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Antagonism of the insulinotropic action of first generation imidazolines by openers of K_{ATP} channels

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$[Ca^{2+}]$, cytosolic calcium concentration

ABSTRACT

The antagonism between K_{ATP} channel-blocking insulinotropic imidazolines – phentolamine, alinidine, idazoxan and efaroxan – and K_{ATP} channel openers, diazoxide and nucleoside diphosphates, was studied in mouse pancreatic islets and B-cells. In inside-out patches from B-cells, 500 μM MgGDP abolished the inhibitory effect of the imidazolines. 300 μM diazoxide further increased channel activity. The depolarizing effect of all imidazolines (100 μM) on the B-cell membrane potential was practically completely antagonized by 300 μM diazoxide. In contrast, diazoxide was unable to decrease the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) which was elevated by phentolamine, whereas the $[Ca^{2+}]_i$ increases induced by the other imidazolines were promptly antagonized. The effects on $[Ca^{2+}]_i$ were reflected by the secretory activity in that the stimulatory effects of alinidine, idazoxan and efaroxan, but not that of phentolamine were antagonized by diazoxide. Metabolic inhibition of intact B-cells by 250 μM NaCN, most likely by a decrease of the ATP/ADP ratio, significantly diminished the K_{ATP} channel-blocking effect of a low concentration of alinidine (10 μM), whereas efaroxan proved to be susceptible even at a highly effective concentration (100 μM). This may explain the oscillatory pattern of the $[Ca^{2+}]_i$ increase typically produced by efaroxan in pancreatic B-cells. In conclusion, the inhibitory effect of imidazolines on K_{ATP} channels, which is exerted at the pore-forming subunit, Kir6.2, is susceptible to the action of endogenous and exogenous K_{ATP} channel openers acting at the regulatory subunit SUR, which confers tissue specificity. With intact cells this antagonism can be obscured, possibly by intracellular accumulation of some imidazolines.

1. Introduction

Imidazolines are investigated as potential oral antidiabetic drugs with an antihyperglycemic, but not a hypoglycemic mode of action [1], because several compounds of this group enhance insulin secretion only in the presence of a stimulatory glucose concentration [2,3]. Sulfonylureas, in contrast, stimulate insulin secretion in the absence or near absence of glucose [4,5]. Thus, insulin secretion can be inappropriately stimulated by sulfonylureas and the inherent risk of hypoglycemias is limiting their therapeutic use [6].

Early on, it was shown that, similar to sulfonylureas, the stimulation of insulin secretion by imidazolines involves the closure of ATP-dependent potassium channels (K_{ATP} channels) [7–9]. Later, it was found that the imidazolines RX871024 and efaroxan have additional sites of action at a late step in stimulus-secretion coupling [10,11]. The contribution of these effects to the insulinotropic characteristics in general and to the glucose-dependency of imidazoline-stimulated secretion in particular is still unclear. In fact, the imidazoline RX 871024 stimulated insulin secretion from K_{ATP}-deficient B-cells but did so at stimulatory and at non-stimulatory glucose

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concentrations [12]. Imidazolines which do not inhibit K_{ATP} channels were presented recently (second generation imidazolines), but appear to be less effective as insulin secretagogues [13,14].

It is well known that K_{ATP} channel-blocking imidazolines antagonize the channel-opening effect of diazoxide and increase diazoxide-inhibited insulin secretion. In fact, the antagonism of a diazoxide-induced suppression of insulin secretion has been used as test system to identify insulinotropic imidazolines [8,15]. However, it was shown that diazoxide had only a very small opening effect on a pre-existent K_{ATP} channel block induced by phentolamine [7,16]. On the other hand, diazoxide significantly reduced the K_{ATP} channel-blocking activity of the imidazoline analogue guanabenz in inside-out patches from B-cells [17] and reduced the ability of phentolamine to raise the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) [15,18].

Two questions led to this investigation: firstly, can the B-cell K_{ATP} channel still respond to changes in cellular energy metabolism and to pharmacological K^+ channel openers in the presence of K_{ATP} channel-blocking imidazolines, which is of obvious relevance for a potential clinical use? Secondly, are there differences within the group of K_{ATP} channel-blocking imidazolines, which correspond to the different glucose dependencies of the insulinotropic effect of phentolamine and efaroxan [19], and to the different pattern of drug-induced desensitization of insulin secretion [20]?

Thus, we characterized the ability of nucleoside diphosphates and diazoxide to antagonize the insulinotropic action of first generation (K_{ATP} channel-blocking) imidazolines. In contrast to sulfonylureas, which inhibit K_{ATP} channel activity by binding to the regulatory subunit of the B-cell K_{ATP} channel, SUR1 [21,22], imidazolines block K_{ATP} channels by interaction with the pore-forming subunit, Kir6.2 [23]. Both channel-opening nucleotides and pharmacological potassium channel openers exert their effect by binding to SUR [21,22].

For comparison, the antagonism of the effects of tolbutamide and quinine was also measured. Tolbutamide is the prototypical first generation sulfonylurea which closes K_{ATP} channels by binding to the SUR subunit. In concentrations above 500 μM tolbutamide also exerts a blocking effect directly on Kir6.2 [22]. Quinine stimulates insulin secretion by inhibiting K_{ATP} channel activity [24,25] and, similar to imidazolines, this effect is exerted at the pore-forming subunit Kir6.2 [26,27].

