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β-Cell toxicity of ATP-sensitive K^+ channel-blocking insulin secretagogues

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Abstract

A prolonged exposure of isolated pancreatic islets to insulin secretagogues, the imidazolines phentolamine, alinidine and idazoxan (100 μM each), the sulfonylurea tolbutamide (500 μM), or the alkaloid quinine (100 μM) resulted in morphological damage of 4–18% of β-cells compared to less than 2% in controls. Thus, the question arose whether K_{ATP} channel-blocking insulin secretagogues are β-cell toxic as has already been suggested for sulfonylureas. The concentration- and time-dependency of the secretagogue-associated toxicity was documented by viability assays in insulin-secreting HIT T15 cells. Treatment for 24 h with idazoxan reduced MTT conversion by 50% at 100 μM and by 98% at 1000 μM. Phentolamine and quinine reduced viability comparably at 1000 μM, but were less toxic at 100 μM. On the other hand, the imidazoline alinidine and the sulfonylurea tolbutamide were only moderately toxic (less than 40% viability loss at 1000 μM). The imidazoline efaroxan appeared even to be non-toxic. Apoptotic DNA fragmentation and DEVD-caspase activation was observed at 100 μM of idazoxan and phentolamine, whereas at 1000 μM signs of necrosis predominated. Alinidine, tolbutamide and quinine treatment did not increase markers of apoptotic cell death. Blocking Ca^{2+} influx by D600 did not diminish secretagogue-associated toxicity. Electron microscopy confirmed the validity of these observations for β-cells in intact mouse islets. In summary, β-cell toxicity of the tested insulin secretagogues varied widely and did not depend on a prolonged Ca^{2+} influx via L-type Ca^{2+} channels. Thus, secretagogue-mediated closure of K_{ATP} channels is apparently not per se β-cell toxic.

Keywords: Pancreatic islets; HIT-cells; Imidazolines; Tolbutamide; Apoptosis; Necrosis

1. Introduction

In a recent investigation, the desensitization of insulin secretion by a panel of structurally diverse inhibitors of ATP-dependent K^+ channels (K_{ATP} channels) was studied. Isolated incubated mouse islets were examined by electron microscopy in order to determine the content of secretory granules. Unexpectedly, islets cultured for 18 h in the presence of the secretagogues did not only show varying degrees of degranulation but also contained a higher number of ultrastructurally damaged β-cells than control-cultured islets [1]. The β-cell damage was most prominent after exposure to the imidazoline idazoxan but there was also an increased number of damaged β-cells following exposure to the sulfonylurea tolbutamide [1].

This observation was reminiscent of a recent report that several imidazolines, a group of investigational antidiabetic drugs, negatively affected viability of insulin-secreting cell lines [2]. In this investigation some imidazolines like idazoxan were found to be markedly toxic, while others like efaroxan were apparently completely devoid of such an effect. Thus, the authors concluded that the toxicity was due to individual properties of the imidazolines and was not the consequence of the common K_{ATP} channel-blocking and insulin-releasing effect. On the other hand, Berggren and co-workers had 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

Abbreviations: K_{ATP} channels, ATP-dependent K^+ channels; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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voltage-dependent Ca^{2+} channels [3]. These authors concluded that a prolonged influx of Ca^{2+} into the β-cell was the critical event triggering β-cell apoptosis.

Since the stimulation of insulin secretion by both, sulfonylureas [4] and imidazolines [5,6], involves closure of K_{ATP} channels, and consequently, depolarization of the plasma membrane with subsequent Ca^{2+} influx via L-type Ca^{2+} channels, the question arises how a long-term exposure to the K_{ATP} channel blocking imidazoline efaroxan [7,8] can be devoid of a β-cell damaging effect. In view of the observations by Efanova et al. [3] one would rather expect that similar to tolbutamide all insulinotropic imidazolines should induce apoptosis and decrease β-cell viability.

This study was undertaken to provide answers to the following questions: firstly, was the desensitization of insulin secretion the consequence of a β-cell toxic effect of the secretagogues? Therefore, we chose the same panel of insulin secretagogues used earlier to investigate the mechanism of desensitization [1,9]. Secondly, is the toxicity of imidazolines comparable to that of other K_{ATP} channel-blocking insulin secretagogues like tolbutamide and quinine? Thirdly, is an increased influx of Ca^{2+} from the extracellular space sufficient to elicit a β-cell toxic effect via induction of apoptosis?

2. Materials and methods

2.1. Materials

Phentolamine was donated by Ciba-Geigy, alinidine by Boehringer Ingelheim and idazoxan and efaroxan by RBI. D600 (methoxyverapamil) was a gift from Knoll and aprotinin from Bayer. Tolbutamide was obtained from Serva. Digitonin, MTT, quinine, sodium dodecylsulfate and tergitol NP40 were purchased from Sigma, collagenase P and dithiotreitol from Boehringer Mannheim, RNase A from Qiagen and DEVD-pNA from Bachem. Cell culture medium RPMI 1640 (without glucose) was from Gibco BRL and fetal calf serum from Biochrom. GFX minicolumns were from Amersham Pharmacia and NuSieve 3:1 agarose from FMC Bioproducts. HEPES was supplied by AppliChem, all other reagents of analytical grade by E. Merck.

