Interference of H_2O_2 with stimulus–secretion coupling in mouse pancreatic β-cells

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1. We have reported previously that in mouse pancreatic β-cells H_2O_2 hyperpolarizes the membrane and increases the ATP-sensitive K^+ current recorded in the perforated patch configuration of the patch-clamp technique. The present study was undertaken to elucidate the underlying mechanisms.

2. The intracellular ATP concentration measured by chemoluminescence was reduced by H_2O_2. The ADP concentration increased in parallel during the first 10 min, resulting in a pronounced decrease in the ATP/ADP ratio.

3. Consistent with these results, glucose-stimulated insulin secretion from isolated islets was inhibited by H_2O_2.

4. Membrane hyperpolarization measured with intracellular microelectrodes in intact islets and inhibition of insulin secretion were counteracted by tolbutamide, indicating that the channels are still responsive to inhibitors and that the ATP concentration is not too low to trigger exocytosis. However, the sensitivity of the β-cells to tolbutamide was reduced after treatment with H_2O_2.

5. H_2O_2 increased the intracellular Ca^{2+} activity ($[Ca^{2+}]_i$) in a biphasic manner. A first transient rise in $[Ca^{2+}]_i$ due to mobilization of Ca^{2+} from intracellular stores was followed by a sustained increase, which was at least partly dependent on Ca^{2+} influx. The first phase seems to reflect Ca^{2+} mobilization from mitochondria.

6. Our results demonstrate that H_2O_2 interferes with glucose metabolism, which influences the membrane potential and ATP-sensitive K^+ current via the intracellular concentration of ATP. These events finally lead to an inhibition of insulin secretion despite an increase in $[Ca^{2+}]_i$.

Several studies have shown that the infiltration of macrophages into the islets of Langerhans during the early stages of insulitis is one of the first steps in the development of insulin-dependent diabetes mellitus (Kolb-Bachofen *et al.* 1988; Lee *et al.* 1988; Hanenberg *et al.* 1989). It is generally believed that macrophages produce large amounts of reactive oxygen species such as hydrogen peroxide (H_2O_2), resulting in an undesired cytotoxic attack against β-cells. Alloxan and streptocotocin, two agents widely used to induce diabetes in animals, also lead to the formation of H_2O_2, which may thus contribute to the manifestation of diabetes (Takasu *et al.* 1991). Pancreatic β-cells are extremely sensitive to oxidative stress because of the low expression and activity of the enzymes defending cells against an assault by oxidants (Malaisse *et al.* 1982; Lenzen *et al.* 1996). Moreover, the GSH/GSSG (reduced/oxidized glutathione) ratio in islets is low compared with other tissues (Ammon *et al.* 1983). It is well known that reactive oxygen species alter β-cell function and finally lead to the destruction of β-cells. They interfere with enzymes involved in glucose metabolism (Welsh *et al.* 1991; Dimmeler *et al.* 1993; Nakazaki *et al.* 1995), inhibit DNA synthesis, and lead to DNA fragmentation, and probably via the activation of the poly(ADP-ribose) polymerase to a decrease of the cellular NAD^+ content (Yamamoto *et al.* 1981; Takasu *et al.* 1991; Fehsel *et al.* 1993; Radons *et al.* 1994). We were interested in investigating whether H_2O_2 interferes with an early step in stimulus–secretion coupling, i.e. at the level of the plasma membrane. In a previous paper we showed that H_2O_2 hyperpolarizes the cell membrane and we assumed that an intracellular factor is involved in this effect (Krippeit-Drews *et al.* 1994*b*). In the present paper we have further elucidated the events underlying membrane hyperpolarization and we have studied how $[Ca^{2+}]_i$ and insulin secretion are influenced by H_2O_2.

METHODS

Cell and islet preparation

The experiments were performed on islets or single cells of fed female NMRI mice (25–30 g), killed by cervical dislocation. For cell membrane potential measurements a piece of pancreas was fixed in a perifusion chamber and islets were micro-dissected by hand. The other experiments were performed on islets isolated by collagenase digestion of the pancreas. For patch-clamp experiments and measurements of $[Ca^{2+}]_i$, islet cells were dispersed in Ca^{2+}-free medium and cultured for up to 4 days in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Plant, 1988). Insulin secretion was determined with freshly prepared islets.

Solutions and chemicals

The extracellular fluid for cell membrane potential measurements was composed of (mM): 120 NaCl, 5 KCl, 2·5 $CaCl_2$, 1·2 $MgCl_2$, 24 $NaHCO_3$, 15 glucose, gassed with 95% O_2 and 5% CO_2 to maintain a pH of 7·4 at 37 °C.

Whole-cell ATP-sensitive K^+ current recordings were performed at 32 °C with nystatin in the pipette solution (150–250 μM). The pipette solution also contained (mM): 10 KCl, 10 NaCl, 70 K_2SO_4, 4 $MgCl_2$, 2 $CaCl_2$, 10 EGTA, 20 Hepes, pH adjusted to 7·15 with KOH. The bath solution was composed of (mM): 140 NaCl, 5 KCl, 1·2 $MgCl_2$, 2·5 $CaCl_2$, 15 glucose, 10 Hepes, pH adjusted to 7·4 with NaOH. The same bath solution was used for the determination of $[Ca^{2+}]_i$ at 37 °C. In these experiments β-cells were identified by the glucose-evoked rise in $[Ca^{2+}]_i$ induced by changing the bath glucose concentration from 0·5 to 15 mM. Insulin secretion and the ATP/ADP ratio were determined at 37 °C in a bath solution with the following composition (mM): 122 NaCl, 4·7 KCl, 1·1 $MgCl_2$, 2·5 $CaCl_2$, 10 Hepes, 15 glucose, supplemented with 0·5% bovine serum albumin. The insulin secretion measurements were started in a bath solution containing 3 mM glucose, and in each experiment the biphasic increase in insulin release following the augmentation of the glucose concentration to 15 mM was registered as a control for the quality of the islets.