2. Materials and methods

2.1. Materials

Phentolamine was kindly donated by Novartis/Ciba-Geigy (Lörrach, Germany) and alinidine by Boehringer Ingelheim (Ingelheim, Germany). Idazoxan and efaroxan were from Tocris (Bristol, UK). Idazoxan was kept tightly sealed and in the dark because of the chemical instability of the dioxane ring structure. Quinine and diazoxide were from Sigma (Taufkirchen, Germany) and tolbutamide from Serva (Heidelberg, Germany). Collagenase P and GDP (dilithium salt) was supplied by Roche Diagnostics (Mannheim, Germany), UDP (trisodium salt) by Sigma and Fura-2/AM by Molecular Probes (Leiden, The Netherlands). Cell culture medium RPMI 1640 was purchased from Gibco BRL (Gaithersburg, MD, USA) and fetal calf serum from Biochrom (Berlin, Germany). ATP was measured using reagent kits from Sigma. All other reagents of analytical grade were from E. Merck (Darmstadt, Germany). Diazoxide was dissolved in dry dimethylsulfoxide (DMSO) to prepare stock solutions of various concentrations. Tolbutamide stock solutions were prepared in 0.1 N NaOH. When nucleoside diphosphates (0.5 mM) were present in the test media 0.2 mM $MgCl_2$ was added to keep the free Mg^{2+} concentration constant.

2.2. Tissues

Islets were isolated from the pancreas of NMRI 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. Islets and 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 °C.

2.3. Measurement of insulin secretion

Batches of 50 freshly isolated NMRI mouse islets were introduced into a purpose-made perifusion chamber thermostated at 37 °C and perifused with a HEPES-buffered Krebs–Ringer medium containing the respective secretagogue. The insulin content in the fractionated effluate (1 ml/min) was determined by ELISA (Mercodia, Uppsala, Sweden).

2.4. Electrophysiological recordings

K_{ATP} channel activity was measured by the patch-clamp technique using the cell-attached and inside-out configurations. The membrane potential was measured using the conventional whole-cell configuration under current clamp condition [28]. Pipettes were pulled from borosilicate glass (2 mm o.d., 1.4 mm i.d., Hilgenberg, Malsfeld, Germany) by a two-stage vertical puller (List Electronic, Darmstadt, Germany) and had resistances between 3 and 6 MΩ when filled with solution. Currents were recorded by an EPC 7 patch-clamp amplifier (List Electronic), low pass-filtered by a 4-pole Bessel filter at 2 kHz and stored on a video tape. The pipette holding potential was 0 mV in cell-attached and +50 mV in inside-out recordings.

The composition of the bath solution for inside-out experiments (intracellular solution) was: 140 mM KCl, 1.0 mM $MgCl_2$, 10 mM EGTA, 2.0 mM $CaCl_2$ and 5 mM Hepes, pH 7.15. The pipette solution in these experiments consisted of 146 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM Hepes and 3 mM glucose, pH 7.4. An ATP-containing solution (bath solution, supplemented with 1.0 mM MgATP and, additionally, 0.8 mM $MgCl_2$) was used to close the K_{ATP} channels completely and to inhibit channel run-down. The composition of the bath solution in cell-attached and whole-cell experiments (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 mode consisted of 146 mM KCl,

1.2 mM $MgCl_2$, 2.6 mM $CaCl_2$, and 10 mM Hepes, pH 7.40, that of whole-cell experiments 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 [29].

All experiments were performed at room temperature (20–23 °C). Data were analysed off-line using pClamp 6.03 software (Axon Instruments, Foster City, CA, USA). In inside-out experiments the current amplitudes were determined as a measure of K_{ATP} channel activity. In cell-attached experiments the channel activity ($N \times P_O$) was calculated as $1/T \times \sum n_i \times t_i$, 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 is the total time for which quantitation was performed, usually 50 s.

2.5. Microfluorimetric measurements of $[Ca^{2+}]_i$

Islets and single islet cells were cultured on glass cover slips in Petri dishes and were used from day 2 to 4 after isolation. Fura-2/AM [30] was loaded at a concentration of 2 μM for 30 min at 37 °C. 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. 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, Munich-Gräfelfing, Germany. All perifusions were performed at 35 °C using a HEPES-buffered Krebs–Ringer medium. Image pairs were taken at intervals as indicated in the figures, illumination time for each image was 800 ms.

2.6. Data handling and statistics

Statistical calculations were performed using Prism and Instat software (Graph-Pad, San Diego, USA). Without further specification "significant" stands for $P < 0.05$ and t-test stands for Student's unpaired two-sided t-test.

3. Results

3.1. Antagonism of secretagogue-induced blockade of K_{ATP} channels by MgGDP and diazoxide

Initially, it was tested whether the blocking effect of imidazolines on K_{ATP} channels in inside-out membranes from B-cells was antagonized by MgGDP and diazoxide. Since the efficacy of diazoxide varies depending on the nucleotide concentration, the effect of diazoxide was quantified when MgGDP was already present in the bath medium. The secretagogues were used at a concentration supposed to give a marked, but not maximally inhibitory effect (Fig. 1). Both, phentolamine and alinidine, as well as tolbutamide and quinine significantly reduced K_{ATP} channel activity and in each case MgGDP (500 μM) exerted a significant opening effect (Table 1). A similar opening effect, albeit somewhat less extensive, was produced by 500 μM MgUDP (data not shown). The presence of diazoxide (300 μM) in addition to MgGDP led to a significant further increase of channel activity in the presence of alinidine and tolbutamide, but not of phentolamine and quinine (Table 1).