2.2. Tissue culture

Islets were isolated from the pancreas of NMRI mice by a conventional collagenase digestion technique. The freshly isolated islets were transferred into cell culture medium RPMI 1640 with 10% fetal calf serum and cultured in a humidified atmosphere of 95% air and 5% CO_2. The tissue culture medium had a non-stimulatory glucose concentration of 5 mM. HIT T15 cells were cultured in RPMI 1640 (10 mM glucose) with 10% FCS.

2.3. Electron microscopy

Collagenase-isolated islets from NMRI mice were pooled and cultured in batches of 50 islets. After tissue culture for 18 h in the presence of the various secretagogues, the islets were fixed for electron microscopy [10] by immersing in a solution of *para*-formaldehyde (2%) and di-glutaraldehyde (2%) in cacodylate buffer (0.1 M, pH 7.3), post-fixing in osmium tetroxide (1%) for 1 h and embedding in epoxy resin. Fifty-nanometer sections of the islets were cut by an ultramicrotome (Ultracut, Leica-Reichert-Jung, Germany), placed on nickel grids and contrast-stained with uranyl acetate and lead citrate. Transmission electron microscopy was performed using a Zeiss EM 9 electron microscope. Five medium-sized islets (diameters about 150 μm) were chosen for ultrastructural examination. The analyzed islets contained 70–100 β-cells per section which were classified as being intact or damaged. Two independent incubations were performed for each condition.

2.4. Viability assessment by MTT conversion

HIT T15 cells were seeded in 96-well plates 1 day before the 24 or 48 h exposure to the secretagogues. The incubation volume was 90 μl. At the end of the test period 10 μl MTT solution (5 mg/ml in PBS) was added and the cells were incubated for 1 h at 37 °C. Thereafter cells were lysed by the addition of sodium dodecylsulfate. The conversion of MTT into magenta-colored formazan, which is dependent on mitochondrial metabolism [11], was measured in a microplate reader at 550 nm against a background at 650 nm. Data from treated cells were presented relative to those from untreated cells which were given as 100% viability.

2.5. DNA fragmentation

Internucleosomal DNA fragmentation was analyzed by a spin column assay [12]. About 10^6 HIT cells were seeded in 3.5 cm dishes 1 day before a 24 h exposure to the tested agents. At the end of the incubation, cells were scraped off the culture dishes, washed in PBS and spun down. The washed cells were resuspended in a Tris/EDTA buffer (pH 7.5) containing RNase A (100 μg/ml). Cells were lysed at neutral pH by adding an equal volume of 1.2% SDS. After 5 min a CsCl solution was added and cell debris and proteins were precipitated after cooling to 4 °C and centrifugation at 14,000 × *g*, each for 15 min. The fragmented DNA in the supernatant was collected on a GFX minicolumn. Fifteen microliters of the eluate (30% of total volume) were loaded per lane on a 1.6% Nusieve agarose TAE gel and separated by electrophoresis at 100 V for 35 min. DNA bands were stained with ethidium bromide and visualized at 312 nm on a transilluminator and recorded by a CCD camera (Biodoc, Biometra, Göttingen).

The intensity of the DNA-ladder pattern was scored visually by three independent observers in 0.5 steps on a scale ranging from 0 to 4. The resulting mean values were rounded to the next 0.5 step.

2.6. Assay of caspase activity

About 10^6 HIT cells were seeded in 6 cm dishes 1 day before the 3 or 24 h exposure to the test agents. At the end of the test period, cells were trypsinized and washed with PBS containing 2 mM PMSF. Then the cells were taken up in caspase buffer (HEPES 10 mM, tergitol-NP40 1%, aprotinin 0.01 mg/ml, EDTA 4 mM; PMSF 2 mM, DTT 15 mM, pH 7.4) and lysed by digitonin (25 mg/l final concentration, exposure 15 min on ice). After centrifugation at 5700 × *g* at 4 °C for 10 min, 200 μl of the supernatant were incubated at 37 °C with 25 μM of the caspase substrate DEVD-pNA [13]. The absorption was measured in 30 min intervals at 405 nm in a microplate photometer. Caspase activity was determined from the linear increase of absorption per time and was expressed as 1 unit/mg protein, 1 unit corresponding to the conversion of 1 pmol substrate per minute.

2.7. Data handling and statistics

Additional calculations and statistics were performed by Prism and Instat software (GraphPad, San Diego, CA, USA). Without further specifications *t*-test stands for Student's unpaired two-sided *t*-test. If not specified otherwise, significance was considered if $P < 0.05$.