Fura-2 AM was obtained from Molecular Probes, FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone), fura-salt, flufenamic acid, ionomycin, gadolinium oxide, thapsigargin and tolbutamide were from Sigma, ATP and ADP were from Boehringer, the BioOrbit 1243-102 ATP-monitoring-kit was from Merlin Diagnostika GmbH (Bornheim-Fersel, Germany) and H_2O_2 was from Hedinger (Stuttgart, Germany). D600 was kindly provided by Knoll AG (Ludwigshafen, Germany). All other chemicals were purchased from Merck in the purest form available.

Recording methods

Membrane potential measurements. The potential difference across the cell membrane was determined using high resistance microelectrodes (Meissner & Schmelz, 1974). The β-cells were identified by the characteristic oscillations of cell membrane potential which they display in the presence of 15 mM glucose at 37 °C.

Patch-clamp recordings. Patch pipettes were pulled from borosilicate glass capillaries (Clark Electromedical, Pangbourne, UK). They had resistances between 3 and 5 MΩ when filled with pipette solution. Membrane currents were recorded 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' software (WaveMetrics, Lake Oswego, Oregon, USA). Patch-clamp recordings were performed at 32 °C. Whole-cell ATP-sensitive K^+ currents were measured in the perforated patch configuration at a holding potential of −70 mV and during 300 ms pulses to −80 and −60 mV at 15 s intervals. Perforation was monitored by the decrease in series resistance (G_s). Perforation was usually adequate for voltage-clamping ($G_s < 30$ MΩ) within 10 min of seal formation.

Measurement of insulin release. After isolation, the islets were placed in batches of 50 in two parallel perifusion chambers (control and test solution). The islets were pre-incubated for 15 min in a medium containing 3 mM glucose at 37 °C. The perifusion flow rate was 0·65 ml min^{-1}. Effluent fractions were sampled at 2 min intervals and insulin was measured by a double-antibody radioimmunoassay with rat insulin as the standard (Linco Research, St Louis, MO, USA).

Measurement of the ATP/ADP ratio. The ATP/ADP ratio was determined by measuring ATP and ADP in the same batch of eight islets. At the end of the incubation period extracellular fluid was sucked off and islets were disintegrated with NaOH–cysteine solution (40 mM–0·5 mM) and stored at −20 °C. For luminescence measurements aliquots of each sample were dissolved with a buffer containing (mM): 20 creatine phosphate, 100 glycine, 1 $MgSO_4$ at pH 9·0, with or without creatine kinase (20 pg ml^{-1}). Aliquots were either neutralized with HCl to pH 7·65 immediately or after an incubation with creatine kinase for 10 min at room temperature (20–25 °C) to convert all ADP to ATP. The ATP concentration was measured in a luciferin/luciferase assay using the ATP-monitoring kit with a luminescence biometer 1253 (Bio-orbit, Merlin, Bornheim-Mersel, Germany).

Measurement of $[Ca^{2+}]_i$. Intracellular Ca^{2+} activity ($[Ca^{2+}]_i$) was measured by the fura-2 method according to Grynkiewicz *et al.* (1985). Single cells or small clusters of cells were loaded with fura-2 AM (5 μM) for 30 min. Intracellular fura-2 was excited with light at 340, 360 and 380 nm wavelengths, generated by directing light from a monochromator light source through appropriate filters in a computer-controlled wheel. The excitation light was then directed through the objective lens (NPlanL × 40, Leica, Bensheim, Germany) by means of a glass fibre light guide and a dichroic mirror. The emitted light was filtered (512 nm) and measured by an ICCD camera. The ratio of the emitted light intensity at 340 nm/380 nm excitation wavelength (F_{340}/F_{380}) was used to calculate $[Ca^{2+}]_i$ according to an *in vitro* calibration with fura-2 salt. The microscope type DMIRB was from Leica. All other hardware and the software needed for fluorescence measurements were from IonOptix (Milton, MA, USA). Alternatively, $[Ca^{2+}]_i$ was measured with equipment and software from TILL photonics (Planegg, Germany) where the light wavelength is adjusted by means of a diffractive grating. The microscope was a Zeiss Axiovert 100 equipped with a PlanNeofluar × 40 objective lens.

Presentation of results

Electrophysiological experiments and Ca^{2+} measurements are illustrated by recordings that are representative of the indicated number of experiments performed with different cells. Cells of at least three different cell preparations have been used for each series of experiments. If possible, means ± S.E.M. are given in the text for the indicated number of experiments. For the other experiments data are presented as means ± S.E.M. in the figures. The statistical significance of differences between means was assessed by a Student's one-sample *t* test or a *t* test for paired values when two samples were compared. Multiple comparisons were made by ANOVA followed by Student–Newman–Keuls test. $P \leqslant 0{\cdot}05$ was considered as indicating a significant difference.

RESULTS

Effects of H_2O_2 on membrane potential and ATP-sensitive K^+ current

Figure 1*A* shows that the addition of 1 mM H_2O_2 hyperpolarized the cell membrane and suppressed the oscillations of the cell membrane potential observed in the presence of 15 mM glucose. The membrane potential measurements were performed with intracellular microelectrodes in intact islets. Previously, we have reported that the hyperpolarization was sustained after removal of H_2O_2 from the bath solution (Krippeit-Drews *et al.* 1994*b*), suggesting that glucose has lost the ability to depolarize the β-cells. Figure 1*A* demonstrates that the addition of 0·1 mM tolbutamide depolarizes the membrane again and small spikes were observed. However, 1 mM of the sulfonylurea further depolarized the membrane and induced spikes of large amplitude. On average, the fraction of plateau phase (percentage of time with spike activity) was 55 ± 6 % under control conditions in 15 mM glucose (calculated for the last 4 min under control conditions) ($n = 4$). Treatment of the cells with 1 mM H_2O_2 hyperpolarized the membrane by −25 ± 5 mV from the plateau potential ($n = 4$). The addition of 0·1 mM tolbutamide for 5–7 min after wash-out of H_2O_2 depolarized the membrane by 18 ± 3 mV, and in three out of four experiments small spikes appeared. Increasing the tolbutamide concentration to 1 mM depolarized the cells further by 10 ± 2 mV, and large spikes could be detected in all four experiments. The small oscillations of the cell membrane potential which can be seen in Fig. 1*A* after addition of H_2O_2 have been observed in 11 out of 19 experiments in which H_2O_2 was tested.

