Contrasting effects of alloxan on islets and single mouse pancreatic β-cells

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Alloxan is used to induce diabetes in animals; however, the underlying mechanisms are still a matter of debate. Alloxan evoked a rapid hyperpolarization of the plasma membrane potential and suppressed electrical activity elicited by 15 mM glucose, thus terminating voltage-dependent Ca^{2+} influx. Accordingly, glucose-induced oscillations in intracellular free Ca^{2+} concentration were abolished. The effect of alloxan on membrane potential could not be reversed by glucose but was reversed by tolbutamide. However, the sensitivity to tolbutamide was decreased after treatment of the cells with alloxan. These effects closely resemble those described earlier for H_2O_2. H_2O_2 and alloxan decreased the mitochondrial membrane potential, indicating a decrease in ATP production and thus interference with cell metabolism. A decrease in ATP synthesis would explain the plasma membrane hyperpolarization observed in intact islets, reflecting the activation of ATP-dependent K^+ channels. Surprisingly, alloxan inhibited the whole-cell K^+_{ATP} current measured in single cells and the single-channel K^+_{ATP} current registered in excised patches. This inhibitory effect of alloxan is not mediated by changes in cell metabolism but seems to be due to direct interactions with the K^+_{ATP} channels via thiol-group oxidation. We have monitored the appearance of reactive oxygen species in single cells and islets treated with alloxan and H_2O_2 for comparison. In contrast to H_2O_2, alloxan induced the appearance of measurable reactive oxygen species only in islets but not in single cells. The results show that alloxan evokes different effects in islets and single cells, giving a possible explanation for inconsistent results reported in the past. It is concluded that alloxan exerts its diabetogenic effect by the production of H_2O_2 in intact islets.

Key words: electrical activity, hydrogen peroxide, intracellular free Ca^{2+} concentration, mitochondrial membrane potential, reactive oxygen species.

INTRODUCTION

Chemical compounds such as alloxan or streptozotocin induce diabetes in animals, which serve as model systems for the study of multiple aspects of the disease. Depending on the administered dose, syndromes similar to non-insulin-dependent diabetes mellitus or insulin-dependent diabetes mellitus can be induced [1].

Although many examples have been described showing that alloxan induces diabetes in animals [2] and impairs glucose-induced insulin secretion from isolated islets [3] and insulin-secreting tumour cells [4], the mechanism of alloxan action is still a matter of debate. It has been reported that alloxan rapidly and selectively accumulates in β-cells in comparison with non-β-cells [5]. The targets affected by alloxan that lead to the inhibition of hormone release are not necessarily the same as those inducing apoptosis and/or necrosis of β-cells. Obviously, several steps in stimulus–secretion coupling are influenced by alloxan, and the target that is preferentially affected by alloxan might depend on the concentration used. Several reports directly or indirectly indicate that alloxan affects the membrane potential and ion channels in β-cells [3,6,7]. It is well known that glucokinase is inhibited by alloxan, the enzyme that is believed to function as the glucose sensor in β-cells [8,9]. Alloxan induces DNA damage that induces the activation of poly(ADP-ribose) synthetase and concomitantly the depletion of the cells of NAD^+ [10]. It is generally believed that the generation of reactive oxygen species (ROS) is involved in the action of alloxan [11]. This would fit with the suggestion that the underlying molecular mechanism of glucokinase inhibition is the oxidation of two adjacent thiol groups in the sugar-binding site of the enzyme [12]. Some authors have postulated that the alloxan effect is due mainly to the formation of H_2O_2 [13,14].

In previous papers we have shown that ROS such as H_2O_2 [15,16] and NO [17,18], which are assumed to contribute to the development of insulin-dependent diabetes mellitus, influence ion-channel activity in β-cells via effects on cell metabolism and via direct interactions with the channels. The present study was intended to elucidate the targets of alloxan at the membrane level and to determine whether its mode of action shows similarities to the action of ROS.

EXPERIMENTAL

Cell and islet preparation

The experiments were performed on islets or single cells of fed female NMRI mice (25–30 g), which had been killed by cervical dislocation. For measurements of cell membrane potential, a piece of pancreas was fixed in a perifusion chamber and islets were microdissected by hand. The other experiments were performed on islets isolated by digestion of the pancreas with collagenase. For patch-clamp experiments and measurements of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and mitochondrial membrane potential ($\Delta\Psi$), islet cells were dispersed in Ca^{2+}-free medium and cultured for up to 4 days in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin [19].

Abbreviations used: a.u., arbitrary units; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; DCF, dichlorofluorescein; DCFH, reduced DCF; DTE, dithioerythritol; ROS, reactive oxygen species.

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Solutions and chemicals

The extracellular fluid for measurements of cell membrane potential was composed of (in mM): 120 NaCl, 5 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 24 $NaHCO_3$ and 15 glucose, gassed with O_2/CO_2 (19:1) to maintain a pH of 7.4 at 37 °C.

Whole-cell K^+_{ATP} current recordings were performed at 32 °C with nystatin (150–250 μM) in the pipette solution. The pipette solution also contained (in mM): 10 KCl, 10 NaCl, 70 K_2SO_4, 4 $MgCl_2$, 2 $CaCl_2$, 10 EGTA and 20 Hepes, adjusted to pH 7.15 with KOH. The bath solution (solution 1) was composed of (in mM): 140 NaCl, 5 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 0.5 glucose and 10 Hepes, adjusted to pH 7.4 with NaOH. This bath solution was also used for the determination of $[Ca^{2+}]_i$, $\Delta\Psi$ and the appearance of ROS at 37 °C; glucose concentration as indicated.

To record currents through single K^+_{ATP} channels at 32 °C, seals were obtained with a pipette solution composed of (in mM): 130 KCl, 1.2 $MgCl_2$, 2 $CaCl_2$, 10 EGTA and 20 Hepes, adjusted to pH 7.4 with KOH. The bath contained solution 1. Before patch excision, the bath solution was replaced by a Mg^{2+}-free solution (solution 2) with the following composition (in mM): 130 KCl, 4.6 $CaCl_2$, 10 EDTA and 20 Hepes, adjusted to pH 7.20 with KOH. The lack of Mg^{2+} and ATP in this solution prevented phosphorylation and probably decreased K^+_{ATP} channel rundown [20,21].

