performed, usually 50 s.

2.4. Microfluorimetric measurements of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$)

Cells from ob/ob mouse islets, which contain around 95% B-cells [11] were cultured on glass cover slips in Petri dishes and were used from day 2–4 after isolation. Fura-2/AM [12] was loaded at a concentration of 2 μM for 30 min at 37°. The cover slip with the attached cells was inserted in a purpose-made perifusion chamber, which was placed on the stage of an upright epifluorescence microscope fitted with a Zeiss Fluar (40×) objective (Oberkochen, Germany). A dual-wavelength illumination system was connected to the microscope by a quartz fiber light guide. The fluorescence (excitation at 340 or 380 nm, emission > 470 nm) was recorded by a slow-scan CCD camera. Illumination system, CCD camera, and imaging software were supplied by TILL Photonics (Planegg, Germany). All perifusions were performed at 37° by using a HEPES-buffered Krebs-Ringer bicarbonate medium. Image pairs were taken at intervals as indicated in the figures; illumination time for each image was 300 ms.

3. Results

3.1. Activity of K_{ATP} channels in intact pancreatic B-cells desensitized by imidazolines, tolbutamide, or quinine

The first question was whether K_{ATP} channels could still be influenced by the secretagogues after induction of desensitization. This was tested by measuring K_{ATP} channel activity in intact single B-cells which had been kept in cell culture under the same conditions as were used to desensitize insulin secretion. After desensitization by 100 μM phentolamine, there was virtually no spontaneous activity ($N * P_O < 0.0001$) of K_{ATP} channels. Even more so, neither diazoxide nor sodium azide were able to overcome the phentolamine-induced complete block of electrical activity (Fig. 1A). After an additional period of 24 h in cell-culture medium without phentolamine, there were again spontaneous channel openings. In comparison with control-cultured cells, this channel activity was still quite low ($N * P_O$ = 0.0008 vs. 0.0395 in control, $n = 3$ each), but increased markedly in response to 250 μM diazoxide and 5 mM sodium azide (data not shown). In addition, channel activity in phentolamine-desensitized B-cells could be brought about by patch excision. B-cells desensitized by alinidine (100 μM) showed a significant ($P = 0.04$, t-test) reduction of K_{ATP} channel activity ($N * P_O = 0.0021$), this channel activity increased about 7-fold in response to diazoxide.

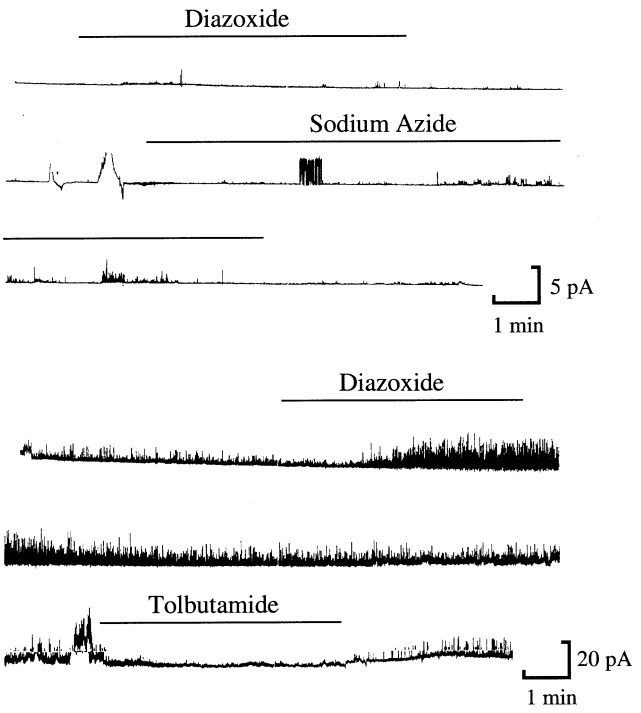

Fig. 1. Activity of K_{ATP} channels in desensitized B-cells. Single B-cells from pancreatic islets were incubated for 18 h in RPMI 1640 (5 mM glucose) with 100 μM phentolamine (A) or 500 μM tolbutamide (B). Then the channel activity in response to 300 μM diazoxide and 2 mM sodium azide (A) or 300 μM sodium azide and 500 μM tolbutamide (B) was measured by using the cell-attached configuration of the patch-clamp technique. Here, upward deflections denote inward currents.