In three experiments (not shown) the hyperpolarization induced by H_2O_2 was compared in the same cell to the hyperpolarization provoked by 100 μM diazoxide, which maximally repolarizes the membrane to close to the K^+ reversal potential by opening the ATP-sensitive K^+ channels.

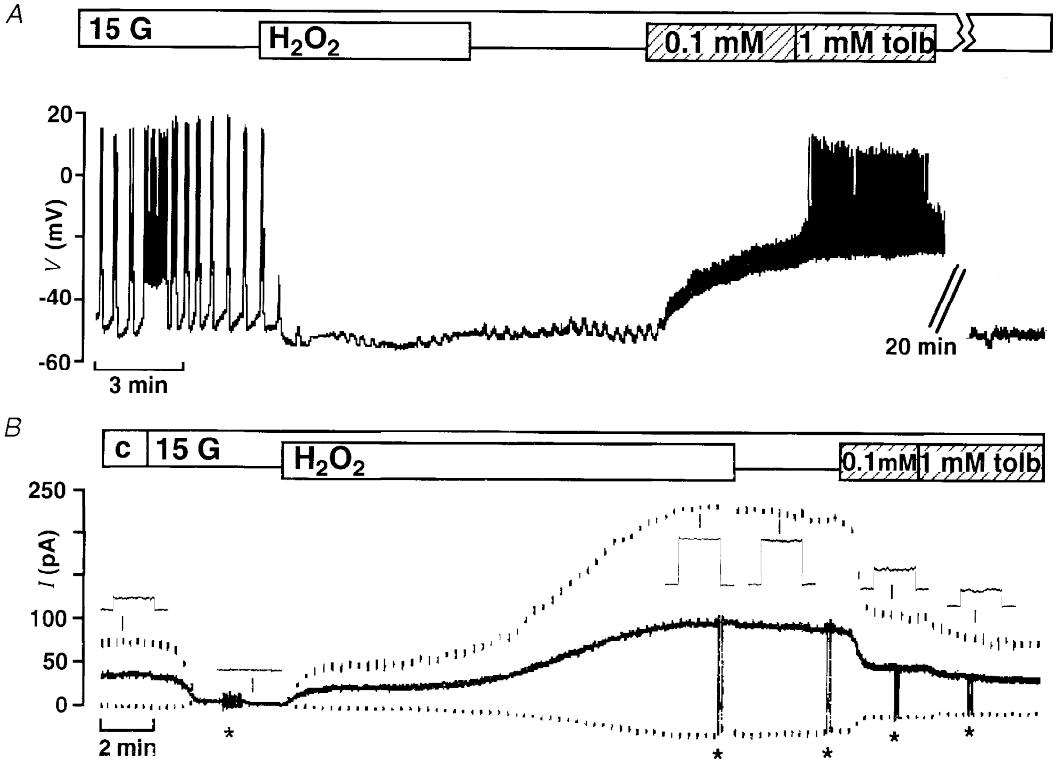

Figure 1. H_2O_2 hyperpolarizes the membrane potential by increasing the ATP-sensitive K^+ current

A, effects of 1 mM H_2O_2 and 0·1 and 1 mM tolbutamide (tolb) on the membrane potential of mouse pancreatic β-cells in the presence of 15 mM glucose (15 G). The H_2O_2-induced hyperpolarization was irreversible but could be counteracted by inhibiting the ATP-sensitive K^+ current with tolbutamide. The record is representative of four experiments with similar results. *B*, ATP-sensitive K^+ current monitored in the perforated-patch mode in the presence of 15 mM glucose (15 G). The holding potential was −70 mV (continuous trace) and every 15 s 300 ms voltage steps to −80 and −60 mV (lower and upper dashed traces, respectively) were applied. H_2O_2 dramatically increased the current. Again the irreversible effect of H_2O_2 was at least partly counteracted by tolbutamide. The insets show the corresponding currents on an extended time scale. At the points marked by asterisks the amplifier was switched from voltage-clamp to current-clamp mode and the membrane potential was registered. The record is representative of five experiments with similar results.

Addition of diazoxide led to a hyperpolarization of -32 ± 2 mV from the plateau potential while H_2O_2 evoked a hyperpolarization of -18 ± 3 mV.

To elucidate the mechanism underlying the hyperpolarization, the effect of H_2O_2 on the ATP-sensitive K^+ current was investigated in the perforated patch configuration with dissociated cells. Figure 1*B* shows a representative recording from this series of experiments, demonstrating that H_2O_2 markedly increased the current. For quantification, the current elicited by a 10 mV depolarizing voltage step from the holding potential of -70 mV was used. In four out of the five experiments in this series the membrane potential was measured simultaneously (switch from voltage-clamp to current-clamp mode is marked by asterisks in the experiment shown in Fig. 1*B*). On average, the current under control conditions (c) in 0·5 mM glucose was 15 ± 6 pA and the membrane potential was -80 ± 2 mV. The current was completely abolished in all experiments when the glucose concentration was raised to 15 mM, the membrane potential depolarized and spikes of up to -25 mV were observed. Subsequent addition of 1 mM H_2O_2 in the presence of 15 mM glucose significantly increased the current to 73 ± 16 pA and hyperpolarized the membrane potential to -80 ± 1 mV. Thus, in contrast to intact islets in single cells, H_2O_2 is able to hyperpolarize the membrane potential to the K^+ reversal potential. After removal of H_2O_2, the current and the membrane potential did not change significantly (69 ± 17 pA and -80 ± 1 mV, respectively). The addition of 0·1 mM tolbutamide did not abolish the current but reduced it to 25 ± 9 pA. The membrane potential was -79 ± 2 mV under these conditions. At 1 mM, the sulfonylurea induced a significant further decrease in the current, to 19 ± 7 pA, and slightly depolarized the membrane to -76 ± 1 mV, but no spikes occurred.