3.2. Repolarization of B-cell membrane potential by diazoxide

To ascertain that diazoxide was able to antagonize the K_{ATP} channel-blocking effect of imidazolines to an extent relevant for subsequent steps in stimulus-secretion coupling, the plasma membrane potential was measured. Here, all secretagogues were used at concentrations known to effectively stimulate insulin secretion. With an extracellular solution containing 5 mM glucose the plasma membrane potential of the B-cells was -73.4 ± 1.1 mV ($n = 22$). All test agents, albeit with different kinetics, induced a depolarization often marked by the occurence of action potential spiking (Fig. 2). The depolarizing effect of the imidazolines (phentolamine, alinidine, efaroxan, idazoxan) was similar in magnitude to that of tolbutamide and proved to be practically completely antagonizable by 300 μM diazoxide (Figs. 2 and 3). The depolarizing effect of tolbutamide (acting on SUR 1) and quinine (acting on Kir6.2) was antagonized to the same extent by diazoxide (Fig. 3). Often, action potential spiking ceased directly after the addition of diazoxide when there was still a marked plateau depolarization.

3.3. Diazoxide effects on secretagogue-induced increases of $[Ca^{2+}]_i$

For these experiments the secretagogues were used at 100 μM, the same concentration as for the membrane potential measurements. Phentolamine, alinidine, efaroxan and quinine all increased $[Ca^{2+}]_i$ of perifused islets in the presence of 5 mM glucose. The amplitude corresponded to that of a strong

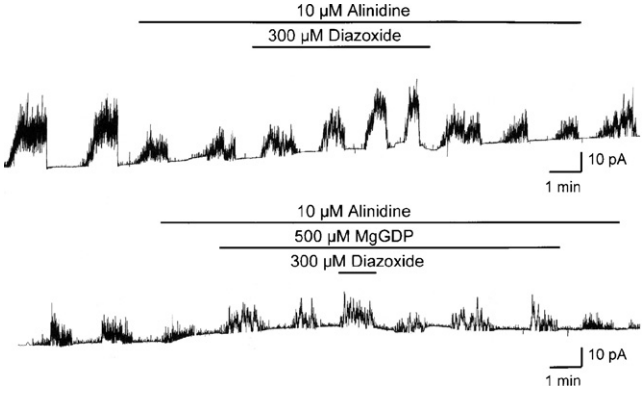

Fig. 1 – Antagonism between the imidazoline, alinidine and K^+ channel openers at K_{ATP} channels in inside-out patches from normal mouse B-cells. Upper trace: diazoxide (300 μM) increased K_{ATP} channel activity in the presence of alinidine (10 μM). Lower trace: MgGDP (500 μM) increased K_{ATP} channel activity in the presence of alinidine (10 μM), there is also an additional opening effect of diazoxide (300 μM). Compounds were applied as indicated by the horizontal bars. 1 mM MgATP was added at regular intervals to block K_{ATP} channels completely and to inhibit channel run-down. To account for channel run-down, channel activity in the presence of MgGDP was referred to the mean value of channel activities before and after MgGDP exposure. Likewise, the effect of diazoxide was referred to the mean value of the channel activities in the presence of MgGDP before and after diazoxide exposure.

Table 1 – Effect of MgGDP and diazoxide on the block of B-cell K_{ATP} channels by imidazolines, tolbutamide and quinine

	Secretagogue	Secretagogue + 500 μM MgGDP	Secretagogue + 500 μM MgGDP + 300 μM diazoxide
10 μM Phentolamine	25.8 ± 3.8	628.3 ± 265.2	95.3 ± 16.1
10 μM Alinidine	43.6 ± 7.3	519.2 ± 161.7	141.3 ± 15.1
50 μM Tolbutamide	17.8 ± 2.6	548.9 ± 204.6	151.2 ± 28.4
10 μM Quinine	63.2 ± 13.8	302.8 ± 47.0	122.4 ± 18.0

The K_{ATP} channel activity was measured using inside-out patches from pancreatic B-cells as shown in Fig. 1. For easier comparison, the channel activity is given in percent of the channel activity under the preceding condition, which was normalized to 100%. Values are mean ± S.E.M. of six to seven experiments. The inhibitory effect of each secretagogue and the opening effect of MgGDP were significant ($P < 0.05$, Wilcoxon test). The opening effect of the additional presence of diazoxide was significant for alinidine and tolbutamide ($P < 0.05$, Wilcoxon test).