Whole-cell Ca^{2+} currents were recorded with a pipette solution containing (in mM): 50 $CsCl_2$, 70 *N*-methyl-D-glucamine, 58 HCl, 4 $MgCl_2$, 3 Na_2ATP, 10 EGTA, 2 $CaCl_2$, 10 Hepes, pH 7.15. The bath solution was composed of (in mM): 115 NaCl, 1.2 $MgCl_2$, 10 $BaCl_2$, 20 tetraethylammonium chloride, 10 Hepes, 0.1 tolbutamide, 15 glucose, pH 7.4. Ba^{2+} was used as charge carrier.

Alloxan was freshly prepared immediately before each use, unless indicated otherwise. Fura 2 acetoxymethyl ester, rhodamine 123 and dichlorofluorescein (DCF) were obtained from Molecular Probes (Eugene, OR, U.S.A.). All other chemicals were purchased from Sigma (Deisenhofen, Germany) and Merck (Darmstadt, Germany) in the purest form available.

Recording methods

Membrane potential measurements

The potential difference across the cell membrane was determined with high-resistance microelectrodes [22]. The β-cells were identified by their characteristic oscillations of cell membrane potential in the presence of 15 mM glucose at 37 °C.

Patch-clamp recordings

Patch pipettes were pulled from borosilicate glass capillaries (Clark Electromedical Instruments, Pangbourne, Reading, Berks., U.K.). They had resistances between 3 and 5 MΩ when filled with pipette solution. Membrane currents were recorded and evaluated with an EPC-9 patch-clamp amplifier and PULSE software (HEKA, Lambrecht, Germany). For off-line analysis, data were also stored on video tape, played back by means of a MacLab4S interface with CHART software (WissTech, Spechbach, Germany) and evaluated with IGOR (WaveMetrics, Lake Oswego, OR, U.S.A.). Patch-clamp recordings were performed at 32 °C. Whole-cell K^+_{ATP} currents were measured in the perforated-patch configuration at a holding potential of −70 mV and during 300 ms pulses to −80 mV and −60 mV at 15 s intervals. Under these experimental conditions the current is almost entirely K^+_{ATP} current, blockable by tolbutamide [23]. Perforation was monitored by the decrease in series resistance, R_s. Perforation was usually adequate for voltage-clamping ($R_s < 30$ MΩ) within 10 min of seal formation.

Single K^+_{ATP} channel currents were recorded in inside-out patches at a membrane potential of −50 mV. Open probabilities (P_o) were calculated for a recording time of 30 s.

Ba^{2+} currents were measured every 15 s during 50 ms pulses to 0 mV from a holding potential of −70 mV.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured by the fura 2 method as described by Grynkiewicz et al. [24]. Equipment and software were provided by Till Photonics (Planegg, Germany). Large cell clusters (several hundred cells) were loaded with fura 2 acetoxymethyl ester (5 μM) for 30 min at 37 °C. Intracellular fura 2 was excited with light of wavelength 340, 360 or 380 nm adjusted by means of a diffraction grating. The excitation light was then directed through the objective (PlanNeofluar 40× objective; Zeiss, Stuttgart, Germany) by means of a glass fibre light guide and a dichroic mirror. The emitted light was filtered (long-pass 515 nm) and measured by a digital camera. The ratio of the emitted light intensity at 340/380 nm excitation wavelength was used to calculate $[Ca^{2+}]_i$ according to a calibration *in vitro* with fura 2 salt.

Measurement of $\Delta\Psi$

$\Delta\Psi$ was measured with the same equipment as for $[Ca^{2+}]_i$. Rhodamine 123 fluorescence was excited at 480 nm and the intensity of the emitted light (LP515 nm) was measured. Large clusters of cells were loaded with rhodamine 123 (10 μg/ml) for 10 min at 37 °C. An increase in rhodamine 123 fluorescence corresponds to a decrease in $\Delta\Psi$ [25].

Measurement of ROS

The appearance of ROS was monitored with the same equipment as for $[Ca^{2+}]_i$. DCF fluorescence was excited at 480 nm and the intensity of the emitted light (LP515 nm) was measured. The membrane-permeant non-fluorescent fluorescein derivative, reduced DCF (DCFH) diacetate, is cleaved by intracellular esterases to membrane-impermeant DCFH and oxidized by intracellular oxidants to DCF, which is fluorescent [26]. Single cells or cell clusters were loaded with DCFH diacetate (20 μM in the bath solution) for 30 min at 37 °C. Results are presented in arbitrary units (a.u.) expressing changes in DCF fluorescence in a single cell or a cell cluster. DCF fluorescence increases with time owing to the non-specific production of oxidants by normal cell metabolism. We therefore fitted a straight line to this non-specific increase in the fluorescence signal and subtracted this line from the whole curve to make clear the changes induced by H_2O_2 or alloxan.

Presentation of results

Electrophysiological experiments, $[Ca^{2+}]_i$, DCF fluorescence and $\Delta\Psi$ measurements are illustrated by recordings that are representative of the indicated number of experiments performed with different cells. Cells from at least three different cell preparations were used for each series of experiments. If possible, means ± S.E.M. are given in the text for the indicated number of experiments. The statistical significance of differences between means was assessed by a one-sample *t* test or Student's *t* test for paired values when two samples were compared. Multiple comparisons were made by analysis of variance followed by the Student–Newman–Keuls test. $P \leqslant 0.05$ was considered as significantly different.

RESULTS

Effects of alloxan on the membrane potential of mouse pancreatic β-cells

To reveal whether alloxan influences stimulus–secretion coupling of pancreatic β-cells at the membrane level, the cell membrane potential of β-cells in intact islets was recorded with intracellular microelectrodes in the presence of 15 mM glucose. Alloxan (5 mM) led to a complete suppression of electrical activity (Figure 1A). The alloxan solution was freshly prepared directly before application in each experiment. On average, the fraction of plateau phase (percentage of time with spike activity) under control conditions was $50 \pm 7\,\%$ in this series of experiments ($n = 5$). Alloxan (5 mM) hyperpolarized the β-cell membrane potential by -8 ± 2 mV from the threshold potential of -53 ± 5 mV to -61 ± 6 mV ($n = 5$, $P < 0.001$) and electrical activity was abolished. This effect could not be reversed by glucose. In the presence of 3 mM alloxan, spike activity was completely suppressed in one out of three experiments and tended to decrease in the other two (Figure 1B). Alloxan (1 mM) led to a slight increase in the fraction of plateau phase from $53 \pm 3\,\%$ under control conditions to $60 \pm 3\,\%$ ($n = 4$, $P < 0.02$) (Figure 1C).