Effect of H_2O_2 on the intracellular ATP and ADP concentration

Since the increase in the ATP-sensitive K^+ current was only observed when the metabolism of the cells was intact (Krippeit-Drews *et al.* 1994), we tested whether 1 mM H_2O_2 influences the ATP/ADP ratio, which is the main intracellular regulator of channel activity (Ashcroft & Rorsman, 1989). Figure 2 shows the effect of 1 mM H_2O_2 on the ATP/ADP ratio (panel *A*) and the ATP (panel *B*) and ADP (panel *C*) concentration in the islets. H_2O_2 reduced the ATP concentration in the presence of 15 mM glucose from $10·2 \pm 0·4$ to $4·8 \pm 0·5$ pM islet^{-1} within 30 min, with the main decrease occurring within the first 10 min. Under control conditions, the ATP concentration did not change within 30 min ($11·0 \pm 1·3$ pM islet^{-1}). The ADP

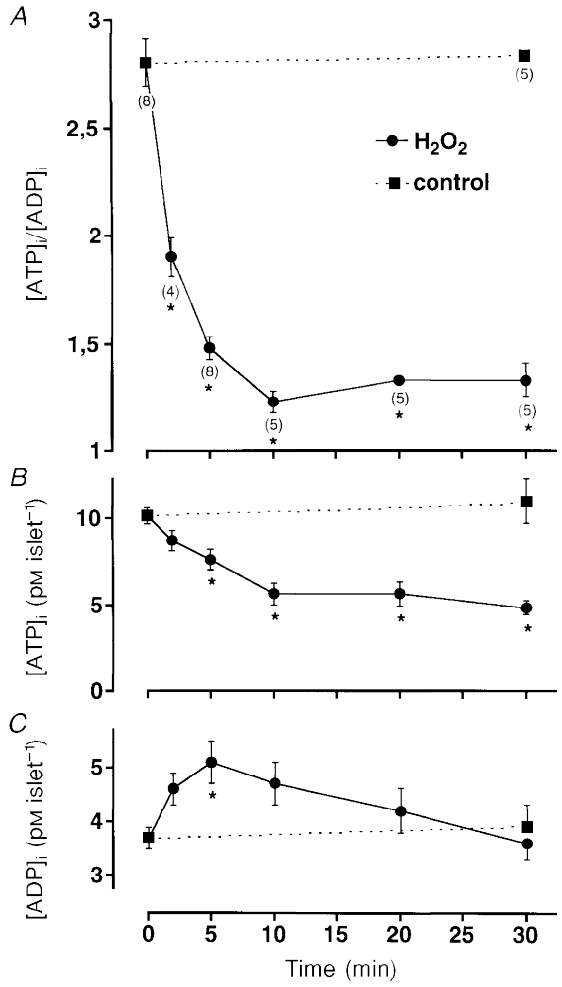

Figure 2. H_2O_2 diminishes intracellular ATP content

A, mean changes in the ATP/ADP ratio monitored with isolated islets after addition of 1 mM H_2O_2 in the presence of 15 mM glucose. Numbers of experiments (n) given in parentheses below the symbols in *A* also apply to corresponding symbols in *B* and *C*. *Values significantly different from controls. *B* and *C*, H_2O_2 induced changes in intracellular ATP (*B*) and ADP (*C*) content. Note that the H_2O_2 effect is biphasic: within the first 5 min the loss in ATP coincided with an increase in ADP whereas afterwards the concentration of both nucleotides decreased.

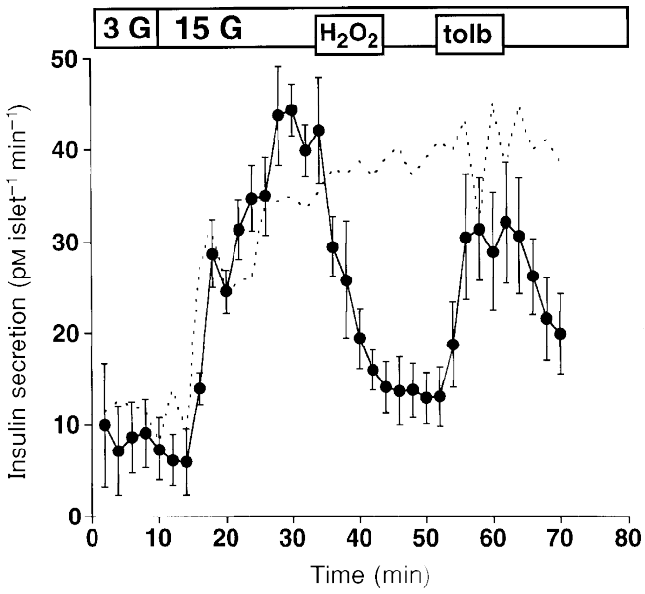

Figure 3. H_2O_2 decreases the insulin secretion from isolated mouse islets

Effects of 1 mM H_2O_2 and 1 mM tolbutamide (tolb) on insulin release from perifused mouse islets. Switching from 3 to 15 mM glucose (3 G, 15 G) elicited the response of the islets to glucose. H_2O_2 irreversibly inhibited glucose-stimulated insulin secretion, an effect which was blunted by tolbutamide. Values are means ± S.E.M. of four experiments. Control experiments without H_2O_2 and tolbutamide are represented by the dashed line.

concentration first increased after addition of H_2O_2 from 3·7 ± 0·2 to 5·1 ± 0·4 pM islet^{-1} within 5 min. Afterwards it decreased again, reaching a value of 3·6 ± 0·3 pM islet^{-1} after 30 min. The changes in the ATP and ADP concentration resulted in a decrease of the ATP/ADP ratio to about half of the control values. The maximum reduction of the ATP/ADP ratio was reached within 10 min.

Influence of H_2O_2 on insulin secretion

The first part of Fig. 3 demonstrates the typical biphasic rise in the rate of insulin secretion in response to an increase of the extracellular glucose concentration from 3 to 15 mM. The addition of 1 mM H_2O_2 to glucose-stimulated islets caused a marked inhibition of insulin release which was not reversible after removal of H_2O_2 ($n = 4$). However, tolbutamide (1 mM) was still able to augment insulin secretion ($n = 4$). After wash-out of tolbutamide, insulin release decreased again, emphasizing that glucose has lost its ability to stimulate hormone secretion from β-cells.