After pretreatment of the B-cells with 100 μM idazoxan or 500 μM tolbutamide (Fig. 1B), there was still considerable activity of the K_{ATP} channels ($N * P_O$ = 0.0055 or 0.0049, $n = 4$ each). The difference between this level of activity and that of control-cultured cells was not significant. In both cases, channel activity increased about tenfold in response to 100 μM diazoxide and decreased in response to 100 μM tolbutamide. Desensitization with quinine (100 μM) yielded virtually the same results as obtained with phentolamine, in that there was no spontaneous K_{ATP} channel activity in intact B-cells and no diazoxide-induced channel opening. Again, this block was reversible after a 24-h period in cell-culture medium without quinine. After this time there was only a small number of channel openings, but a clear increase in response to diazoxide and azide (data not shown). Desensitization by 40 mM K^+ in the cell-culture medium did not lead to significant changes in K_{ATP} channel activity (data not shown).

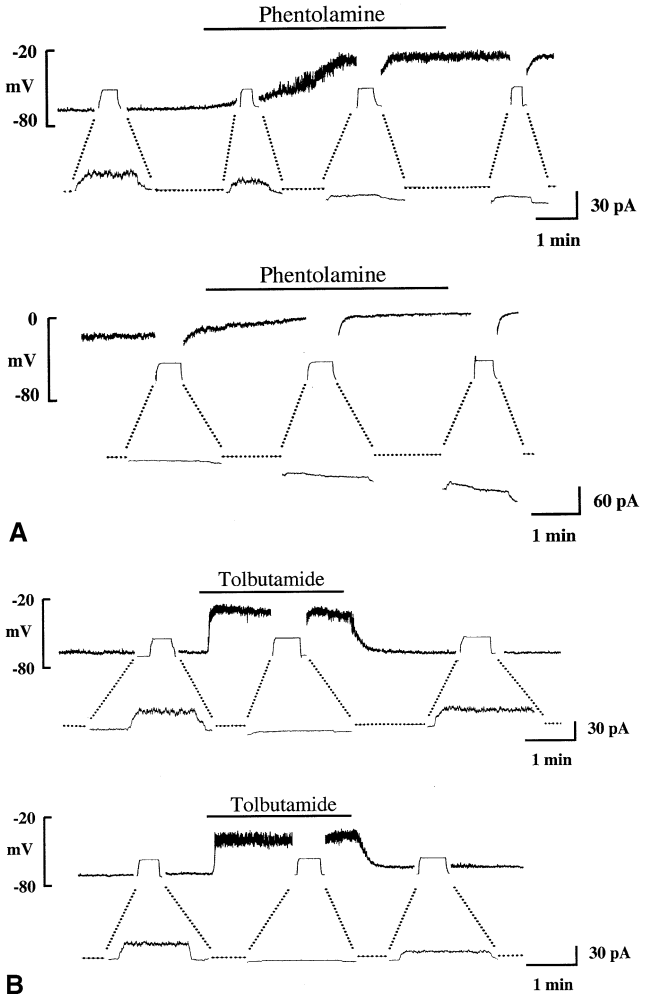

Fig. 2. Changes of membrane potential and whole-cell currents in pancreatic B-cells in response to 100 μM phentolamine (A) and 500 μM tolbutamide (B). The upper graphs show the response of control-cultured B-cells, the lower graphs the response of B-cells desensitized by culturing in presence of the respective secretagogue. The membrane potential (upper traces) was measured using the whole-cell configuration of the patch clamp technique under current clamp condition. Before, during and after exposure to the test agents the whole-cell current (lower traces) was measured by switching to voltage clamp at a holding potential of −50 mV.