K^+ depolarization (Fig. 4). Then the glucose concentration was raised to 10 mM, which led to a transient decrease of $[Ca^{2+}]_i$ followed by a regain of the elevated $[Ca^{2+}]_i$ levels. Alinidine and particularly efaroxan produced an oscillatory $[Ca^{2+}]_i$ increase while phentolamine and quinine produced a more sustained increase. When diazoxide (300 μM) was added to the perifusion medium $[Ca^{2+}]_i$ decreased rapidly in the case of alinidine and efaroxan (Fig. 4, left panels), whereas phentolamine- and quinine-induced increases of $[Ca^{2+}]_i$ were not significantly affected by diazoxide (Fig. 4, right panels). The same response pattern, i.e. antagonism of the effects of alinidine and efaroxan, but not of phentolamine and quinine, could be seen with single B-cells perifused with 5 mM glucose (not shown).

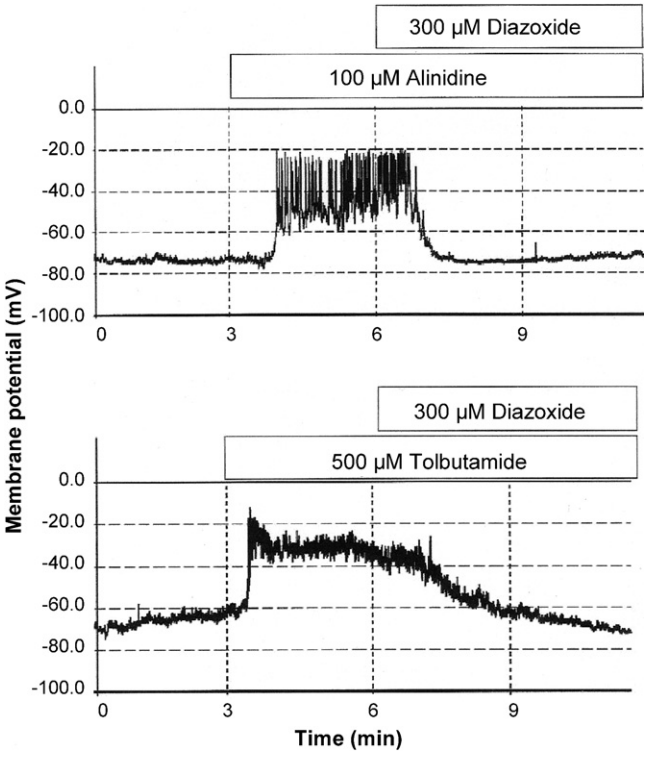

Fig. 2 – Repolarizing effect of diazoxide on B-cells depolarized by alinidine (upper panel) and tolbutamide (lower panel). The membrane potential was measured using the conventional whole-cell configuration under current clamp condition. Compounds were applied as indicated by the horizontal bars. Alinidine (100 μM) had a markedly depolarizing effect with a fast onset of action, inducing action potentials on top of a plateau potential, which was immediately antagonized by diazoxide (300 μM). The depolarization by tolbutamide (500 μM) was similarly fast in onset, the antagonism by diazoxide (300 μM) required more time for completion.

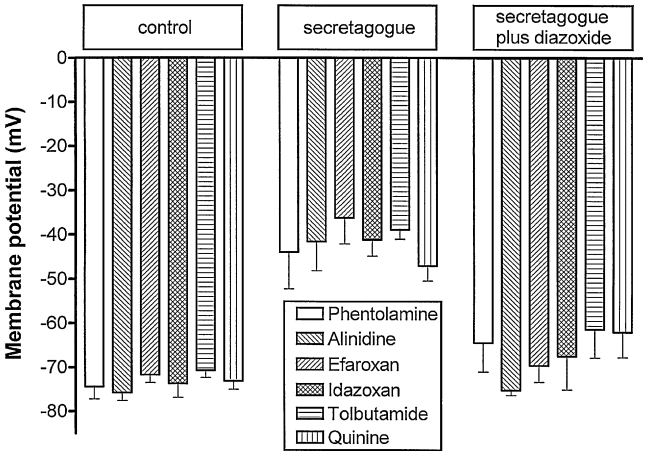

Fig. 3 – Comparison of the antagonistic effects of diazoxide (300 μM) on the depolarization induced by phentolamine, alinidine, efaroxan, idazoxan, tolbutamide and quinine (100 μM each, tolbutamide 500 μM). The membrane potential was measured using the conventional whole cell-technique under current clamp condition as shown in Fig. 2. The control value is the resting membrane potential immediately before the addition of the secretagogues. Values are mean ± S.E.M. of four to five experiments. Each secretagogue significantly depolarized the B-cell membrane and in each case this effect was significantly antagonized by diazoxide.

3.4. *Antagonism of secretagogue-induced insulin secretion by diazoxide*

Since some imidazolines like efaroxan do not stimulate insulin secretion at 5 mM glucose, the antagonism between diazoxide and imidazolines on insulin secretion was investigated using a glucose concentration of 10 mM in all experiments. The secretagogues were used at the same concentration as for the membrane potential and $[Ca^{2+}]_i$ measurements. In each case the addition of the secretagogue to the perifusion