Because the effect of 5 mM alloxan could not be reversed by glucose (Figure 1A), it seemed likely that alloxan interfered either with cell metabolism or directly with ion channels. To test whether the channels were still in function after treatment of the cells with alloxan we used the sulphonylurea tolbutamide, which directly inhibits K^+_{ATP} channels. As shown in Figure 2 (upper panel), with intact islets the alloxan-induced hyperpolarization could be reversed by tolbutamide. This indicates an effect of alloxan on cell metabolism rather than a direct effect on ion channels. However, the effectiveness of tolbutamide was decreased. On average, the membrane potential after treatment with alloxan was -63 ± 5 mV ($n = 7$). At 0.1 mM, the sulphonylurea depolarized the membrane potential by 20 ± 2 mV ($P < 0.001$). In contrast with cells not treated with alloxan (results not shown), clear spike activity occurred only in two out of the seven experiments. A higher concentration of tolbutamide (1 mM) further depolarized the membrane potential by 8 ± 2 mV ($P < 0.01$) and induced continuous spike activity in six out of the seven cells tested. Membrane hyperpolarization and decreased sensitivity to tolbutamide provoked by alloxan closely resembles the action of H_2O_2 in pancreatic β-cells, as described previously (Figure 2, lower panel) [16].

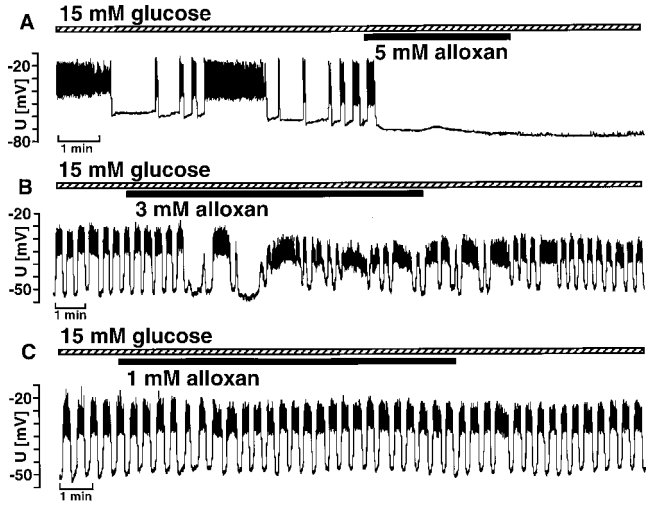

Figure 1 Alloxan inhibits glucose-induced electrical activity in intact islets

Concentration dependence of the effect of alloxan on the membrane potential of mouse pancreatic β-cells measured in intact islets in the presence of 15 mM glucose. (**A**) Alloxan (5 mM) hyperpolarized the membrane and abolished electrical activity irreversibly; (**B**) 3 mM alloxan partly inhibited electrical activity; (**C**) 1 mM alloxan was almost ineffective. The recordings are representative of eight, three and four experiments with similar results for the protocols used in (**A**), (**B**) and (**C**) respectively.

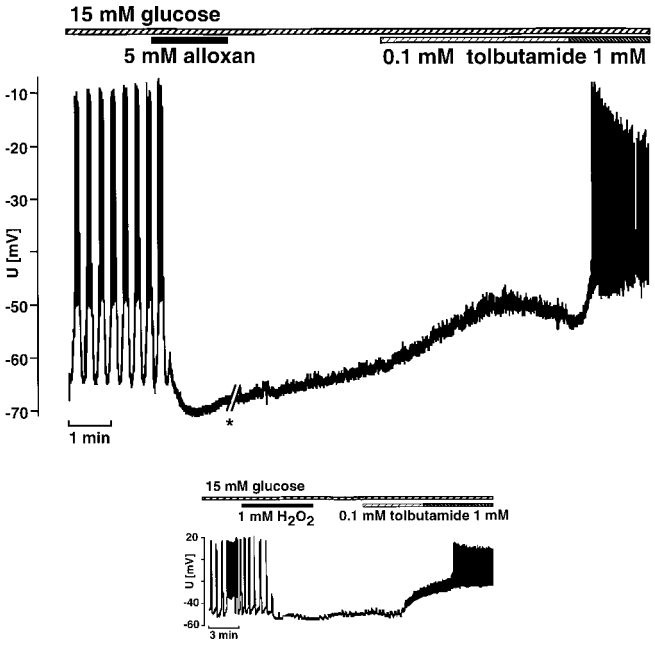

Figure 2 Effect of alloxan on membrane potential could be counteracted by tolbutamide

Upper panel: glucose-induced (15 mM) electrical activity measured in intact islets was abolished by hyperpolarizing the membrane potential with 5 mM alloxan. The effect was counteracted by inhibiting the K^+_{ATP} current with tolbutamide. The recording is representative of seven experiments with similar results. The asterisk indicates a gap of 2 min. Lower panel (reproduced from [16] with permission. © 1999 The Physiological Society.): H_2O_2 had the same effects as alloxan under these conditions. Note that the tolbutamide sensitivity of the current was decreased in both cases as compared to untreated cells.

It has been known for many years that a high glucose concentration can protect β-cells against the action of alloxan [27–29]. Therefore the lack of an inhibitory effect of 1 mM alloxan on the membrane potential (Figure 1C) could be due to the presence of 15 mM glucose in these experiments. This possible interaction between glucose and alloxan was tested in intact islets by adding 1 mM alloxan in the presence of 3 mM glucose. The subsequent addition of 15 mM glucose would not be expected to depolarize the cells if 1 mM alloxan had an inhibitory effect at a low glucose concentration. However, a clear depolarization and spike activity was observed under these conditions (Figure 3). On average, the fraction of plateau phase was $53 \pm 6\,\%$ in the presence of 15 mM glucose ($n = 4$). Perfusing the islets with a solution containing 3 mM glucose stopped electrical activity and the cell membrane potential hyperpolarized by -8 ± 1 mV ($P < 0.02$) from the threshold potential. The subsequent addition of 1 mM alloxan for 2–3 min in the presence of 3 mM glucose did not induce a significant change in membrane potential (1 ± 1 mV). However, the cell membrane depolarized in response to a high concentration of glucose (15 mM), and the fraction of plateau