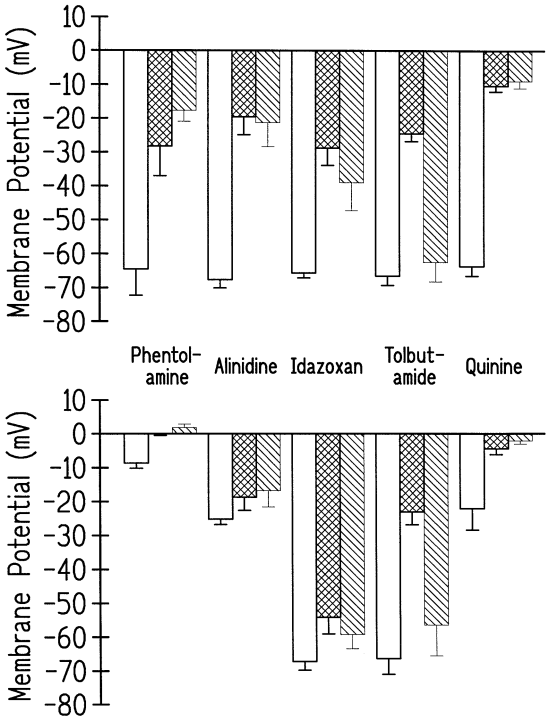

Fig. 3. Effects of insulin secretagogues on membrane potential of control-cultured (upper panel) and secretagogue-desensitized (lower panel) B-cells. Isolated B-cells were cultured for 18 h in cell-culture medium RPMI 1640 containing 5 mM glucose and, additionally, 100 μM of the named secretagogues (tolbutamide 500 μM) or no secretagogue (control culture). The effect of the secretagogues on the membrane potential of these cells was then measured as depicted in Fig. 2. The open bars represent values measured immediately before exposure to the secretagogue, the cross-hatched bars values during exposure and the lined bars values after return to basal extracellular medium. The data are means ± SEM of four to six experiments.

3.2. Effect of secretagogue-induced desensitization on membrane potential and whole-cell current

The observation that K_{ATP} channel activity of B-cells was strongly inhibited by long-term exposure to insulin secretagogues suggested that this treatment should result in an increased insulin secretion, rather than in the observed reduction. To clarify how insulin secretion was dissociated from K_{ATP} channel activity, the B-cell membrane potential was determined by using the whole-cell mode of the patch clamp technique under current clamp condition. Before, during and after exposure to the test agents the whole-cell current was measured by switching to voltage clamp at a holding potential of −50 mV (Fig. 2A–B).

Though control-cultured B-cells had a membrane potential of about −66 mV (Fig. 3, upper panel), the membrane potential of phentolamine-desensitized B-cell was only −8.8 mV (Fig. 3, lower panel). Nevertheless, there was still a significant reaction when 100 μM phentolamine was added to the bath medium, in that the membrane potential decreased further to 0.1 mV and remained there when phentolamine was withdrawn. Alinidine desensitization was also accompanied by a marked reduction of the membrane potential to −25.2 mV, whereas the membrane potential of idazoxan-preincubated B-cells was unchanged (−67.3 mV). In both cases renewed exposure to the respective secretagogue led to a significant depolarization, the extent of which was smaller than under control condition, namely to −18.7 mV in the case of alinidine and to −54.2 mV in the case of idazoxan. A preincubation with tolbutamide affected neither the resting membrane potential nor the extent of depolarization upon renewed exposure nor the reversibility of depolarization. Quinine-desensitized cells in contrast showed a marked loss of the membrane potential (−22.1 mV after preincubation), which was further reduced to −4.4 mV by renewed quinine incubation. As found with phentolamine

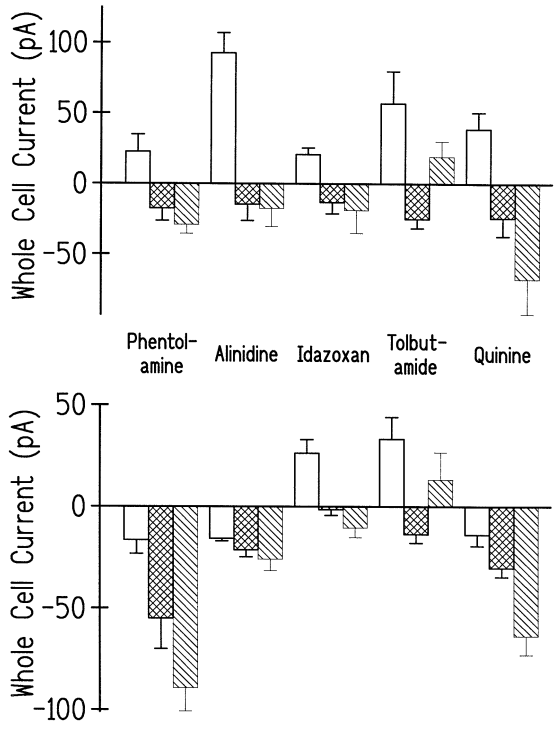

Fig. 4. Effects of insulin secretagogues on whole-cell currents of control-cultured (upper panel) and desensitized (lower panel) B-cells. Isolated B-cells were cultured for 18 h in cell-culture medium RPMI 1640 containing 5 mM glucose and, additionally, 100 μM of the named secretagogues (tolbutamide 500 μM) or no secretagogue (control culture). The effect of the secretagogues on the plasma membrane current of these cells was then measured as depicted in Fig. 2. The open bars represent values measured immediately before exposure to the secretagogue, the cross-hatched bars values during exposure and the lined bars values after return to basal extracellular medium. The data are means ± SEM of four to six experiments.

and alinidine, this depolarization was not reversible in the time frame of the experiments.