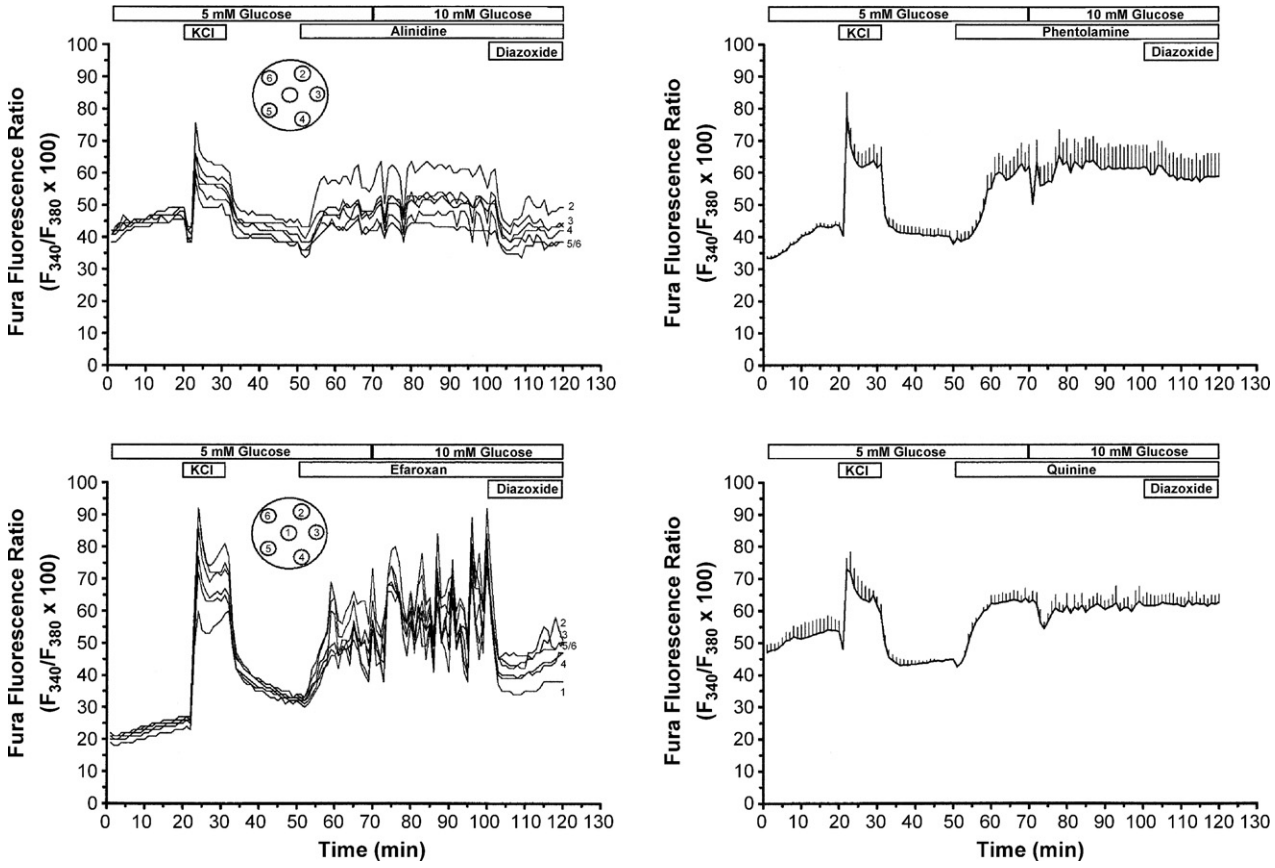

Fig. 4 – Effects of diazoxide on the increase of $[Ca^{2+}]_i$ induced by phentolamine, alinidine, efaroxan and quinine. Intact islets were loaded with Fura 2/AM and perifused with Krebs–Ringer medium containing 5 mM glucose, which was raised to 10 mM glucose after 70 min. A K^+ depolarization preceded the exposure to the secretagogues and served as a positive control. After a 50 min exposure to 100 μM of the secretagogues, 300 μM diazoxide was added. Diazoxide decreased the $[Ca^{2+}]_i$ levels elevated by alinidine and efaroxan (left graphs), but not those elevated by phentolamine or quinine (right graphs). To depict the $[Ca^{2+}]_i$ oscillations induced by alinidine and efaroxan and the immediate cessation by addition of diazoxide, single experiments are shown in the left graphs. The traces are derived from islet subregions as indicated and are representative for four experiments. In the right graphs mean values ± S.E.M. of four to five experiments are shown.

significantly enhanced secretion. The enhancement of secretion by alinidine, idazoxan, efaroxan was effectively antagonized by 300 μM diazoxide. In each case the secretion in the combined presence of the secretagogue and diazoxide was significantly smaller than in the presence of the secretagogue alone (Fig. 5A). Here, diazoxide decreased insulin secretion below the level established by 10 mM glucose alone (Fig. 5A). The onset and velocity of decrease was comparable to the effect of diazoxide on tolbutamide-induced insulin secretion (not shown). In marked contrast, the enhancement of secretion evoked by phentolamine or quinine could not be significantly decreased by a 40 min perifusion with diazoxide (Fig. 5B).