3. Results

3.1. Ultrastructural features of secretagogue-induced β-cell toxicity

In islets cultured for 18 h in the presence of a maximally depolarizing concentration of the test agents (500 μM for tolbutamide, 100 μM for the other compounds) there was a higher number of damaged β-cells (4–18%) than in control-cultured islets (1.5%). In control-cultured islets (Fig. 1A) the occurrence of damaged β-cells was confined to the islet periphery whereas in secretagogue-exposed islets centrally located β-cells were also affected. After exposure to idazoxan 17.6% of the β-cells were damaged or appeared even necrotic (Fig. 1B). In some cells, swollen mitochondria and intracytoplasmic vacuolization were seen as signs of a principally reversible damage. In the majority of the affected cells ruptures of organelle membranes and of the plasma membrane were found, which are signs of irreversible damage. In the vast majority of β-cells (≥85%) the alterations were typical for a necrotic mode of cell death, but there was also a sizable fraction where the morphological features were compatible with apoptosis (≤15%). Comparable alterations in the β-cell morphology were also found, albeit less frequently, after incubation with two other imidazolines, phentolamine (6.2%, Fig. 1C) and alinidine (4.8%). Quinine induced changes in 9.6% of the β-cells, tolbutamide only in 3.8%. In the latter case the nuclear heterochromatin condensation and marginalization was particularly prominent (Fig. 1D). There were no signs of a compensatory increased mitotic replication. It is noteworthy that in none of the sections damaged non-β-cells could be found.

3.2. Viability assessment by MTT conversion

The time course and concentration dependency of the secretagogue-associated toxicity was assessed by measuring MTT metabolism of insulin-secreting HIT cells following exposure to the secretagogues for 24 or 48 h. After 24 h marked differences between the toxicity of the secretagogues were apparent (Fig. 2A). Idazoxan caused no significant reduction in MTT conversion at 10 μM, while at 100 μM viability was reduced to 50% and at 1000 μM it was abolished. At 100 μM phentolamine was significantly less toxic than idazoxan, whereas at 1000 μM it also led to a virtually complete loss of MTT conversion. The toxicity of alinidine increased only moderately between 10 and 1000 μM, similar to tolbutamide, which led to a significant 15% reduction of MTT metabolism only at 1000 μM. Quinine was non-toxic at 10 and 100 μM, while at 1000 μM a nearly complete loss of viability was observed.

An exposure for 48 h (Fig. 2B) displayed even more pronounced differences between the secretagogues. Idazoxan abolished MTT conversion already at 100 μM and phentolamine reduced viability by 40% at 100 μM. In contrast, the toxicity of alinidine and tolbutamide was only slightly more pronounced than after 24 h with 50% or more of the cells surviving even at the highest concentrations. Quinine killed all cells at 1000 μM, but in contrast to idazoxan the toxic effect of 100 μM was not much augmented after the longer exposure period.

3.3. Detection of apoptosis by DNA fragmentation and DEVD-caspase activity

Since viability of the HIT cells was not significantly affected by a 24 h exposure to 10 μM of the secretagogues, only the concentration range from 100 to 1000 μM was tested for a potential DNA cleavage. A 24 h exposure to 100 μM of either idazoxan or phentolamine resulted in the typical apoptotic ladder pattern of internucleosomally fragmented DNA (Fig. 3A). At 1000 μM phentolamine produced a massive accumulation of irregular, large DNA fragments (Fig. 3B). Comparably, the intensity of the DNA ladder pattern was more irregular at 1000 μM of idazoxan than at 100 μM. DNA laddering by tolbutamide (1000 μM) and alinidine (100–1000 μM) was much weaker and did not significantly differ from control lanes (Fig. 3A). A