Corresponding to the changes in the membrane potential, the desensitization affected the whole-cell current. In the resting state, control-cultured B-cells had a net outward current ranging in magnitude from 22–93 pA per cell (Fig. 4, upper panel). Addition of secretagogues then changed this into a net inward current ranging from −15 pA to −24 pA. After culturing for 18 h in the presence of phentolamine, alinidine, or quinine, the net outward current had changed into an inward current, whereas B-cells that had been cultured in the presence of tolbutamide or idazoxan still showed a net outward current that was not significantly different from that of the corresponding control-cultured cells. Upon re-exposure to the respective secretagogue, tolbutamide-, and idazoxan-desensitized B-cells reacted principally like control-cultured cells with a net inward current. Reexposure of the phentolamine-, alinidine-, and quinine-desensitized cells increased significantly the magnitude of net inward current (Fig. 4, lower panel).

3.3. Effect of secretagogue-induced desensitization on secretagogue-induced increase of $[Ca^{2+}]_i$

The electrophysiological measurements showed that the secretagogues still exerted their primary effect when a desensitization of secretion had developed. The fluorescence of Fura-loaded B-cells was measured to see whether a depolarization was still coupled to an increase in $[Ca^{2+}]_i$ in the desensitized state. Renewed stimulation of phentolamine-desensitized B-cells with phentolamine failed to produce an increase of $[Ca^{2+}]_i$. Even a subsequent depolarization with 40 mM K^+ failed to affect $[Ca^{2+}]_i$ (Fig. 5A). With alinidine-desensitized cells, there was a moderate increase of $[Ca^{2+}]_i$ in response to alinidine, this response being much weaker than that of control-cultured B-cell. In contrast to phentolamine-desensitized B-cells, alinidine-preincubated cells responded to a K^+-depolarization with a marked increase of $[Ca^{2+}]_i$ (Fig. 5B). The presence of tolbutamide during the preincubation led to a modest reduction of the initial rise of $[Ca^{2+}{}_i]$ when the B-cells were stimulated again with tolbutamide (Fig. 5C). Similarly, the preincubation with quinine abolished the initial overshoot of the $[Ca^{2+}]_i$ increase elicited by perfusion with quinine, but the remaining increase was still of considerable magnitude. With both, quinine-preincubated and control-cultured B-cells, $[Ca^{2+}]_i$ remained elevated after withdrawal of quinine from the perifusion (Fig. 5D). The small increase of $[Ca^{2+}]_i$ caused by idazoxan was abolished after preincubation. These idazoxan-pretreated B-cells were nevertheless able to react to a K^+ depolarization with a strong increase of $[Ca^{2+}]_i$ (Fig. 5E).

3.4. Effect of secretagogue-induced desensitization on depolarization-induced increase of $[Ca^{2+}]_i$

The above measurements of $[Ca^{2+}]_i$ suggested that at least the desensitization by phentolamine might directly affect the K^+ depolarization-induced increase of $[Ca^{2+}]_i$, but it was unclear whether this effect was generated during the long-time depolarization in the cell culture or was acutely produced by re-exposure to the secretagogues. To assess how pretreatment with the secretagogues affected the ability of the voltage-dependent Ca^{2+} channels to react to a depolarization, the $[Ca^{2+}]_i$ of secretagogue-desensitized B-cells in response to a depolarizing concentration of K^+ (40 mM) was measured.