3.5. *Effect of metabolic blockade on imidazoline- and tolbutamide-induced block of K_{ATP} channels*

To compare the effect of metabolic inhibition on sulfonylurea- and imidazoline-induced inhibition of K_{ATP} channels, intact pancreatic B-cells were exposed to imidazolines or to tolbutamide, then sodium cyanide (250 μM) was added to the perifusion medium and finally, the K_{ATP} channel-blocking insulin secretagogues were washed out and channel activity was measured in the presence of cyanide alone (Fig. 6). It was ascertained that 250 μM cyanide significantly decreased the ATP content of statically incubated islets (data not shown). At the given concentrations all secretagogues were significantly effective to inhibit K_{ATP} channel activity (Table 2). The inhibition of channel activity by a low concentration of alinidine (10 μM) and by a high and a low concentration of efaroxan (100 or 10 μM, respectively) was significantly relieved by metabolic blockade. The effect on the tolbutamide-induced block of channel activity was more variable, but still significant, while the antagonism of 10 μM phentolamine was not quite significant with the given number of experiments. When the secretagogues were washed out in the continuing presence of NaCN, a significant further increase of channel activity was noted with alinidine, efaroxan and tolbutamide, but not with phentolamine (Table 2). This increase was less extensive than that produced by NaCN in control B-cells (data not shown), suggesting that the wash-out was not yet complete.

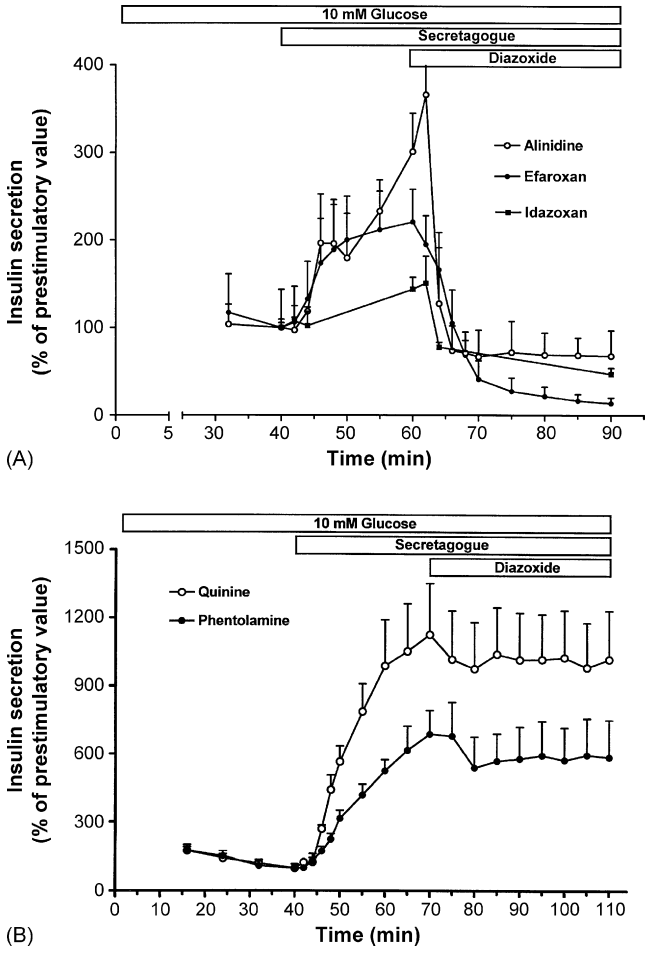

Fig. 5 – Effects of diazoxide on the enhancement of insulin secretion by imidazolines. (A) Antagonism of the insulinotropic effect of alinidine, efaroxan and idazoxan. The secretion of isolated, perifused mouse islets in the presence of 10 mM glucose was normalized to 100%. From 40 to 90 min the respective secretagogue (100 μM) was present, from 60 to 90 min diazoxide (300 μM) was additionally present. Values are mean ± S.E.M. of four to five perifusion experiments. Each secretagogue caused a significant increase of secretion during a 20 min perifusion period and in each case diazoxide significantly antagonized the insulinotropic effect. (B) No antagonism by diazoxide of the insulinotropic effect of phentolamine and quinine. Mouse islets were perifused with Krebs–Ringer medium containing 10 mM glucose throughout the experiment. From 40 to 110 min the respective secretagogue (100 μM) was present, from 70 to 110 min diazoxide (300 μM) was additionally present. Values are mean ± S.E.M. of four perifusion experiments.