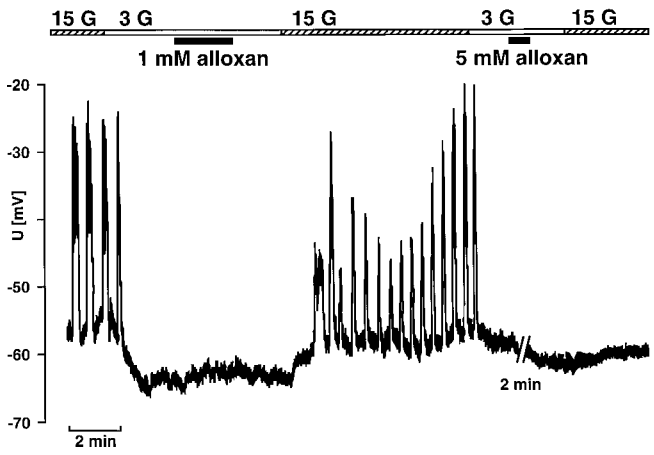

Figure 3 Effects of alloxan on membrane potential at low glucose concentration

Electrical activity induced by 15 mM glucose (15 G) in intact islets was suppressed when the extracellular glucose concentration was decreased to 3 mM (3 G). Under these conditions, 1 and 5 mM alloxan had no effect, but 15 mM glucose could restore electrical activity only after treatment with the lower concentration of alloxan. It therefore seems that the inefficiency of 1 mM alloxan shown in Figure 1 was not due to the high glucose concentration. The experiment shown is representative of four with similar results for the individual manoeuvres.

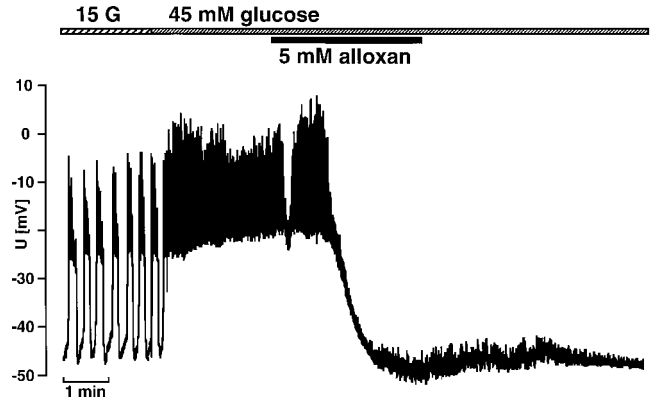

Figure 4 High glucose concentration does not prevent the alloxan effect on membrane potential

Switching the extracellular glucose concentration from 15 mM (15 G) to 45 mM led to a depolarization of the cells and continuous spike activity in intact islets. The high glucose concentration did not prevent the hyperpolarization induced by 5 mM alloxan. The experiment is representative of four with similar results.

phase was $47 \pm 8\,\%$ ($n = 4$, not significant compared with control). With the use of the same protocol but 5 mM alloxan instead, the depolarization induced by 15 mM glucose was prevented (Figure 3; $n = 4$).

It has been reported in several studies that the effects of glucose and alloxan are competitive [8,27]. To study in more detail a possible protection against the alloxan effect by glucose in intact islets we examined whether 5 mM alloxan was still effective in the presence of a very high concentration of glucose (45 mM). As shown in Figure 4, alloxan still hyperpolarized the β-cell membrane potential. On average, the fraction of plateau phase in this series of experiments was $53 \pm 3\,\%$ in the presence of 15 mM glucose. The addition of 45 mM glucose depolarized the membrane potential to the plateau potential with continuous spike

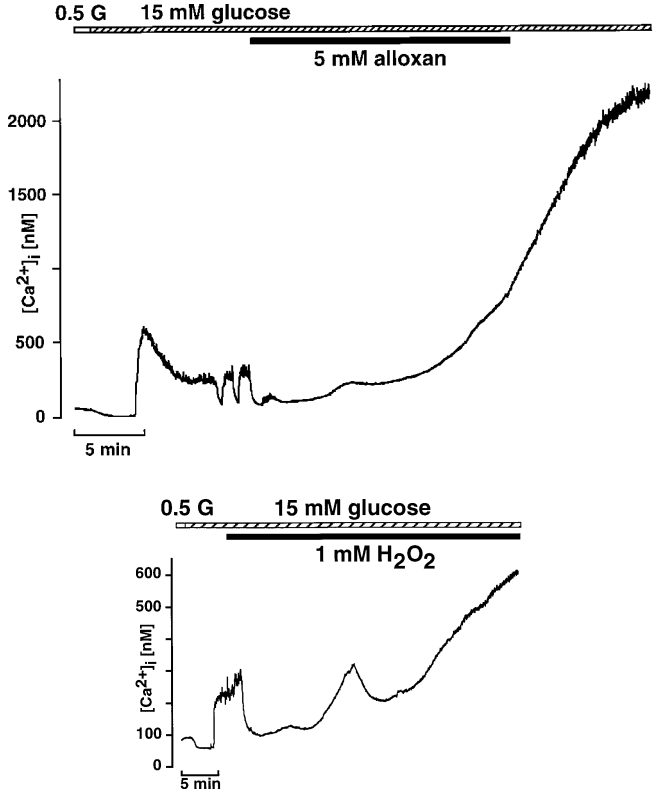

Figure 5 Effect of alloxan on $[Ca^{2+}]_i$

Upper panel: switching the extracellular glucose concentration from 0.5 mM (0.5 G) to 15 mM led to the well-known triphasic changes in $[Ca^{2+}]_i$. The subsequent addition of 5 mM alloxan led to a marked decrease in $[Ca^{2+}]_i$ followed by a biphasic increase in $[Ca^{2+}]_i$: a first transient phase was followed by a large sustained increase. The experiments were performed with large cell clusters. Lower panel: the very similar effect of H_2O_2 on $[Ca^{2+}]_i$. One experiment representative of eight with similar results is shown.

activity ($n = 4$, $P < 0.001$). Alloxan (5 mM) completely abolished electrical activity and hyperpolarized the cell membrane potential by -26 ± 4 mV from the plateau potential to -62 ± 5 mV (Figure 4; $n = 4$, $P < 0.01$). These two series of experiments presented in Figures 3 and 4 clearly show that glucose did not protect β-cells against the hyperpolarization induced by alloxan.