Effect of H_2O_2 on $[Ca^{2+}]_i$

Figure 4 shows one example for the biphasic effect of H_2O_2 on $[Ca^{2+}]_i$. Under control conditions (c) the bath solution contained 0·5 mM glucose. Increasing the glucose

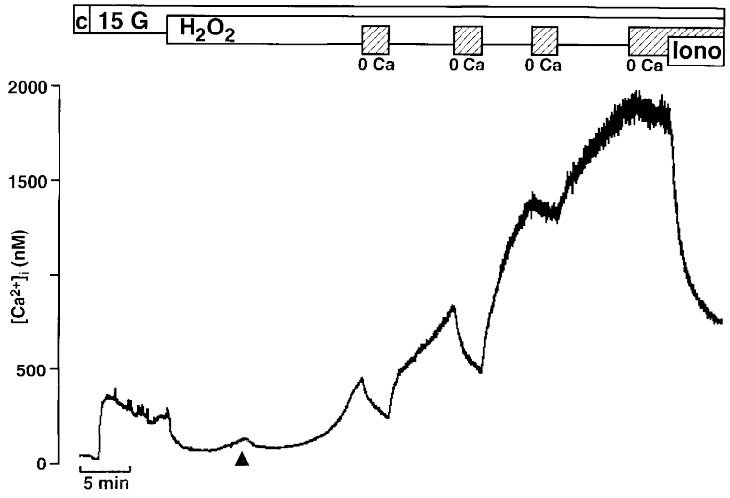

Figure 4. Effect of H_2O_2 on $[Ca^{2+}]_i$

Switching the extracellular glucose concentration from 0·5 mM (c = control) to 15 mM (15 G) led to an increase in $[Ca^{2+}]_i$. The subsequent addition of 1 mM H_2O_2 led to a marked drop in $[Ca^{2+}]_i$ followed by a biphasic increase in $[Ca^{2+}]_i$, a first transient phase (see arrowhead) was followed by a large sustained increase. At the time intervals marked by the hatched bars Ca^{2+} was removed from the extracellular bath solution and 1 mM EGTA was added. After a steady-state level of $[Ca^{2+}]_i$ had been reached, the Ca^{2+} ionophore ionomycin (1 μM) was applied in the absence of extracellular Ca^{2+}. The experiment shown is representative of five with similar results.

concentration to 15 mM resulted first in a small decrease in $[Ca^{2+}]_i$ as described previously (Gylfe, 1989). Thereafter, $[Ca^{2+}]_i$ markedly increased, and often this rise was followed by small oscillations of $[Ca^{2+}]_i$. On average, $[Ca^{2+}]_i$ was 141 ± 22 nM under control conditions and increased to a maximum of 443 ± 22 nM with 15 mM glucose ($n = 10$). The addition of H_2O_2 first led to a drop in $[Ca^{2+}]_i$ as a result of the closure of L-type Ca^{2+} channels induced by the hyperpolarization. Thereafter, a transient increase in $[Ca^{2+}]_i$ (marked by the arrowhead in Fig. 4) was observed, which was followed by a marked sustained increase. In the experiment shown in Fig. 4, which is representative of five similar experiments, the dependency of the second steep rise on extracellular Ca^{2+} has been studied. During the rising phase, $[Ca^{2+}]_i$ decreased after removal of extracellular Ca^{2+}, but not to basal levels. The effect of extracellular Ca^{2+} withdrawal diminished with increasing $[Ca^{2+}]_i$ and was almost abolished in the steady state. Ca^{2+} ionophores like ionomycin or A23187 (1 μM) still markedly decreased $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}. An unspecific leakage of fura-2 out of the cells or an increase in the F_{340}/F_{380} ratio due to an augmentation of reduced pyridine nucleotides (Pralong *et al.* 1990; Gilon & Henquin, 1992) can be excluded because H_2O_2 did not change the fura-2 signal when the excitation wavelength was 360 nm ($n = 5$). In addition, H_2O_2 did not induce any change in the fluorescence signals of fura-salt (5 μM) when tested *in vitro* in the presence of 300 nM free Ca^{2+} ($n = 3$, not shown).

Figure 5 shows that the second increase in $[Ca^{2+}]_i$ induced by H_2O_2 is not blockable by either 100 μM D600 (Fig. 5*A*, $n = 4$), an inhibitor of L-type Ca^{2+} channels, or by 100 μM Gd^{3+} (Fig. 5*B*, $n = 4$) or flufenamate (Fig. 5*C*, $n = 4$). Gd^{3+} and flufenamate are blockers of non-selective cation channels and of the Ca^{2+} release-activated Ca^{2+} current (I_{crac}).