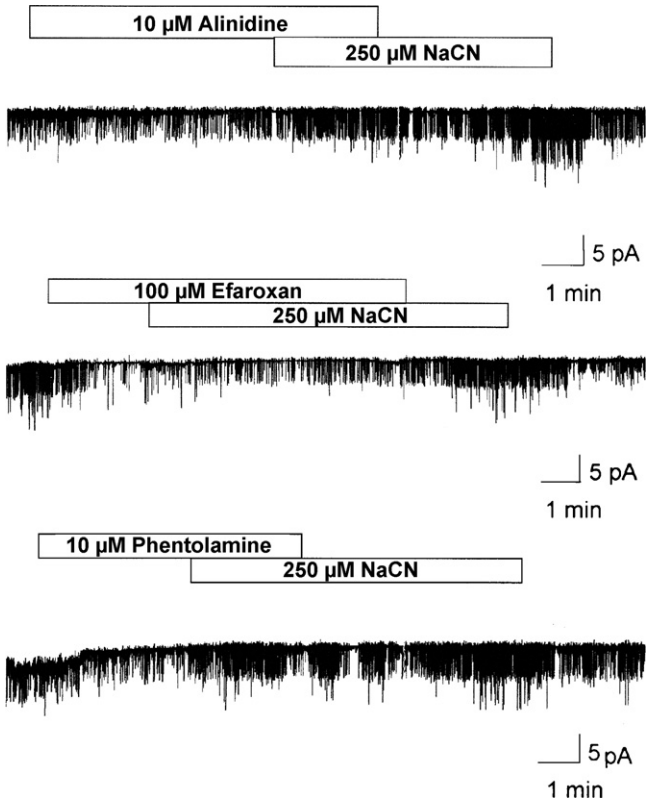

Fig. 6 – Effect of metabolic blockade by NaCN (250 μM) on the inhibition of K_{ATP} channels by alinidine, efaroxan and phentolamine in intact mouse B-cells. K_{ATP} channel activity was measured in the cell-attached configuration of the patch clamp-technique. Compounds were applied as indicated by the horizontal bars. Representative traces of four to five experiments each.

4. Discussion

In this investigation it could be shown that the imidazoline-induced block of K_{ATP} channels in pancreatic B-cells is subject to the opening action of nucleoside diphosphates and diazoxide and that this antagonism is relevant for the insulinotropic effect. The observations reported in the literature so far were contradictory. This can now be explained by the marked differences among the imidazolines, which become relevant when intact B-cells and islets are used.

The measurement of K_{ATP} channel activity in inside-out patches from B-cells clearly showed that the blocking effect of low concentrations of imidazolines can be antagonized by MgGDP. This concurs with an earlier observation where MgADP slightly but significantly antagonized the block by the α-agonistic imidazoline clonidine [31]. For the present study MgGDP was chosen as channel opener because its action is less complex than that of MgADP. In the absence of Mg^{2+}, nucleoside diphosphates exert an inhibitory effect in addition to an opening effect on K_{ATP} channels [29]. GDP has a much lower inhibitory efficacy than ADP and thus MgGDP, in contrast to MgADP, is a virtually pure K_{ATP} channel opener [32]. The opening effect results from binding of the Mg-complexed dinucleotides to SUR1 whereas the inhibitory effect results from an interaction of the nucleotide with Kir6.2 [33]. To check whether the opening effect of nucleoside diphosphates was also detectable under conditions relevant for secretion (intact B-cells, maximally effective concentration of the imidazolines) we used NaCN and measured K_{ATP} channel activity in the cell-attached mode (see below).

Table 2 – Effect of 250 μM NaCN on the block of K_{ATP} channels by imidazolines and by tolbutamide in intact pancreatic B-cells

	Secretagogue	Secretagogue + 250 μM NaCN	250 μM NaCN
10 μM Phentolamine	41.0 ± 6.1 (n = 5)	158.7 ± 54.3 (n = 5) (P = 0.08)	184.1 ± 84.2 (n = 5) (P = 0.7)
10 μM Alinidine	31.7 ± 10.1 (n = 6)	169.0 ± 43.5 (n = 5) (P = 0.03)	389.3 ± 206.4 (n = 4) (P = 0.3)
100 μM Alinidine	2.0 ± 0.7 (n = 6)	5.4 ± 2.3 (n = 5) (P = 0.2)	107.6 ± 35.4 (n = 5) (P = 0.03)
10 μM Efaroxan	45.4 ± 4.8 (n = 9)	96.1 ± 17.6 (n = 5) (P = 0.02)	403.9 ± 145.2 (n = 5) (P = 0.08)
100 μM Efaroxan	10.8 ± 3.1 (n = 7)	183.4 ± 51.1 (n = 5) (P = 0.02)	3444.1 ± 1195.8 (n = 5) (P = 0.04)
500 μM Tolbutamide	14.7 ± 6.3 (n = 6)	106.1 ± 59.3 (n = 6) (P = 0.03)	3585.4 ± 1488.9 (n = 6) (P = 0.004)

The K_{ATP} channel activity was measured using the cell-attached mode as shown in Fig. 6. Channel activity is expressed in percent of the activity under the control condition (extracellular medium), values are mean ± S.E.M. of the given number of experiments. The inhibitory effect of the secretagogues was significant in each case ($P < 0.05$, Welch's t-test). The opening effect of NaCN in the presence of the secretagogue was significant for 10 μM alinidine, for both concentrations of efaroxan ($P < 0.05$, Welch's t-test) and for tolbutamide ($P < 0.05$, Wilcoxon test). The subsequent wash-out of the secretagogue led to a further increase, which was significant for 100 μM alinidine, 100 μM efaroxan ($P < 0.05$, Welch's t-test) and for tolbutamide ($P < 0.01$, Wilcoxon test). The increase after wash-out of 10 μM efaroxan was marginally significant ($P = 0.08$, Welch's t-test). After wash-out of 10 μM alinidine there was an increase in each experiment, a significant difference was not attained with the given number of experiments due to large variability of the increase.

Using the inside-out configuration it was also possible to verify an opening effect of diazoxide, however, this effect was not significant for phentolamine and quinine. This could mean that either MgGDP had already achieved a maximal channel activation or that phentolamine and quinine could not be antagonized by diazoxide. The latter view would be concurrent with earlier investigations, which had shown that diazoxide had at best a small opening effect on phentolamine-blocked K_{ATP} channels [7,16] and that diazoxide did not antagonize the quinine-induced channel block [25]. The measurement of the B-cell membrane potential gave a clear-cut answer to this problem: the depolarizing effect of all imidazolines and of quinine could be antagonized by diazoxide. This permits the conclusion that in principle diazoxide, which acts by binding to SUR1 [21,22], can antagonize the effects of K_{ATP} channel blockers irrespective of whether they act via SUR1 or Kir6.2.