Effects of alloxan on $[Ca^{2+}]_i$

The hyperpolarization of the cell membrane potential by 5 mM alloxan observed in intact islets suggests that $[Ca^{2+}]_i$ is decreased by alloxan. Figure 5 (upper panel) shows one representative registration of $[Ca^{2+}]_i$ out of eight. These experiments were performed with very large cell clusters (several hundred cells) or islets. Increasing the glucose concentration from 0.5 to 15 mM first led to a small decrease in $[Ca^{2+}]_i$, due to Ca^{2+} sequestration into stores [30] followed by a rapid increase and Ca^{2+} oscillations. After the addition of 5 mM alloxan, $[Ca^{2+}]_i$ decreased rapidly and oscillations stopped, as expected from the measurement of membrane potential. However, in a later phase, $[Ca^{2+}]_i$ increased markedly in a biphasic manner. Again, this behaviour of the signal strongly resembled that obtained with H_2O_2 (Figure 5, lower panel) [16]. The H_2O_2-induced transient increase in $[Ca^{2+}]_i$ is due mainly to Ca^{2+} release from mitochondria, whereas the sustained level is caused by Ca^{2+} influx [16].

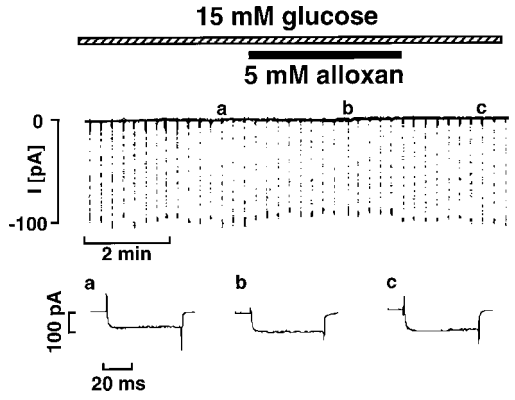

Figure 6 Effect of alloxan on currents through voltage-dependent Ca^{2+} channels

Alloxan (5 mM) induced a slight and reversible inhibition in the current through voltage-dependent Ca^{2+} channels with Ba^{2+} as the charge carrier. The experiments were performed with single cells. Currents were elicited every 15 s by 50 ms voltage steps from −70 to 0 mV. The lower traces show the current at points a, b and c on an extended time scale. One representative experiment out of 11 is shown.

Effects of alloxan on voltage-dependent Ca^{2+} channels

The alloxan-induced hyperpolarization of the cell membrane potential observed in intact islets might theoretically be due, at least in part, to the closure of Ca^{2+} channels. Thus we tested alloxan on the current through voltage-dependent Ca^{2+} channels with Ba^{2+} as the charge carrier. These experiments were performed on single cells. Alloxan (5 mM) induced a slight and reversible inhibition of the current (Figure 6); however, this cannot explain the marked hyperpolarization found in microelectrode recordings on whole islets. The lower traces in Figure 6 show the current at points a, b and c on an extended time scale. The current in 11 experiments was -101 ± 16 pA under control conditions and -81 ± 15 pA ($P < 0.001$) after the addition of 5 mM alloxan. After wash-out of alloxan the current amplitude increased to -90 ± 15 pA ($P < 0.01$).

Effect of alloxan on $\Delta\Psi$

Because the registrations of cell membrane potential suggested an effect of alloxan on cell metabolism, we tested the action of the compound on $\Delta\Psi$, which can be taken as a measure of ATP production [18,25,31]. Large clusters (several hundred cells) or islets were used for these experiments and changes in $\Delta\Psi$ were registered by the fluorescence of rhodamine 123. Figure 7 (upper panel) shows one representative experiment out of six. The increase in the glucose concentration from 0.5 to 15 mM first led to a strong decrease in rhodamine fluorescence, reflecting the hyperpolarization of $\Delta\Psi$ due to increased ATP production. With a short delay, the increase in glucose concentration triggered Ca^{2+} influx, which led in turn to a slight depolarization of $\Delta\Psi$ before a steady state was reached. This behaviour has been described in detail recently [31]. In the presence of 15 mM glucose, 5 mM alloxan led to a strong and sustained rise in rhodamine 123 fluorescence, i.e. depolarization of $\Delta\Psi$, indicating a blockade of ATP production. This could explain the hyperpolarization of the cell membrane potential evoked by alloxan, because the decrease in intracellular ATP concentration would lead to an increase in the hyperpolarizing K^+_{ATP} current. Again the results are very similar to those registered with H_2O_2 (Figure 7, lower panel).

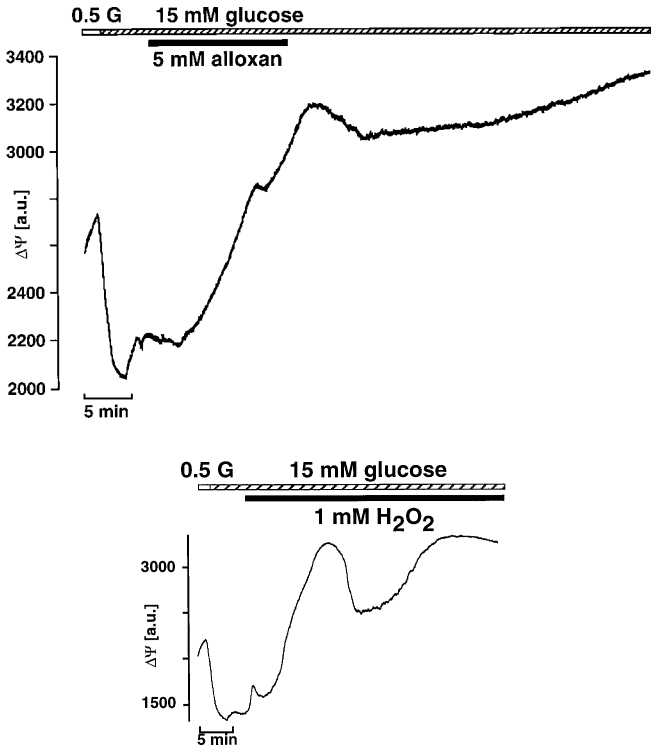

Figure 7 Alloxan and H_2O_2 depolarize $\Delta\Psi$ strongly

Upper panel: Increasing the extracellular glucose concentration from 0.5 mM (0.5 G) to 15 mM led to a decrease in rhodamine 123 fluorescence, indicating a hyperpolarization of $\Delta\Psi$ due to increased ATP production. A partial depolarization of $\Delta\Psi$ before the addition of alloxan was due to the glucose-induced increase in $[Ca^{2+}]_i$ [31]. Alloxan (5 mM) and H_2O_2 (1 mM) (lower panel) depolarized $\Delta\Psi$ both drastically and in a sustained manner. The experiments were performed with large cell clusters. One representative experiment out of six (alloxan) or four (H_2O_2) is shown.