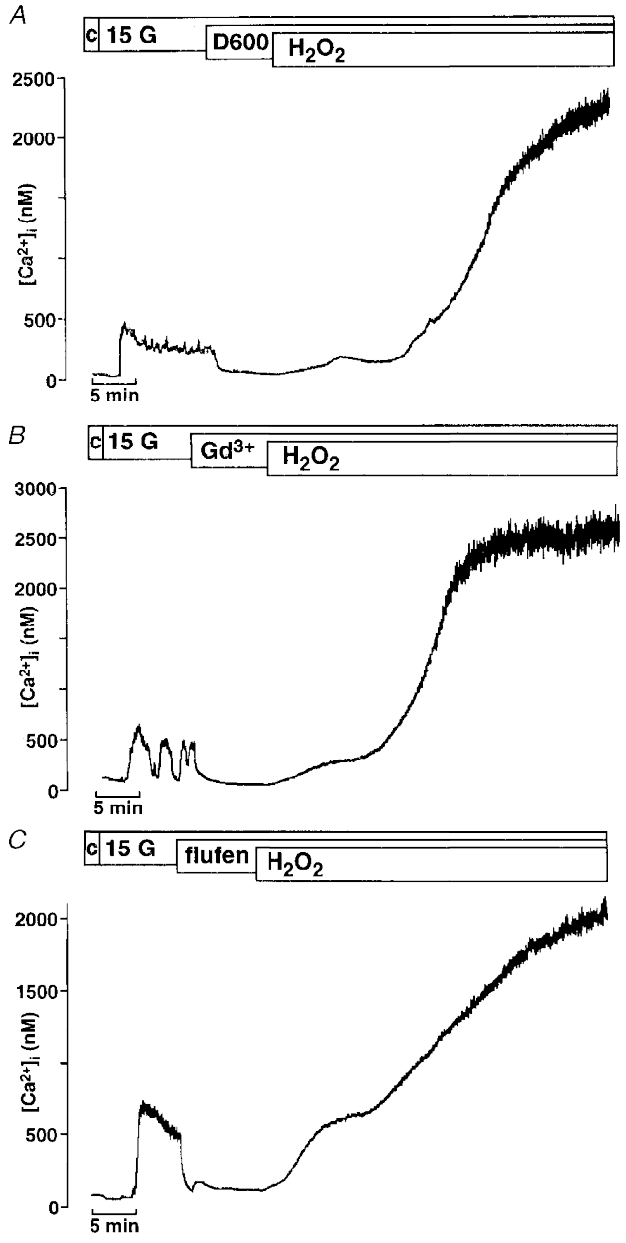

Figure 5. Influence of D600, Gd^{3+} and flufenamate on H_2O_2-induced changes in $[Ca^{2+}]_i$

In all experiments $[Ca^{2+}]_i$ was first increased by switching from 0·5 mM glucose (c = control) to 15 mM glucose (15 G) in the bath solution. Neither the L-type Ca^{2+} channel blocker D600 (100 μM; *A*) nor the inhibitors of non-selective cation channels and the Ca^{2+} release-activated Ca^{2+} current I_{crac}, Gd^{3+} (100 μM; *B*) or flufenamate (flufen, 100 μM; *C*), suppressed the second marked rise in $[Ca^{2+}]_i$ induced by 1 mM H_2O_2. Each experiment presented in this figure is representative of four experiments with similar results.

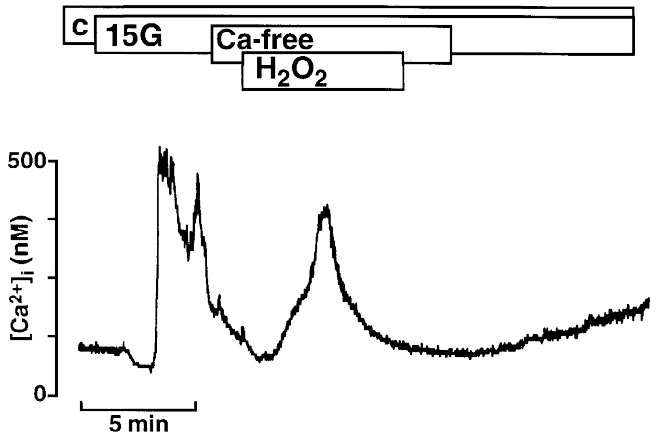

Figure 6. Effect of H_2O_2 on $[Ca^{2+}]_i$ in Ca^{2+}-free solution

Raising the glucose concentration from 0·5 mM (c = control) to 15 mM (15 G) increased $[Ca^{2+}]_i$. A subsequent switch to Ca^{2+}-free solution (1 mM EGTA) restored $[Ca^{2+}]_i$ to control values. Under these conditions the addition of 1 mM H_2O_2 led to a transient increase in $[Ca^{2+}]_i$, pointing to Ca^{2+} release from intracellular sites. The experiment shown is representative of nine with similar results.

Figure 6 demonstrates that the first transient rise was also observed in a Ca^{2+}-free bath solution. It was not different from the effect observed in Ca^{2+}-containing extracellular medium. On average, the first increase in $[Ca^{2+}]_i$ induced by H_2O_2, expressed as the area under the curve, was 425 ± 45 nM min in the bath solution with Ca^{2+} ($n = 10$) and 475 ± 70 nM min in the Ca^{2+}-free bath solution ($n = 9$).

As shown in Fig. 7*A* in one representative experiment out of four, pretreatment of the β-cells with 1 μM thapsigargin for 30 min did not suppress the first transient increase in $[Ca^{2+}]_i$ induced by H_2O_2 (right arrowhead). Due to the irreversible inhibition of the Ca^{2+} pumps by thapsigargin, the initial decrease in $[Ca^{2+}]_i$ is abolished (left arrowhead). In contrast, 100 μM of FCCP, a mitochondrial uncoupler which reduces the mitochondrial membrane potential and Ca^{2+} uptake into the mitochondria driven by the membrane potential, clearly blunted the first phase of the H_2O_2 effect (Fig. 7*B*, $n = 5$). The mean value of the area under the curves after addition of H_2O_2 in the presence of FCCP was 217 ± 49 nM min compared with 425 ± 45 nM min with H_2O_2 alone.