As shown in Fig. 6, it turned out that desensitization by all test agents diminished the increase of $[Ca^{2+}]_i$ in response to a K^+ depolarization. Although this reduction was rather small with tolbutamide-desensitized cells, consisting of a lower peak value during the initial overshoot of $[Ca^{2+}]_i$ increase (Fig. 6B), phentolamine-desensitized cells were completely unresponsive to a K^+ depolarization (Fig. 6A). Alinidine- and quinine-desensitized B-cells reacted promptly to the K^+ depolarization, but both the peak value and the plateau of $[Ca^{2+}]_i$ increase were clearly lower than

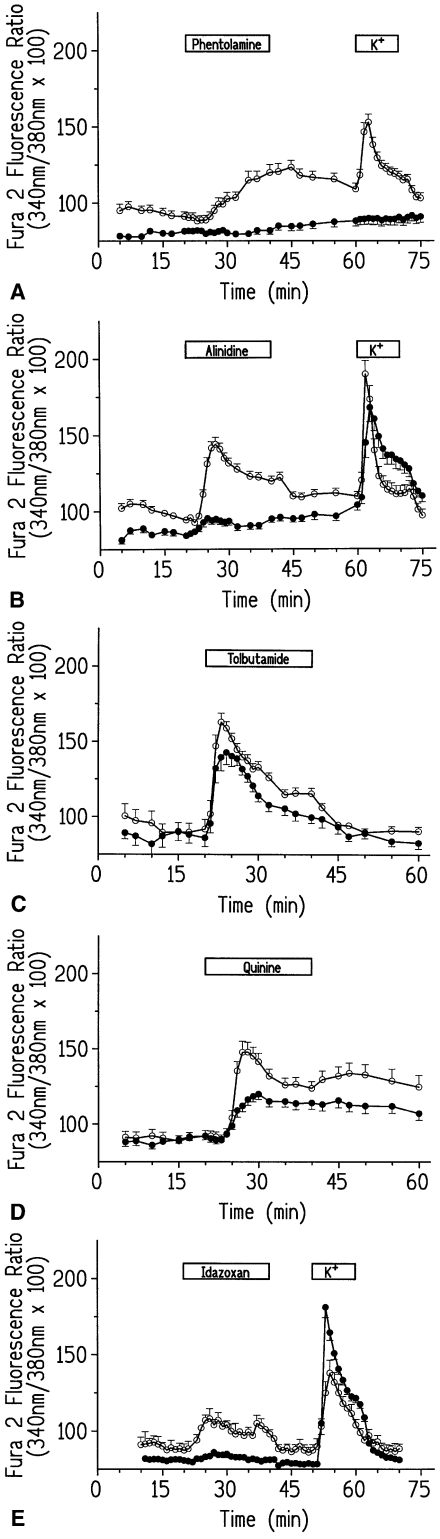

Fig. 5. Effect of insulin secretagogues on the cytosolic Ca^{2+} concentration of secretagogue-desensitized B-cells. Single B-cells from pancreatic islets were incubated for 18 h in RPMI 1640 (5 mM glucose) containing either 100 μM phentolamine (A), 100 μM alinidine (B), 500 μM tolbutamide (C), 100 μM quinine (D), or 100 μM idazoxan (E). Then the cells were loaded with Fura 2 and perifused with a Krebs-Ringer medium containing the respective secretagogue at the same concentration. Open circles denote measurements with control-cultured B-cells. The data are means ± SEM of 23–36 cells from three to four experiments.