Effects of alloxan on K^+_{ATP} currents

Most of the experiments described above indicate an effect of alloxan on metabolism, with striking similarities to the effect of H_2O_2 [16]. We therefore speculated that the cell membrane hyperpolarization could be caused by the opening of K^+_{ATP} channels due to the decreased cytosolic ATP content, as has been shown for H_2O_2 (Figure 8A, lower panel) [16]. We therefore tested the effect of alloxan on the whole-cell K^+_{ATP} current measured in the perforated-patch configuration with intact metabolism. These experiments were performed with single cells obtained from dispersed islets, as generally used in patch-clamp experiments. To our surprise we found an inhibition of the current amplitude (Figure 8A, upper panel) instead of the expected increase. In 16 experiments the mean current amplitude was 12.6 ± 1.4 pA under control conditions; it decreased to 3.2 ± 0.5 pA ($P < 0.001$) after treatment of the cells with 5 mM alloxan. After wash-out of alloxan the current remained inhibited (Figure 8B) but increased after addition of 300 μM of the K^+ channel opener diazoxide and the amplitude mounted up to 15.4 ± 3.0 pA ($n = 8$, $P < 0.001$). In all experiments with alloxan described so far, the alloxan solution was freshly prepared

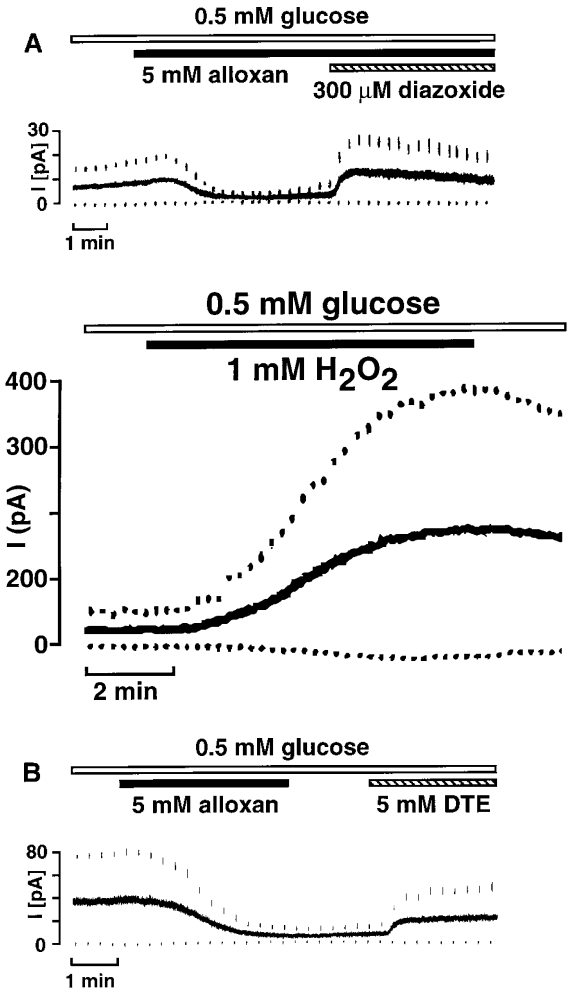

Figure 8 Alloxan decreases the K^+_{ATP} current in single β-cells

(**A**, **B**) K^+_{ATP} current was monitored on single cells in the perforated-patch mode in the presence of 0.5 mM glucose. The holding potential was −70 mV (solid line); every 15 s, 300 ms voltage steps to −80 mV and −60 mV (lower and upper broken traces respectively) were applied. Alloxan (5 mM) markedly decreased the current. Lower panel in (**A**): H_2O_2 strongly increased the K^+_{ATP} current, in contrast with alloxan (modified from [15]: Pflügers Arch. Eur. J. Physiol. H_2O_2-induced hyperpolarization of pancreatic β-cells. Krippeit-Drews, P., Lang, F., Häussinger, D. and Drews, G. **426**, 552–554, Figure 2, 1994. © 1994 Springer-Verlag). Upper panel in (**A**): the alloxan effect was counteracted by diazoxide (300 μM). One representative experiment out of 16 (eight with diazoxide) is shown. (**B**) The effect was reversed by the reducing agent DTE (5 mM). One representative experiment out of six is shown.

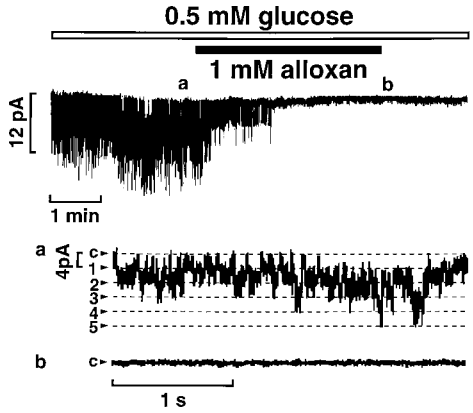

Figure 9 Effect of alloxan on single-channel K^+_{ATP} currents in excised membrane patches

Alloxan (1 mM) irreversibly abolished currents through K^+_{ATP} channels in inside-out patches of β-cell membranes. The experiments were performed with single cells. The holding potential was −50 mV. Under control conditions up to five channels opened simultaneously in this experiment, which was representative of eight with similar results. The lower panels show current traces at points a and b on an extended time scale.