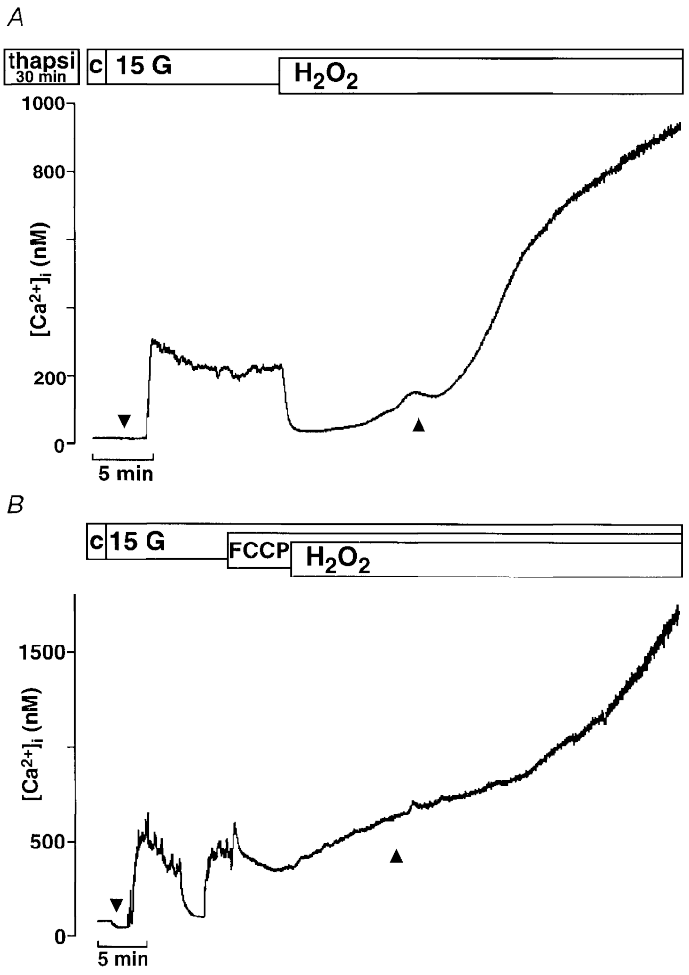

Figure 7. Effect of the Ca^{2+} pump inhibitor thapsigargin and the mitochondria uncoupler FCCP on the first phase increase in $[Ca^{2+}]_i$ induced by H_2O_2

A, the cells were pre-incubated for 30 min with 1 μM thapsigargin. The effectiveness of the treatment is demonstrated by the complete suppression of the first initial decrease in $[Ca^{2+}]_i$ after increasing the glucose concentration from 0·5 to 15 mM (compare traces at ▼ in *A* and *B*). Ca^{2+} store depletion by thapsigargin did not prevent the first rise in $[Ca^{2+}]_i$ induced by H_2O_2 (▲). The experiment shown is representative of four with similar results. *B*, addition of 100 μM FCCP in the presence of 15 mM glucose suppressed the first phase of the H_2O_2-evoked rise in $[Ca^{2+}]_i$ (compare traces at ▲ in *A* and *B*). The experiment shown is representative of five with similar results.

DISCUSSION

H_2O_2 plays an important role as a cytotoxic molecule attacking pancreatic β-cells and leading to alteration of function and eventually β-cell destruction. It is released from activated macrophages, infiltrating the islets during early stages of insulitis, and it is also produced by agents which chemically induce diabetes like alloxan and streptozotocin (Takasu *et al.* 1991). However, relatively little information is available on the mechanism by which H_2O_2 interferes with insulin secretion in β-cells. In the present paper we have shown that H_2O_2 inhibits insulin secretion from pancreatic islets by decreasing the ATP/ADP ratio, an effect leading to the opening of ATP-sensitive K^+ channels and membrane hyperpolarization. Moreover, we observed that H_2O_2 biphasically increased the intracellular Ca^{2+} activity. Thus, H_2O_2 may induce a deviation from the strong coupling between $[Ca^{2+}]_i$ and insulin secretion normally found in β-cells.

We observed an irreversible inhibition of glucose-induced insulin secretion by 1 mM H_2O_2. An inhibition of glucose-stimulated insulin release by H_2O_2 at concentrations between 0·1 and 0·5 mM has also been reported for rat (Kim *et al.* 1994) and human (Jahr *et al.* 1995) islets. We propose that the rapid inhibition of insulin secretion induced by H_2O_2 is due to membrane hyperpolarization which is caused by the opening of ATP-sensitive K^+ channels. The activation of ATP-sensitive K^+ current after addition of H_2O_2 was only observed in the perforated patch configuration with intact cell metabolism but, as previously reported, not in the standard whole-cell configuration (Krippeit-Drews *et al.* 1994*b*). Similar results were obtained by Nakazaki *et al.* (1995) who investigated the effect of H_2O_2 on single ATP-sensitive K^+ channels. They found an activation of the channels by H_2O_2 in the cell-attached mode but not in inside-out membrane patches.

Comparison of the action of H_2O_2 and diazoxide revealed that in intact islets 1 mM H_2O_2 did not hyperpolarize the membrane potential to the K^+ reversal potential and even small oscillations of the membrane potential persisted in some experiments. In contrast, in single cells, H_2O_2 hyperpolarized the membrane potential to −80 mV and accordingly spike activity was completely suppressed in all experiments. In an intact islet, an average membrane potential is registered from all coupled cells and we assume that, compared with single cells in intact islets, H_2O_2 does not have unhampered access to all cells. Consistent with this assumption is the observation that the effectiveness of tolbutamide in reversing the hyperpolarizing effect of H_2O_2 is more pronounced in intact islets, where the membrane potential stayed above the K^+ equilibrium potential after additon of H_2O_2. Microelectrode and patch-clamp experiments clearly show that the effectiveness of tolbutamide is strongly reduced after addition of H_2O_2 in the presence of 15 mM glucose. In cells not treated with H_2O_2, 100 μM of the sulfonylurea in the presence of 15 mM glucose is sufficient to induce continuous large spikes (Debuyser *et al.* 1991).

It seems reasonable to speculate that the intracellular factor mediating the activating effect of H_2O_2 on the ATP-sensitive K^+ current is a reduction of ATP, and we have demonstrated that H_2O_2 indeed lowered the ATP concentration in pancreatic β-cells. It has been shown for a variety of other cells such as endothelial cells, platelets and $P388D_1$ cells that H_2O_2 diminishes the intracellular ATP concentration (Holmsen & Robkin, 1977; Spragg *et al.* 1985; Hyslop *et al.* 1988). Thus, it is likely that H_2O_2 also influences ATP-sensitive K^+ channels in other tissues. It has been speculated that the relaxation induced in rabbit tracheal smooth muscles by H_2O_2 is mediated by ATP-sensitive K^+ channels (Gupta & Prasad, 1992). Opening of ATP-sensitive K^+ channels by H_2O_2 has been detected in patch-clamp experiments in ventricular myocytes (Goldhaber *et al.* 1989) and in the renal epithelial cell line LLC-PK1 (Filipovic & Reeves, 1997). In the latter study a concomitant reduction in the cellular ATP concentration has been shown. Several studies have shown that H_2O_2 interferes with enzymes of the glycolytic pathway (Brodie & Reed, 1987; Hyslop *et al.* 1988; Chatham *et al.* 1989; Goldhaber *et al.* 1989) and of oxidative phosphorylation (Hyslop *et al.* 1988; Goldhaber *et al.* 1989; Zhang *et al.* 1990). Nakazaki *et al.* (1995) have used various secretagogues to antagonize the ATP-sensitive K^+ channel activation induced by H_2O_2 in rat β-cells. From the relative potencies of 2-ketoisocaproic acid, glyceraldehyde and glucose in counteracting the H_2O_2 effect, they concluded that the enzymes in mitochondria are less sensitive to H_2O_2 than the glycolytic enzymes.