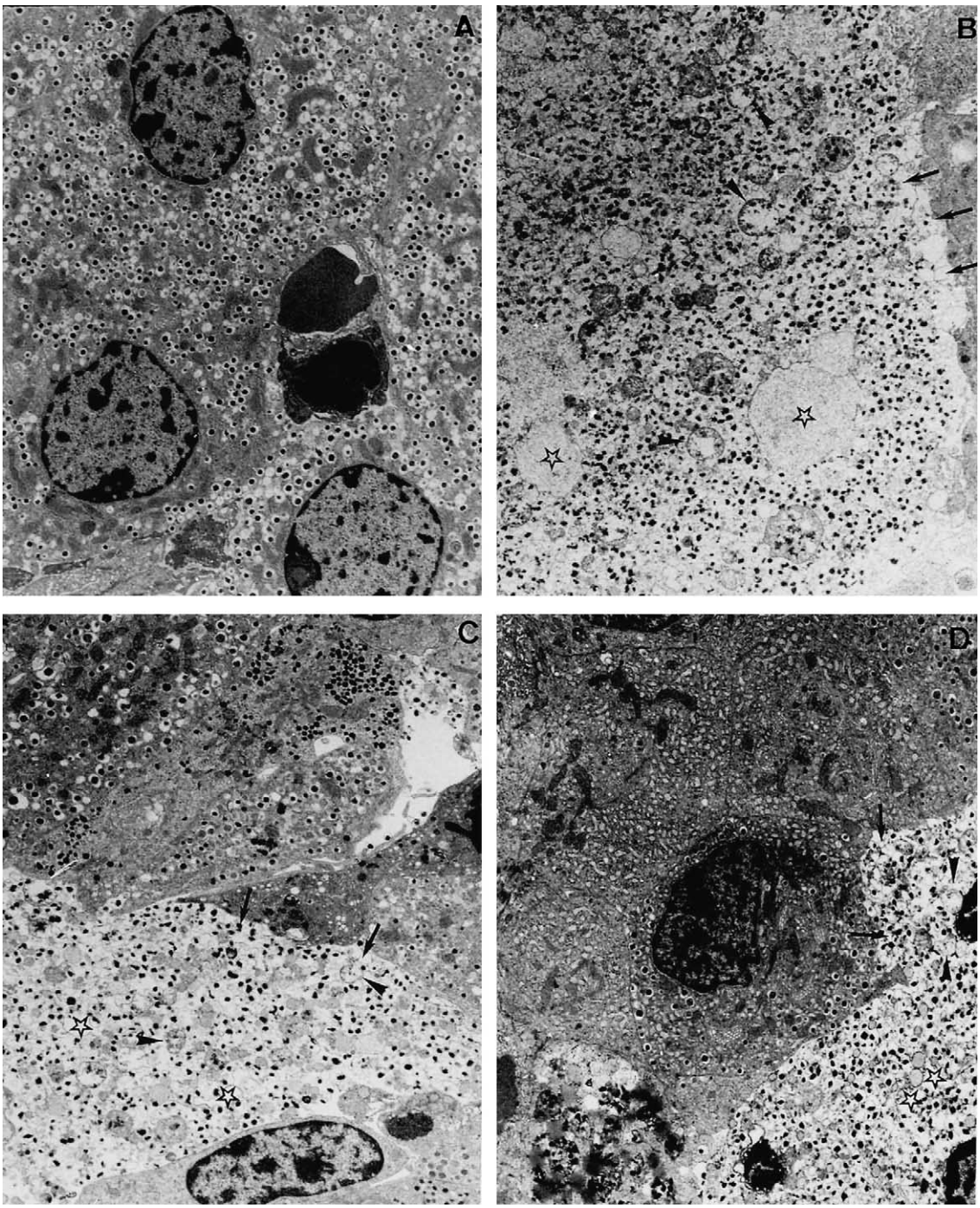

Fig. 1. Ultrastructure of pancreatic islets exposed for 18 h to insulin secretagogues acting by closure of the K_{ATP} channel. (A) Control-incubated islet with well-preserved and well-granulated β-cells. (B) In islets incubated in the presence of 100 μM of idazoxan, clusters of severely damaged β-cells could be found. (C) Incubation of islets with 100 μM of phentolamine led to less extensive damage in single β-cells. The non-β-cells visible in the upper part are not affected. (D) Occasionally, damaged β-cells could also be found after exposure to 500 μM of tolbutamide. Note the strong degranulation after tolbutamide exposure. Ruptures of the plasma membrane are marked by arrows, swollen mitochondria by arrowheads and intracytoplasmic vacuolization by asterisks. Magnification 5800×.

semi-quantitative evaluation of the gels is given in Table 1. The most intensive DNA laddering occurred after exposure to the phosphatase inhibitor cantharidic acid, which was used as a positive control. No DNA laddering was visible when cells were exposed to idazoxan and phentolamine for only 3 h.

A 24 h incubation in the presence of the insulin secretagogues significantly increased DEVD caspase activity by 2–2.5-fold in HIT cells treated with 100 μM phentolamine and 100 μM idazoxan (Fig. 4). However with 1000 μM of these imidazolines virtually no measurable caspase activity was left. Alinidine and tolbutamide treatment did not

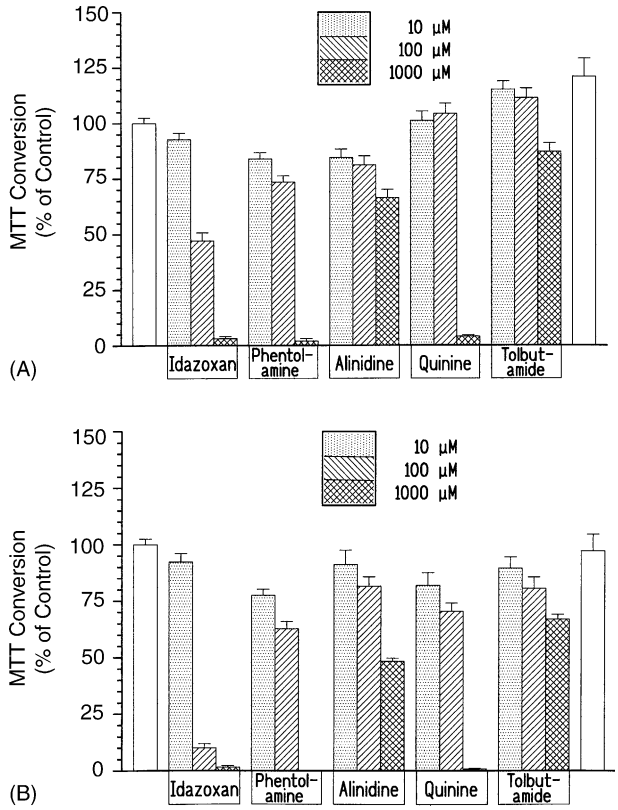

Fig. 2. Reduction of MTT metabolism by exposure of HIT cells to insulin secretagogues for 24 h (A) or 48 h (B). The open column on the left represents the control value (incubation in RPMI 1640 with 5 mM glucose), the open column on the right represents the control value for the tolbutamide incubation, since the tolbutamide stock solution was prepared using 1N NaOH. All effects at 100 and 1000 µM were significant except for those of 100 µM quinine and tolbutamide at 24 h. The values are means ± S.E.M. of six experiments.