directly before addition to the preparation. In the membrane potential experiments, alloxan that was not added immediately after preparation was ineffective. However, in experiments in which the whole-cell K^+_{ATP} current was registered, alloxan even inhibited the current from 12.3 ± 2.7 to 3.1 ± 0.9 pA ($P < 0.02$) when the alloxan solution was prepared several minutes before use ($n = 5$) (results not shown). One possible explanation for these observations is that different decomposition products of alloxan are active in intact islets and in single cells. In intact islets the effects of alloxan resemble those of H_2O_2, which interferes with cell metabolism (Figure 2, lower panel, and Figure 6, lower panel) [16], and in single cells the effects resemble those of thiol reagents, which directly inhibit the K^+_{ATP} current [32]. The inhibitory effects of thiol-group reagents can be reversed by reducing agents such as dithioerythritol (DTE) [33]. To corroborate the hypothesis that alloxan affects in single cells the thiol groups that are important for channel regulation, we tested whether the observed inhibition of the K^+_{ATP} current induced by alloxan could be reversed by DTE (Figure 8B). The experiments were performed on single cells with the same technique as in Figure 8(A). In this series of experiments, 5 mM alloxan decreased the current amplitude from 30.3 ± 4.0 to 4.5 ± 0.7 pA ($n = 6$, $P < 0.001$). Alloxan was removed before the addition of DTE because it turned out that the simultaneous application of both agents has marked effects on the pH of the bath solution. After wash-out of alloxan the current amplitude did not change (5.2 ± 1.1 pA). The addition of 5 mM DTE increased the K^+_{ATP} current amplitude again to 13.7 ± 2.8 pA ($n = 6$, $P < 0.05$).

To establish whether the surprising effect of alloxan on single cells was really due to a direct interaction with the K^+_{ATP} channels, we tested alloxan on excised patches in the inside-out configuration. Under these conditions 1 mM alloxan did indeed abolish single-channel currents (Figure 9). The effect was not reversible after wash-out. The lower panels show the recordings at points a and b on an extended time scale. On average, the open probability was decreased from 0.21 ± 0.02 to 0.03 ± 0.02 ($n = 8$, $P < 0.001$).

Action of alloxan on the membrane potential recorded from single cells

From the results on K^+_{ATP} currents registered in single cells, one would not expect that alloxan would induce a hyperpolarization of the β-cell membrane in single cells. We therefore performed experiments in which the cell membrane potential was measured on single cells in the current-clamp mode of the perforated-patch technique. In the presence of 15 mM glucose the membrane potential was depolarized and spike activity occurred (Figure 10). In contrast with intact islets (see Figures 1–4), 5 mM alloxan did not influence the electrical activity induced by glucose in single cells ($n = 9$). This series of experiments excludes the possibility that the discrepancy in the action of alloxan in single cells (Figure 10) and whole islets (Figures 1–4) was simply due to

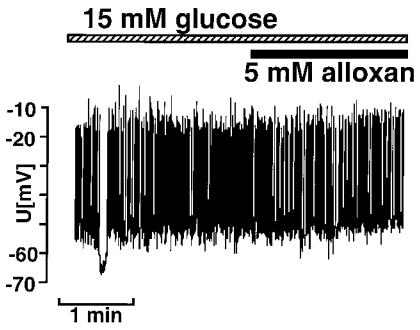

Figure 10 Effect of alloxan on the membrane potential in single cells

Membrane potential registration measured in the current-clamp mode with the perforated-patch configuration on single cells. In the presence of 15 mM glucose the membrane potential was depolarized and spike activity occurred. The addition of 5 mM alloxan did not change the pattern of electrical activity under these conditions. One representative experiment out of nine is shown.

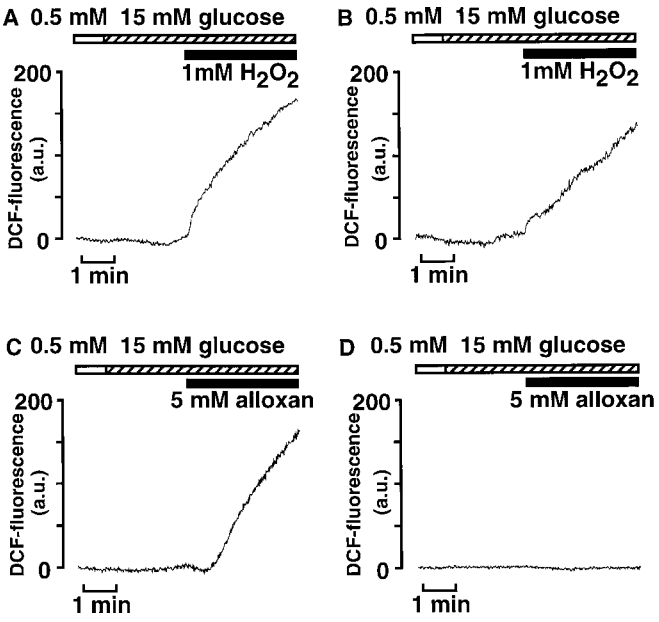

Figure 11 Effects of alloxan and H_2O_2 on DCF fluorescence as an indicator for intracellular ROS appearance

An increase in extracellular glucose concentration from 0.5 to 15 mM did not induce changes in DCF fluorescence. (**A**, **B**) The addition of 1 mM H_2O_2 evoked a rise in intracellular ROS concentration, regardless of whether it was given to cell clusters (**A**) or single cells (**B**). (**C**, **D**) Alloxan (5 mM) led to an increase in DCF fluorescence only when given to a cell cluster (**C**), but not in single cells (**D**). This indicates that a single cell is not capable of producing measurable amounts of H_2O_2 from alloxan. (**A**) and (**B**) are each representative of five similar experiments, (**C**) is one of eight similar experiments and (**D**) is one of 14.

different glucose concentrations and thus to possible differences in the metabolic state of the preparations.

Effects of alloxan on the formation of ROS

The fact that alloxan evoked opposite effects in single cells or large clusters and islets suggested that the discrepancy might arise from differences in alloxan metabolism in the two different preparations (see above). All our results obtained with large clusters of β-cells or islets supported the suggestion that alloxan affects β-cell function by the formation of ROS [11], especially H_2O_2 [13,14]. DCF is often used to detect intracellular ROS formation or appearance, including that of H_2O_2 [26]. We monitored DCF fluorescence with alloxan and with H_2O_2 for comparison in single β-cells and large cell clusters or whole islets. As shown in Figure 11, an increase in the glucose concentration from 0.5 to 15 mM did not influence DCF fluorescence. H_2O_2 increased the DCF fluorescence independently of whether cell clusters (or islets) (Figure 11A) or single cells (Figure 11B) were used in the experiments. This indicated the intracellular appearance of H_2O_2 in both preparations. On average, DCF fluorescence increased within 110 s by 100 ± 34 a.u. in the presence of 1 mM H_2O_2 in islets ($n = 5$) and by 84 ± 30 a.u. in single cells ($n = 5$). These changes in DCF fluorescence are not different statistically. With alloxan a comparable increase in DCF fluorescence was seen solely in islets (Figure 11C). The mean value of the increase in DCF fluorescence induced by 5 mM alloxan was 165 ± 41 a.u. ($n = 8$). By contrast, in single cells the same alloxan concentration was without effect on the DCF fluorescence ($n = 14$), indicating that single β-cells are not capable of producing measurable amounts of ROS in the presence of alloxan (Figure 11D).