Recently, Herson & Ashford (1997) have shown that H_2O_2 depolarizes the membrane of the rat insulin-secreting cell line CRI-G1. They suggest that this depolarization is due to the activation of a non-selective cation channel. Although we did not observe the activation of an inward current by H_2O_2 in our preparation, we cannot entirely rule out that it exists in normal pancreatic β-cells. However, according to our experiments with normal mouse β-cells and those of Nakazaki *et al.* (1995) with normal rat β-cells, the predominant effect of H_2O_2 is activation of ATP-sensitive K^+ channels, since the cell membrane hyperpolarizes after addition of H_2O_2 and insulin secretion is inhibited.

Membrane hyperpolarization would close voltage-dependent Ca^{2+} channels and thus reduce $[Ca^{2+}]_i$. However, after an initial drop, H_2O_2 led to a biphasic increase in $[Ca^{2+}]_i$. The initial transient increase was also observed in Ca^{2+}-free extracellular solution. Since the areas under the curve, calculated in order to quantify and compare the effect of H_2O_2 on $[Ca^{2+}]_i$ in Ca^{2+}-free and Ca^{2+}-containing solutions, were equal, we assume that the elevation of $[Ca^{2+}]_i$ during this phase is exclusively due to mobilization of Ca^{2+} from intracellular sites. The observation that this initial increase is clearly reduced by the mitochondrial uncoupler FCCP but not by the Ca^{2+} pump inhibitor thapsigargin strongly

suggests that it is caused by mobilization of Ca^{2+} from mitochondria. The second dramatic increase in $[Ca^{2+}]_i$ provoked by H_2O_2 seems to be, at least in part, due to Ca^{2+} influx. At high $[Ca^{2+}]_i$ the effect of the removal of extracellular Ca^{2+} was diminished or even abolished, suggesting that Ca^{2+} efflux pathways are insufficient or even inhibited under these conditions. The strong decrease in $[Ca^{2+}]_i$ observed after the addition of an ionophore supports this assumption. It seems unlikely that the second increase in $[Ca^{2+}]_i$ induced by H_2O_2 is due to a direct activation of L-type Ca^{2+} channels, since H_2O_2 did not alter the activity of the voltage-dependent Ca^{2+} current in mouse β-cells (Krippeit-Drews *et al.* 1994*a*) and the second rise in $[Ca^{2+}]_i$ was not blocked by D600. One has to assume that other Ca^{2+} influx pathways are activated by H_2O_2. However, the second increase in $[Ca^{2+}]_i$ evoked by H_2O_2 is not blocked by Gd^{3+} or flufenamate, two inhibitors of unspecific cation channels and I_{crac}. Moreover, since we did not observe a current corresponding to the increase in $[Ca^{2+}]_i$, either the current conductance may be very small or the influx pathway may be electroneutral. An elevation of $[Ca^{2+}]_i$ induced by H_2O_2 has been observed in a variety of other cells such as smooth muscle cells (Roveri *et al.* 1992; Krippeit-Drews *et al.* 1995), endothelial cells (Doan *et al.* 1994), mesangial cells (Shaw *et al.* 1995; Meyer *et al.* 1996), a B-cell line (Qin *et al.* 1996), neuronal cells (Whittemore *et al.* 1995), myocytes (Nakamura *et al.* 1993) and renal tubular cells (Ueda & Shah, 1992), and the effect has been associated with H_2O_2-induced cell injury in, for example, renal tubular cells (Ueda & Shah, 1992), myocytes (Nakamura *et al.* 1993), glomerular mesangial cells (Shaw *et al.* 1995) and neuronal cells (Whittemore *et al.* 1995). The augmentation of $[Ca^{2+}]_i$ has been attributed to influx across the plasma membrane (Meyer *et al.* 1996), to mobilization from intracellular stores (Ueda & Shah, 1992) or to both mechanisms (Roveri *et al.* 1992; Doan *et al.* 1994; Krippeit-Drews *et al.* 1995; Shaw *et al.* 1995; Qin *et al.* 1996).

The observation that H_2O_2 decreased the ATP concentration raises the question of whether H_2O_2 also influences an ATP-dependent step in stimulus–secretion coupling distal to the membrane potential. Experiments carried out on permeabilized insulin-secreting cells have indicated that withdrawal of ATP from the cytoplasm resulted in a dramatic reduction in exocytosis (Regazzi *et al.* 1995). It has been suggested that the readily releasable pool of granules (i.e. releasable without consumption of ATP) is small in pancreatic β-cells and that ATP hydrolysis is required to translocate granules from the reserve pool to the readily releasable pool (Rorsman, 1997). However, the suggestion that the ATP concentration is too low to trigger exocytosis normally after addition of H_2O_2 is not supported by our experiments with tolbutamide, since the sulfonylurea is, at least at high concentrations, able to reverse the H_2O_2-induced inhibition of insulin secretion. Alternatively, the compartmentation of the cells could account for the discrepancy between the Ca^{2+} signal and insulin secretion. Recently, it has been suggested that the Ca^{2+} concentration in the vicinity of the secretory sites may principally control the secretory machinery (Augustine & Neher, 1992; Bokvist *et al.* 1995), indicating a different connection between Ca^{2+} mobilization and various Ca^{2+} influx pathways and secretion. In pancreatic β-cells, L-type Ca^{2+} channels are clustered in the part of the cell containing the secretory granules (Bokvist *et al.* 1995). However, the H_2O_2-induced increase in $[Ca^{2+}]_i$ is mediated by a pathway different from that leading to opening of L-type Ca^{2+} channels and thus the Ca^{2+} activity in the direct vicinity of the granules may not be sufficient.

The present paper reveals that a reduction in ATP concentration plays a central role in the suppression of insulin release by H_2O_2, since it influences secretion at the level of the plasma membrane.

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