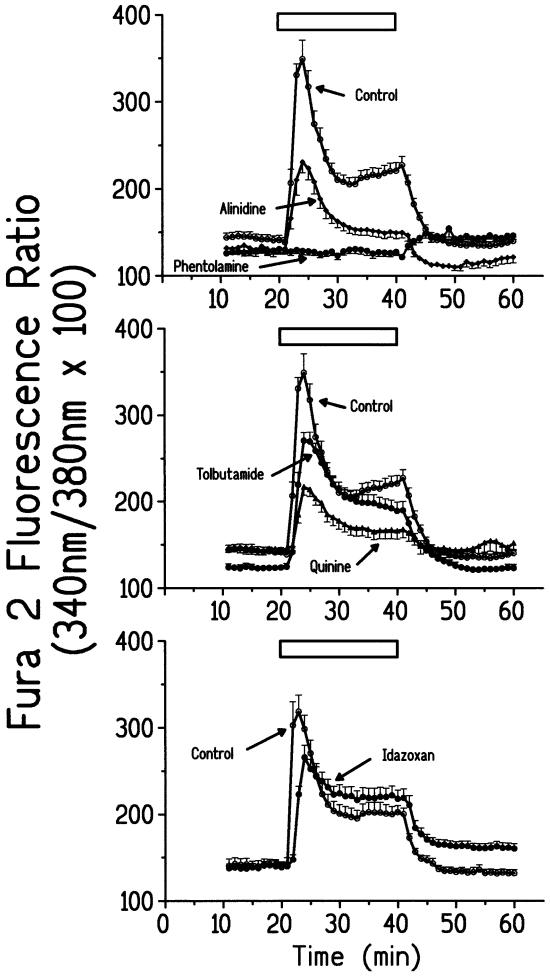

Fig. 6. Effect of a K^+ depolarization on the cytosolic Ca^{2+} concentration of secretagogue-desensitized B-cells. Single cells from pancreatic islets were incubated for 18 h in RPMI 1640 (5 mM glucose) containing either 100 μM phentolamine or 100 μM alinidine (upper graph), 500 μM tolbutamide or 100 μM quinine (middle graph), or 100 μM idazoxan (lower graph). Then the cells were loaded with Fura 2 and perifused with a Krebs-Ringer medium containing 40 mM K^+ as indicated by the open bar. Open circles denote measurements with control-cultured B-cells. The data are means ± SEM of 27–41 cells from four experiments.

in control (Fig. 6A and B). The steady state increase of $[Ca^{2+}]_i$ by quinine was not reversible. A preincubation with idazoxan had effects similar to those of tolbutamid in that the peak value of $[Ca^{2+}]_i$ increase was diminished, whereas the steady-state elevation of $[Ca^{2+}]_i$ was slightly higher than in control-cultured cells (Fig. 6C). Because many imidazolines are ligands at α-receptors, a possible role of B-cell α_2-receptors in influencing the Ca^{2+} channel function was tested by culturing B-cells in the presence of the α-adrenoceptor agonist, clonidine (1 μM), or the irreversible α-adrenoceptor antagonist, benextramine (15 μM). Under both conditions, the K^+ depolarization-induced increase of $[Ca^{2+}]_i$ was not appreciably affected (data not shown).

When B-cells were desensitizated by exposure to 40 mM K^+ and then re-exposed to a high K^+ concentration the initial peak value of $[Ca^{2+}]_i$ increase was clearly dimin-

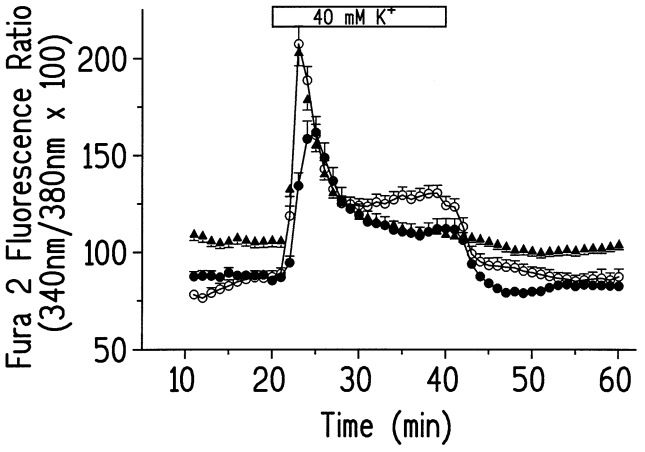

Fig. 7. Role of depolarization and Ca^{2+} influx in the desensitization of B-cells. Single cells from pancreatic islets were incubated for 18 h in RPMI 1640 (5 mM glucose) containing either a normal K^+ concentration (control, open circles), 40 mM K^+ (closed circles), or 40 mM K^+ plus 10 μM nifedipine (triangles). Then the cells were loaded with Fura 2 and perifused with a Krebs Ringer medium containing 40 mM K^+ as indicated. The data are means ± SEM of 18–25 cells from three experiments.

ished, but the prolonged elevation of $[Ca^{2+}]_i$ was only minimally affected (Fig. 7). By use of 10 μM nifedipine Ca^{2+} entry during desensitization by 40 mM K^+ was inhibited. On re-exposure to high K^+, B-cells pretreated in such a way showed a peak value of $[Ca^{2+}]_i$, which was as high as in control-cultured B-cells, starting however from a somewhat elevated resting $[Ca^{2+}]_i$ level (Fig. 7).

4. Discussion

The first question to be answered in this study was whether there is a uniform mechanism of desensitization by imidazolines and whether this mechanism corresponds to an agonist-induced homologous desensitization [3]. Assuming that the inhibition of K_{ATP} channel activity is the common primary effect elicited by binding of the imidazolines to their receptor in the B-cell [5,13], one would expect that in this case K_{ATP} channel activity should no longer be susceptible to the blocking effect of imidazolines. Instead, a persistent complete block of K_{ATP} channel activity was found in phentolamine-desensitized B-cells and a strongly reduced level of channel activity in alinidine-desensitized B-cells. The preserved ability of imidazolines to keep K_{ATP} channels closed precludes the interpretation that the desensitization of secretion by these compounds represents an agonist-induced homologous desensitization. That does not rule out that the B-cell contains imidazoline-receptive structures in addition to the pore-forming subunit of the K_{ATP} channel [7,14].