DISCUSSION

Alloxan induces insulin-dependent or non-insulin-dependent diabetes mellitus in animals, depending on the concentration used and on the specific treatment of the animals [1]. The inhibition of insulin secretion by alloxan is well documented [3,4,34] but the underlying mechanisms are yet unclear. Moreover, it is still unknown which processes are involved in the destruction of β-cells by alloxan. It is assumed that the formation of ROS, including H_2O_2, is involved in the action of alloxan on β-cells [11,13,14]. The participation of ROS in the inhibition of β-cell function can explain why alloxan exerts specific effects on β-cells. First, in comparison with other cells of higher organisms, β-cells show a very low activity and expression of enzymes that normally protect cells against oxidative stress, including catalase, glutathione peroxidase, cytosolic Cu^{2+}/Zn^{2+} dismutase and mitochondrial Mn^{2+} dismutase [35,36]. Secondly, the overexpression of antioxidative enzymes in transgenic mice or RINm5F cells protects against an assault by alloxan, H_2O_2 or hypoxanthine/xanthine oxidase [37,38].

As mentioned above, some studies suggest that one of the compounds formed from alloxan is H_2O_2 [13,14]. The present study elicited some striking similarities between the action of alloxan and H_2O_2 [15,16]: (1) suppression of glucose-induced electrical activity and hyperpolarization of the cell membrane that could not be reversed by glucose but was reversed by tolbutamide; (2) a decrease in the sensitivity of the cells to tolbutamide; (3) blockade of glucose-induced oscillations in $[Ca^{2+}]_i$ and a consecutive biphasic increase in $[Ca^{2+}]_i$ (none of these changes in $[Ca^{2+}]_i$ seem to be due to a direct effect of the compounds on voltage-dependent L-type Ca^{2+} currents); and (4) depolarization of $\Delta\Psi$, which indicates a decrease in ATP production. These similarities do not prove, but strongly suggest, a common mode of action. Accordingly, a sequence of events is deduced from these results for the action of alloxan in pancreatic islets comparable with that described earlier for H_2O_2 [16]: alloxan decreases ATP production, leading to the opening of K^+_{ATP} channels and to cell membrane hyperpolarization. The latter event closes voltage-dependent Ca^{2+} channels, decreases $[Ca^{2+}]_i$ and suppresses Ca^{2+} oscillations and eventually leads to the inhibition of insulin secretion. It is uncertain whether this cascade of events finally ends in the destruction of β-cells.

However, it has been shown that ATP depletion in cells can be an initial step in necrotic and apoptotic cell death [39,40]. Moreover, it has been reported that the depolarization of $\Delta\Psi$ can itself lead to apoptosis via the opening of the permeability transition pore of mitochondria and subsequent swelling of the organelles. The swelling can disrupt the outer mitochondrial membrane, thereby releasing several factors such as cytochrome *c*, which in turn can activate caspase 3, which can lead to the induction of the apoptosis pathway [41]. Further similarities in the action of H_2O_2 and alloxan have been reported in other studies examining downstream signals in the apoptosis cascade. Both compounds lead to DNA fragmentation in isolated islets [13] and NAD^+ depletion [10,14].

The mechanism suggested above for the action of alloxan is confirmed by experiments performed under similar conditions, i.e. with intact mouse islets. Alloxan transiently increased ^{86}Rb efflux at 3 and 15 mM glucose and markedly delayed the decrease in ^{86}Rb efflux normally evoked by 15 mM glucose [3]. These results are consistent with an opening of K^+_{ATP} channels. Glucose-induced insulin secretion was completely abolished under these conditions. Suppression of electrical activity and hyperpolarization of the membrane potential by alloxan has been reported previously by Carroll et al. [6]. The authors proposed the activation of K^+_{ATP} channels as a possible mechanism for the action of alloxan.

Unexpectedly, we could not demonstrate any opening of K^+_{ATP} channels in the presence of alloxan in patch-clamp experiments performed on single β-cells. On the contrary, an inhibition of the K^+_{ATP} current was observed with alloxan, which is clearly in conflict with the sequence of events proposed above. Moreover, it is contradictory to the effects obtained with H_2O_2. The latter compound stimulated the K^+_{ATP} current in single cells but, as expected from the ATP depletion, only when the cell metabolism was intact [15,16]. We assume that this inconsistency occurs because in intact islets an active compound is generated from alloxan (probably H_2O_2) that is not, or not in an effective concentration, produced in single cells. This hypothesis is based on the following observations. (1) In contrast with H_2O_2, ROS accumulation after alloxan treatment can only be observed in islets or large clusters but not in single cells. (2) The inhibition of the K^+_{ATP} current in single cells was also observed with 'aged' alloxan solution, whereas effects on islets were observed only with freshly prepared alloxan solution. In this context it is noteworthy that 'aged' alloxan does not inhibit insulin secretion from islets [3]. (3) The inhibitory effect of alloxan on the K^+_{ATP} current was observed in the perforated-patch configuration and in inside-out patches and was reversed by DTE, indicating a direct interaction of alloxan with the K^+_{ATP} channel by thiol-group oxidation in single cells [32]. By contrast, the action of alloxan in intact islets resembles that of H_2O_2, which is different from the effect observed with classical thiol-group reagents [15,32]. Moreover, it has been shown by Takasu et al. [13] in rats that H_2O_2 is generated in intact islets treated with alloxan but not in the absence of islets or in broken cells.

We conclude that alloxan exerts different effects in tissue and in single cells. This might explain discrepancies in the literature about the actions of alloxan. Under physiological conditions there are striking similarities to the action of H_2O_2, which is very likely to be (one of) the active compound(s) generated by alloxan. The depolarization of $\Delta\Psi$, leading to ATP depletion in the cell with consequent membrane hyperpolarization and decrease in $[Ca^{2+}]_i$, is assumed to be one mechanism relevant to the impairment of β-cell function by alloxan. The disturbed mitochondrial integrity might trigger a signal cascade leading to (apoptotic) cell death.

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