Table 1
Semi-quantitative evaluation of DNA fragmentation in HIT cells induced by insulin secretagogues

Compound	Concentration (µM)	Intensity rating
Idazoxan	100	1.0
	1000	0.0 (F)
Phentolamine	100	1.0
	300	2.0
	1000	1.0 (F)
Alinidine	100	0.0
	300	0.0
	1000	0.0
Quinine	100	0.0
	1000	0.5 (F)
Tolbutamide	1000	0.5
Cantharidic acid	10	2.5
Control		0.0 (0.24)

The intensity of the DNA-ladder pattern on agarose gels (such as shown in Figs. 3 and 5) was scored visually in 0.5 steps on a scale ranging from 0 to 4. Mean values were calculated and rounded to the next 0.5 step. Values ≥1.0 suggest apoptotic DNA cleavage. (F) denotes the presence of large irregular DNA fragments (phentolamine > quinine > idazoxan) as occurs in necrosis or late apoptosis. Cantharidic acid, an inhibitor of phosphatases and a known inducer of apoptosis in HIT cells, was used as a positive control. The number of experiments ranged from 2 to 8.

increase caspase activity (Fig. 4). Quinine had no significant effect at 100 µM, but again at 1000 µM the detectable caspase activity was strongly reduced. The most marked increase of caspase activity was produced by cantharidic acid (about three-fold). When HIT cells were exposed to idazoxan and phentolamine for only 3 h, caspase activity

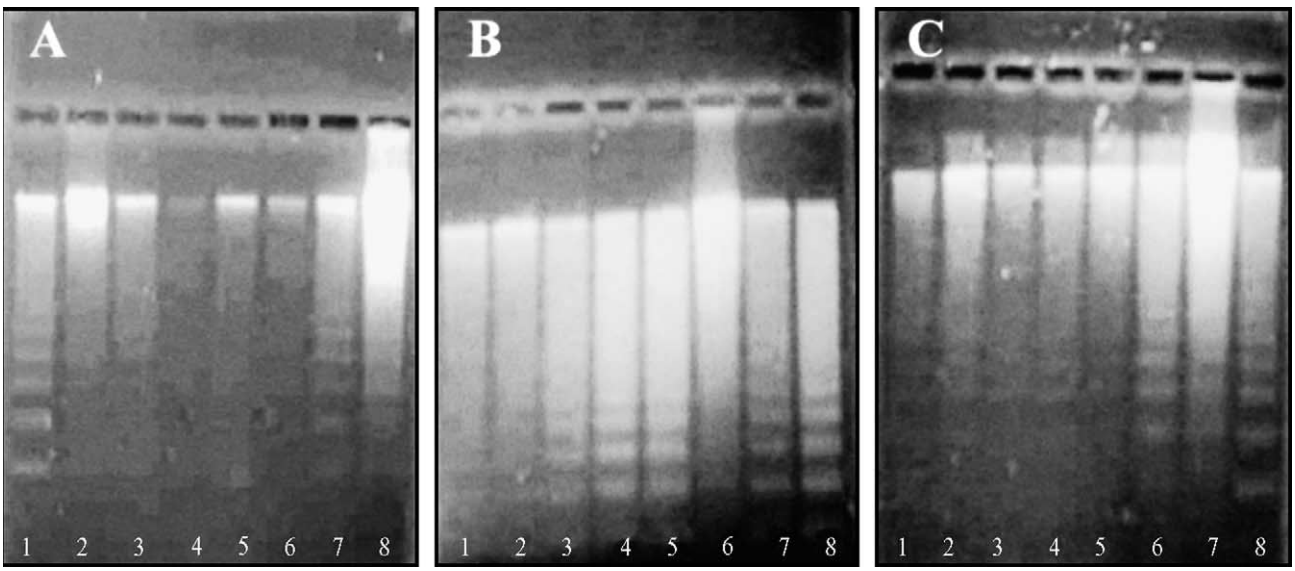

Fig. 3. Fragmentation of HIT cell DNA by insulin secretagogues. (A) Effects of idazoxan (1000 and 100 µM, lanes 2 and 3), tolbutamide (1000 µM, lane 5) phentolamine (100, 300 and 1000, lanes 6–8) in comparison with cantharidic acid = positive control (10 µM, lane 1), and control incubation (lane 4). (B) Concentration dependence of the effect of phentolamine (100, 300, 600, 1000 µM, lanes 3–6), compared with cantharidic acid (5 and 10 µM, lanes 7 and 8) and control incubations (lanes 1 and 2). (C) Concentration dependence of the effect of alinidine (100, 300, 600, 1000 µM, lanes 2–5) compared with phentolamine (300 and 1000 µM, lanes 6 and 7), cantharidic acid (10 µM, lane 8) and control incubation (lane 1).