The observed effects on K_{ATP} channel activity and $[Ca^{2+}]_i$ were not specific for the imidazoline insulin secretagogues. A virtually complete block of K_{ATP} channel was not only induced by phentolamine-pretreatment but also by quinine, whereas a pretreatment with tolbutamide or with idazoxan left no significant effect on K_{ATP} channel activity and membrane potential. The most likely explanation for these striking differences is that the test agents have vastly different kinetics of action. Phentolamine has mostly been described to act slowly and nearly irreversibly (at least in the time frame of the experiments) on K_{ATP} channel activity, $[Ca^{2+}]_i$ and on insulin secretion [15,16] and the same is true for quinine [17–19]. In contrast, tolbutamide acts with a very fast onset and offset [20,21]. Similarly, the effects of idazoxan were shown to be promptly reversible upon withdrawal of the compound and the offset of action of alinidine, although clearly slower than that of idazoxan, was still faster than that of phentolamine [22]. So one can hypothesize that the 30-min time interval between the end of cell culture and the start of measurements is sufficient for tolbutamide and idazoxan to dissociate from their binding sites, whereas up to 24 h are required for phentolamine and quinine to dissociate to an extent that K_{ATP} channel activity can resume. In addition, the observation that patch excision led to a low-level channel activity in phentolamine-desensitized B-cells suggests that this compound is accumulated intracellularly, a mechanism that is held at least partly responsible for the similarly slow kinetics of glibenclamide [20].

Because K_{ATP} channels were virtually completely blocked by phentolamine- and quinine-desensitization one may wonder how these compounds caused a further depolarization of the membrane potential upon re-exposure. In this context it has to be remembered that in contrast to sulfonylureas these compounds are not specific for K_{ATP} channels. In addition to its blocking effect on Na^+ channels quinine is acting on voltage dependent and Ca^{2+} dependent K^+ channels in B-cells [18,19,23,24] and phentolamine was shown to inhibit voltage dependent K^+ channels in B-cells [25]. Thus, it may well be that on re-exposure these compounds block channels that promote the repolarization of the plasma membrane.

The next question was at which step the stimulus-secretion coupling is down-regulated during secretagogue desensitization and whether the mechanism is the same for all secretagogues. In the case of phentolamine, the complete lack of $[Ca^{2+}]_i$ increase is sufficient to explain the loss of secretory responsiveness in spite of an unchanged content of insulin [1]. Here, the problem arose whether the voltage-dependent Ca^{2+} channels were directly affected by phentolamine accumulated in the B-cell or whether they were inactivated by the persistent depolarization [26]. A long-term depolarization as such during the pretreatment had only minor consequences on the subsequent depolarization-induced $[Ca^{2+}]_i$ increase as was evident from the effective K^+ depolarization after pretreatment with tolbutamide or high K^+. However, when the magnitude of the depolarization-induced $[Ca^{2+}]_i$ increase after preincubation with the various secretagogues (Fig. 6) was plotted as a function of the membrane potential of secretagogue-preincubated B-

cells (Fig. 3, lower panel) a very good correlation was obtained (correlation coefficient = −0.989, $P = 0.0014$). So the degree of depolarization immediately before re-stimulation determines which increase of $[Ca^{2+}]_i$ is maximally possible. The variable degree of depolarization after pretreatment with the secretagogues reflects most likely the different extent of inhibition of K_{ATP} channel activity, depending in turn on the dissociation velocity of the secretagogues as discussed above.