There is, however, an obvious discrepancy between the results of the membrane potential measurements on one side and results of the $[Ca^{2+}]_i$- and insulin secretion measurements on the other side. The $[Ca^{2+}]_i$ increase and the secretion enhancement by phentolamine and quinine were practically not affected by diazoxide, even though the depolarization was largely antagonized. The most likely explanation for this is the difference in the cellular integrity. The current clamp measurements were performed in the standard whole-cell configuration where the cytosol is exchanged by the pipette solution within a few minutes [34] whereas the $[Ca^{2+}]_i$ and insulin secretion measurements were performed with intact B-cells.

Earlier, it was reported that the insulinotropic effect of glibenclamide, which is known to accumulate within islet cells, could not be antagonized by the additional presence of 100 μM diazoxide [35], even though an antagonism clearly exists at the level of the KATP channels. We assume that a similar mechanism underlies the inability of diazoxide to antagonize the $[Ca^{2+}]_i$-elevating and insulin-releasing effect of quinine and phentolamine. Alternatively, both compounds may activate signalling mechanisms in the intact cell, which counteract the effect of diazoxide, an effect which would not be shared by the other insulin secretagogues. In both cases formation of the whole-cell configuration would lead to a marked dilution of the cytoplasm, allowing diazoxide to become effective.

The slow spontaneous reversibility of the K_{ATP} channel block by phentolamine [7,31] and quinine [25,36] is concurrent with the former explanation as is the observation that phentolamine and quinine but not alinidine produced a virtually irreversible block of B-cell K_{ATP} channels after a long-term exposure to these imidazolines, which could be overcome by diazoxide only after patch excision [37]. The alternative hypothesis that K_{ATP} channel independent effects exerted by imidazolines [10,11] could be responsible for the lack of antagonism cannot explain why the insulinotropic effects of efaroxan and alinidine are easily antagonized. In these cases diazoxide lowered the secretory rate below the level established by 10 mM glucose prior to the addition of the imidazolines, suggesting that the ATP-mediated effect of glucose was antagonized as well.

In view of the ability of MgGDP and MgUDP to antagonize the K_{ATP} channel blocking effect of imidazolines it was expected that inhibition of oxidative phosphorylation by sodium cyanide would antagonize the imidazoline block in intact B-cells. Metabolic blockade, most likely by a decrease of the cytosolic ATP/ADP ratio, is known to open K_{ATP} channels and to reduce their susceptibility to block by sulfonylureas [38,39]. In our experiments the concentration of the imidazolines proved to be of critical importance. There was no significant antagonism when alinidine was used at 100 μM, whereas at 10 μM, an about half-maximally effective concentration, there was a significant increase in K_{ATP} channel activity by metabolic blockade. Remarkably, the effect of efaroxan was susceptible to metabolic blockade even at a nearly maximally effective concentration (100 μM). Alinidine blocks K_{ATP} channels via Kir6.2 [27], the same is true for phentolamine [23] and efaroxan [40]. Thus, it can be concluded that the block of K_{ATP} channels at Kir6.2 is in principle susceptible to metabolic blockade.

This conclusion is at variance with that of a report comparing the effect of metabolic blockade of B-cells on K_{ATP} channel block by glibenclamide and cibenzoline. In contrast to the channel block by glibenclamide that by cibenzoline, an imidazoline compound with antiarrhythmic and insulinotropic properties, was only minimally affected by metabolic

blockade [39]. Since cibenzoline inhibits K_{ATP} channels via Kir6.2 [41] the authors concluded that metabolic blockade would cause an interruption in signal transmission between SUR1 and Kir6.2 but would not be able to affect drug action at Kir6.2. Since there is a close structural similarity between phentolamine and cibenzoline, the latter compound may exert similarly slow and virtually irreversible effects as phentolamine, accounting for the apparent discrepancy.

The fast reversibility of the K_{ATP} channel block by efaroxan, the immediate antagonism by diazoxide and the antagonism by metabolic blockade of even a high concentration of efaroxan point to a ready dissociation of efaroxan from its binding site at Kir6.2. Thus, the oscillations of $[Ca^{2+}]_i$ produced by efaroxan but not phentolamine at basal glucose concentrations (this paper, 40), could reflect metabolically driven oscillations of the ATP/ADP ratio which affect the efficacy of K_{ATP} channel blockade by efaroxan. The frequency of the efaroxan-induced oscillations in the presence of 5 mM glucose (ca. 0.3 min^{-1}) is in the range of the slow $[Ca^{2+}]_i$ oscillations induced by stimulatory glucose concentrations [42], which would be compatible with this hypothesis.

To conclude, the block of B-cell type K_{ATP} channels by first generation imidazolines can be antagonized by nucleoside diphosphates and diazoxide. Thus, the pharmacology of the imidazoline block of K_{ATP} channels is influenced by the properties of SUR, which confers tissue specificity, even though the blocking action of imidazolines is exerted at Kir6.2.

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