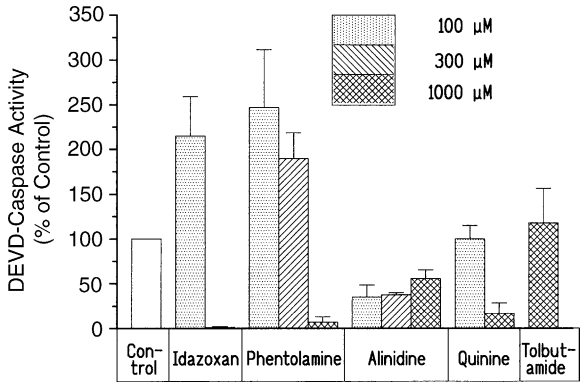

Fig. 4. DEVD-caspase activity in HIT cells exposed for 24 h to insulin secretagogues. The control value of each experiment was normalized to 100%. The increase of caspase activity by 100 μM of idazoxan and 100 μM of phentolamine was significant ($P < 0.05$, ANOVA with Dunnett's post-hoc test). Exposure to 10 μM cantharidic acid served as a positive control, this treatment resulted in a significant increase to 284 ± 36%. The values are means ± S.E.M. of 3–6 experiments.

Fig. 5. Role of Ca^{2+} influx on the fragmentation of HIT cell DNA by insulin secretagogues. Fragmentation induced by 100 μM idazoxan (lane 2), 300 μM or 1000 μM phentolamine (lanes 4 and 6) was not reduced by 50 μM of the blocker of L-type Ca^{2+} channels, D600 (lanes 3, 5 and 7, respectively). Lane 1 = control incubation.

was increased at 1000 μM of these secretagogues (phentolamine two-fold, idazoxan 1.3-fold) but not at 100 μM.

3.4. Role of Ca^{2+} influx for secretagogue-induced β-cell toxicity

The potential contribution of the depolarization-induced Ca^{2+} influx to the secretagogue-induced toxicity was investigated by treating HIT cells with a high potassium concentration (40 mM). This exposure resulted in a moderate decrease in viability as measured by MTT conversion (74 ± 7% of control after 24 h) which did not display much further progress after 48 h (68 ± 5%). DNA laddering under this condition did not differ from control. Surprisingly, the combination of K^+ depolarization plus 50 μM of D600, a blocker of L-type Ca^{2+} channels, led only to a slightly smaller reduction of MTT conversion (viability 82% after 24 h and 76% after 48 h). DNA fragmentation under this condition appeared to be even somewhat increased. D600 by itself had only a negligible effect on MTT conversion and DNA fragmentation (data not shown). There was also no significant antagonistic effect of D600 on the reduction of viability by the secretagogues, including tolbutamide. In the same line, the marked DNA fragmentation caused by a 24 h treatment with phentolamine (300 and 1000 μM) or idazoxan (100 μM) was unaffected by the presence of D600 (Fig. 5) as was DEVD caspase activity (data not shown). The use of the K^+ channel opener diazoxide (300 μM) was also unable to reduce toxicity or DNA fragmentation caused by phentolamine or idazoxan.

Since the depolarization-induced Ca^{2+} influx is the common denominator of all tested secretagogues its role in potential β-cell toxicity was also checked by ultrastructural examination of isolated mouse islets cultured for 18 h in RPMI medium containing either 40 mM K^+ or the respective secretagogue. For each condition a parallel incubation containing in addition 50 μM D600 was performed. Electron microscopy revealed that the number of damaged or necrotic β-cells (1.7 ± 0.5% in control-cultured islets) increased to 3.5 ± 0.8% after 18 h of exposure to 40 mM KCl. As in secretagogue-exposed islets (Fig. 1D) only β-cells were affected. With the given number of observations (10 islets from two incubations) this increase achieved only marginal significance ($P = 0.07$, t-test). The percentage of damaged cells was unchanged (3.3%) by the concomitant presence of 50 μM D600 in the incubation medium (Fig. 6A). Likewise, D600 did not reduce the occurrence of β-cell damage by exposure to 100 μM idazoxan (15.6% in the presence of D600 versus 17.6% in its absence) or by 100 μM phentolamine (7.5% +D600 versus 6.2% −D600) or by 500 μM tolbutamide (3.8% +D600 versus 3.6% −D600; Fig. 6B).

3.5. Lack of β-cell toxicity of the imidazoline efaroxan

Since none of the secretagogues in our test panel was completely devoid of a β-cell toxic effect, we tested whether the reportedly non-toxic imidazoline efaroxan [2] affected HIT cell viability under our conditions. The MTT-metabolizing capacity of HIT cells did not significantly differ from control after a 48 h incubation in the presence of 10, 100 or even 1000 μM efaroxan. In the same experiments tolbutamide and alinidine at 1000 μM moderately, but significantly decreased viability by 23 ± 2% and 33 ± 4%, respectively.