Surprisingly, there was no significant correlation between the $[Ca^{2+}]_i$ increases elicited by re-exposure to the secretagogues and the $[Ca^{2+}]_i$ increases by K^+ depolarization of secretagogue-pretreated B-cells. The main factor contributing to this lack of correlation proved to be idazoxan: like after tolbutamide pretreatment, the membrane potential after idazoxan pretreatment was not different from control, but in contrast to tolbutamide the magnitude of depolarization upon renewed exposure to idazoxan was markedly reduced. This observation fits well to the loss of $[Ca^{2+}]_i$ increase after idazoxan pretreatment and thus it seems that on the level of signal transduction there is a desensitization also by idazoxan pretreatment, which differs in its mechanism from that of the other imidazolines. Possibly, this does not translate into a significant desensitization of secretion because of the rather small insulinotropic effect of idazoxan on control-incubated islets and the less favourable signal-to-noise ratio in secretion measurements. Other groups found that idazoxan was ineffective as a stimulator of insulin secretion [27], which concurred with reports on negligible effects on K_{ATP} channels and $[Ca^{2+}]_i$ [28,29] leading to the hypothesis that idazoxan was not a ligand for the islet imidazoline receptor [3]. Our present data suggest that idazoxan is an active imidazoline compound, albeit of modest efficacy. The small increase in $[Ca^{2+}]_i$ in spite of a marked depolarizing effect points to an inhibitory effect at the step of $[Ca^{2+}]_i$ regulation and may be related to the marked glucose dependency of the insulinotropic effect of this compound [22].

$[Ca^{2+}]_i$, however, cannot be the only mediator of secretagogue-induced desensitization of insulin secretion. This becomes clear by comparing the effects of phentolamine and tolbutamide. The secretory rates in the desensitized state were not much different, namely 33% vs. 39% of the respective values of control-cultured islets (see pages ___–___, this issue), whereas the $[Ca^{2+}]_i$ increases were vastly different: a complete suppression in the case of phentolamine vs. a small reduction of the peak value in the case of tolbutamide. Thus, the main mechanism of desensitization by tolbutamide seems to be located distally to the Ca^{2+} entry. It remains to be clarified whether the low number of secretory granules (see pages 1685–1694, this issue) or functional changes in the exocytotic machinery are responsible for the diminished response to the tolbutamide-induced $[Ca^{2+}]_i$ increase. Earlier morphometric studies [30, 31] are in support of the former view, but in view of the high insulin content of desensitized islets [1] a down-regulation of the secretory machinery by high $[Ca^{2+}]_i$ may well play a role. A decrease in energy metabolism could also affect insulin secretion distally to Ca^{2+} influx [32,33], even though there was no hint from our functional data as to a reduced ATP supply after exposure to the secretagogues.

With regard to the mechanism of tolbutamide desensitization our conclusion is at variance from that of a recent investigation, where a reduction in the number of K_{ATP} channels in the plasma membrane was proposed to be responsible for the sulfonylurea-induced desensitization of secretion [34]. In this investigation, a markedly reduced K_{ATP} channel activity, a persistent partial depolarization of the plasma membrane and a lack of $[Ca^{2+}]_i$ increase on renewed stimulation were found, similar to our results obtained with phentolamine. However, our conclusion concurs with an earlier report by Rabuazzo *et al.* [2], who had investigated the sulfonylurea-induced desensitization using measurements of $^{86}Rb^+$ and $^{45}Ca^{2+}$. A large part of this discrepancy may be due to the vastly different kinetics of the sulfonylureas used in these studies. Kawaki *et al.* [34] used glibenclamide as a test agent which, like phentolamine, has slow kinetics of action, whereas Rabuazzo *et al.* [2] had used both tolbutamide and glibenclamide and found marked differences between the effects of these two sulfonylureas.

As a conclusion of the observations presented in this and the companion paper, a two-stage model is proposed how insulin secretagogues which stimulate secretion by depolarization and Ca^{2+} influx desensitize insulin secretion. The first stage is the regulation of $[Ca^{2+}]_i$, where the secretagogue-induced increase is strongly reduced or even absent. This is caused either by a persistent depolarization at the time of a renewed stimulation by the secretagogues (in the case of phentolamine, alinidine and quinine) or by a loss of depolarizing capability (in the case of idazoxan). The second stage becomes visible when the increase of $[Ca^{2+}]_i$ upon renewed stimulation is only slightly reduced, but the secretion is nevertheless strongly diminished (as in the case of tolbutamide). Here, the desensitization is mainly due to a reduced ability of Ca^{2+} to stimulate insulin release. It remains to be clarified whether the distal mechanism of desensitization is not apparent with the non-sulfonylureas compounds simply because the block is located at a more proximal step or whether it represents a sulfonylurea-specific mechanism, possibly as a consequence of the reported direct effects on the secretory granules [35,36]. The difference between the ultrastructural appearence of tolbutamide-exposed islets and that of islets exposed to other secretagogues with fast kinetics [1] may point in that direction.

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