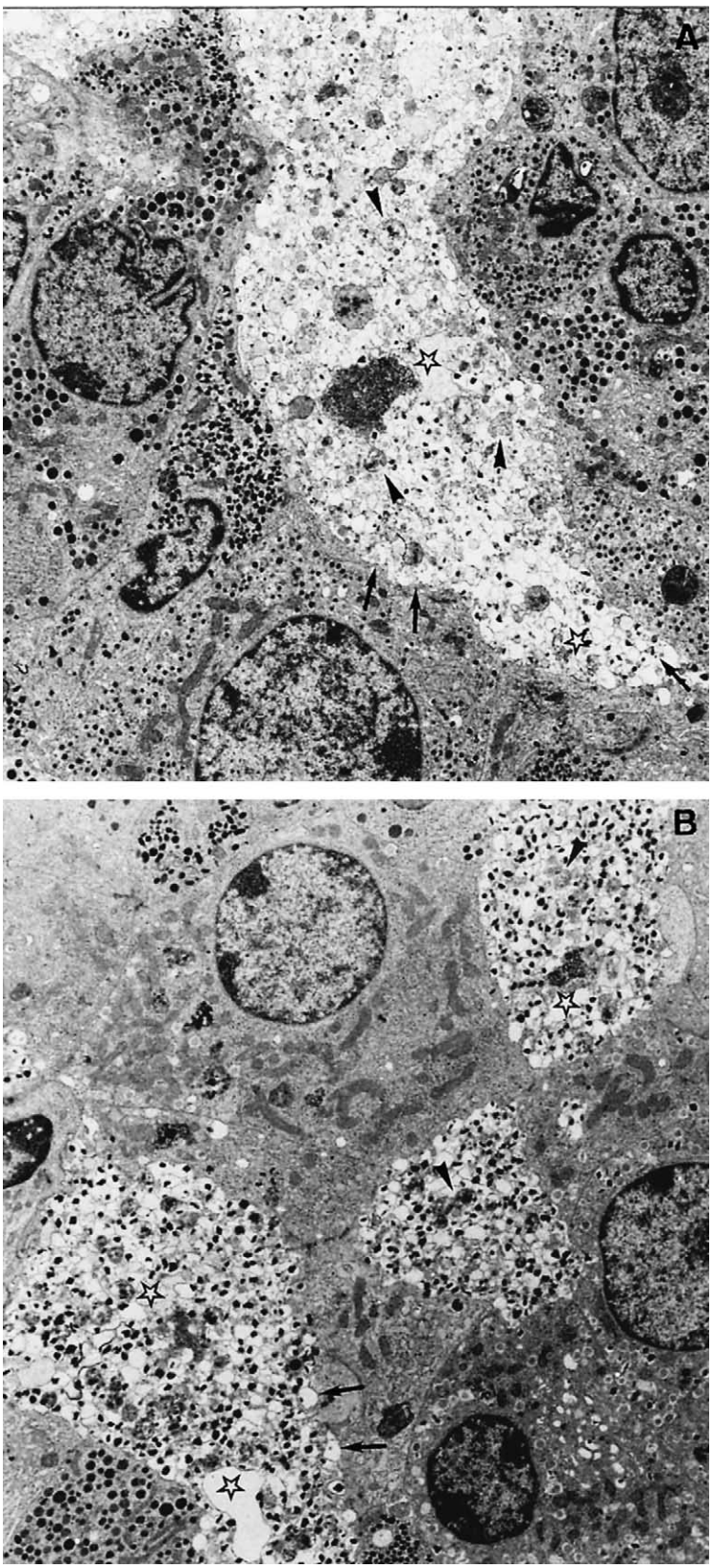

Fig. 6. Ultrastructure of pancreatic islets cultured for 18 h under depolarizing conditions in the presence of 50 μM D600, a blocker of L-type Ca^{2+} channels. Islet cells were depolarized by 40 mM K^+ (A) or by 500 μM tolbutamide (B). Under both conditions, single β-cell necroses occurred occasionally and were distributed evenly throughout the islets. Non-β-cells were not affected. Ruptures of the plasma membrane are marked by arrows, swollen mitochondria by arrowheads and intracytoplasmic vacuolization by asterisks. Magnification 5800×.

4. Discussion

The insulin secretagogues selected initially for this study affected pancreatic β-cells with the following order: idazoxan ⩾ quinine > phentolamine > alinidine = tolbutamide > control. The absence of damaged A- and D-cells in secretagogue-exposed islets permits the conclusion that the tested compounds exert a β-cell-specific toxicity. Therefore, and since the biochemical characterization of the β-cell toxic effect required comparatively large amounts of tissue, HIT T15 cells, a clonal β-cell line was used for further experiments.

In order to ascertain that HIT cells were an appropriate model it was first checked whether the number of damaged β-cells within islets correlated with the loss of viability of HIT cells following exposure to the test agents. A very good and highly significant correlation was obtained ($r = -0.97$, $P = 0.007$) in case quinine was excluded. The toxicity of quinine was unusual in that it displayed a very steep concentration dependency, leading from a non-significant reduction of viability at 100 μM to a nearly complete loss at 1000 μM. Thus, small differences between β-cells and HIT cells in the cellular accumulation of this compound may lead to large differences in apparent toxicity. The observation that HIT cells were somewhat more susceptible to the toxicity of the secretagogues than normal β-cells within islets concurs with a recent report. Here marked effects of imidazolines, especially idazoxan, on the viability of insulin-secreting cell lines were noted, while viability assays revealed at best only modest effects in pancreatic islets [2].

Internucleosomal cleavage of DNA is a late event in apoptosis preceded by activation of caspases [14,15]. Cleavage of proteins with the recognition sequence DEVD is regarded to be specific for caspase-3 and -7, effector caspases activated downstream of mitochondrial events in the intrinsic or mitochondrial pathway of apoptosis [16,17]. Recently, we had shown that phosphatase inhibitors are potent inducers of apoptosis in HIT cells [18,19]. Thus, we included the phosphatase inhibitor cantharidic acid as a positive control in the studies of DNA cleavage and caspase activity.

Only the imidazolines idazoxan and phentolamine, but none of the other insulin secretagogues induced internucleosomal DNA cleavage and an increase in caspase activity. The occurrence of large DNA fragments at 1000 μM of idazoxan and phentolamine suggests that the internucleosomal DNA cleavage, which predominates in apoptosis, was superseded by an irregular DNA cleavage. Such an appearance is observed in necrosis or in late apoptosis where cells also have lost membrane integrity as in primary necrosis [20]. In accordance with this observation idazoxan and phentolamine increased caspase activity at 100 μM, while at 1000 μM caspase activity was nearly reduced to zero. At this concentration MTT conversion and thus viability was also virtually abolished by these compounds. A possible explanation is that caspases 3 and 7, which are located in the mitochondria and the cytosol [21], were released from the cells by defects in the plasma membranes at the high concentrations after the incubation period of 24 h. Such a behavior [22] could be the consequence of primary necrosis or of late apoptosis with secondary necrosis which appears likely since caspase activity was increased in cells treated with 1000 μM of idazoxan and phentolamine for only 3 h. Taken together, the data suggest that idazoxan and phentolamine at 100 μM induced apoptosis whereas at concentrations exceeding 500 μM the damage was so extensive and progressed so fast that under the usual experimental conditions markers of necrotic cell death predominated.

An implicit contradiction appeared to be between the observation that the K_{ATP} channel blocking sulfonylurea tolbutamide-induced apoptosis in β-cells because of a depolarization-induced Ca^{2+} influx [3] and the observation that some imidazolines which also block K_{ATP} channels and induce Ca^{2+} influx [7,23,24] were apparently completely non-toxic for clonal β-cells [2]. A prolonged increase in the cytosolic Ca^{2+} concentration is presumed to play a central role in cell death [25,26]. Thus, a long-lasting depolarization by insulin secretagogues and, consequently, increase in cytosolic Ca^{2+} concentration, could well have detrimental consequences. However, our observations that D600 was ineffective in reducing the secretagogue-associated toxicity and also the toxicity by K^+ depolarization suggested that in both cases β-cell damage was not mediated by a Ca^{2+} influx through L-type channels. In addition, the lack of toxicity of efaroxan, which concurs with data by Morgan and co-workers [2] argues against a role for depolarization-induced Ca^{2+} influx as a common denominator of secretagogue-associated toxicity. Interestingly, efaroxan was recently described to exert an anti-apoptotic effect on β-cells [27], which might offset the consequences of an increased cytosolic Ca^{2+} concentration. Other authors, however, could not confirm such an anti-apoptotic property of efaroxan [28].

With respect to tolbutamide, our data suggest that the weak β-cell toxic effect of the sulfonylurea was neither dependent on Ca^{2+} influx nor displayed distinct signs of apoptosis. A reason for the discrepancy between our data and those of Efanova et al. [3] is not immediately apparent, since the tolbutamide concentration, the concentration of glucose in the medium (5 mM) and the exposure time were comparable. Perhaps, NMRI mouse islets used in the present study are less susceptible than the ob/ob mouse islets used by Efanova et al. The moderate β-cell toxicity observed in our study is not likely to be clinically relevant, but taking into account that the treatment with tolbutamide is for years, a long-term toxic effect of tolbutamide cannot be completely ruled out.

In conclusion, the initial questions of this study can be answered as follows: (i) With the possible exception of idazoxan, the percentage of damaged β-cells was too small to account for the strongly diminished secretion during

desensitization by these secretagogues [1]. (ii) Toxicity of imidazolines was very variable. Two compounds, idazoxan and phentolamine were markedly β-cell-toxic, one compound, alinidine, had moderate effects closely similar to those of the clinically used sulfonylurea tolbutamide, while a fourth imidazoline, efaroxan, appeared to be non-toxic. (iii) The β-cell toxicity of the secretagogues was apparently not the consequence of their blocking effect on K_{ATP} channels and the ensuing depolarization and Ca^{2+} influx via L-type Ca^{2+} channels. A depolarization-induced Ca^{2+} influx by itself was only moderately toxic. Thus, the β-cell toxicity of depolarizing insulin secretagogues does not seem to be a group effect but has to be evaluated for each individual agent. Pancreatic β-cells are known to be more susceptible to various mechanisms of cell damage than pancreatic A- and D-cells, which may be related to the low expression of antioxidative enzymes in β-cells and in β-cell-derived insulin-secreting cell lines [29,30].

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