Involvement of ATP-Sensitive K^+ Channels in Free Radical–Mediated Inhibition of Insulin Secretion in Rat Pancreatic β-Cells

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To explore the mechanisms of inhibition of insulin secretion in pancreatic β-cells by oxygen free radicals, we studied the effects of H_2O_2 on membrane currents using the patch-clamp technique. Exposure of β-cells to H_2O_2 (≥30 μmol/l) increased the activity of ATP-sensitive potassium (K^+_{ATP}) channels without changing the single channel conductance in cell-attached membrane patches. Action currents observed during superfusion of 11.1 mmol/l glucose were suppressed. In inside-out membrane patches, the activity of K^+_{ATP} channels was not influenced by H_2O_2. In conventional whole-cell clamp experiments using a pipette solution containing 3 mmol/l ATP, H_2O_2 did not influence the membrane currents. However, H_2O_2 did activate the K^+_{ATP} channel current in perforated whole-cell clamp configurations. The increased K^+_{ATP} channel current was reversed by subsequent exposure to 11.1 mmol/l 2-ketoisocaproic acid. In cell-attached membrane patches, the K^+_{ATP} channel current evoked by exposure to 30 μmol/l H_2O_2 was inhibited by exposure to 11.1 mmol/l glyceraldehyde, whereas the channel was again activated by exposure to 0.3 mmol/l H_2O_2. Subsequent superfusion of 11.1 mmol/l 2-ketoisocaproic acid inhibited the channel; this effect was counteracted by exposure to 10 mmol/l H_2O_2. Transient inhibition of K^+_{ATP} channels with provocation of action potentials was observed after washout of 100 μmol/l H_2O_2 during superfusion of 2.8 or 11.1 mmol/l glucose. We conclude that H_2O_2 has no direct effect on the K^+_{ATP} channels but that it indirectly activates the channels when it is exposed to β-cells under conditions in which the cellular metabolism is physiologically regulated. *Diabetes* 44:878–883, 1995

Oxygen free radicals are thought to be a major cause of β-cell dysfunction in animals with diabetes induced by alloxan (ALX) or streptozotocin (STZ) (1,2). Since islet cells possess low concentrations of oxygen free radical–scavenging enzymes (3), their cellular function is easily damaged (4,5). The highly reactive hydroxyl radical ($\cdot OH$) is generated via the iron-mediated Fenton reaction (6) from the superoxide anion radical ($\cdot O_2^-$) and H_2O_2, which is known to be produced in pancreatic islets by STZ or ALX (1,2). Therefore, exogenous H_2O_2, which can permeate through lipid membranes, exerts deleterious effects via $\cdot OH$ or peroxidase actions, including effects on the viability of cells and the activity of various enzymes (7,8). It is known that H_2O_2 inhibits the glycolytic pathway (9–11) and oxidative phosphorylation (10,12) and decreases intracellular NAD by activating nuclear enzymes in association with breakage of DNA strands (13,14), resulting in a decrease in intracellular ATP concentrations (10,15,16). Likewise, it has been reported that free radicals activate Na^+-Ca^{2+} exchange and inhibit Na^+-K^+-ATPase and Ca^{2+}-ATPase (17–19). Free radicals also directly inhibit the ATP-sensitive K^+ (K^+_{ATP}) channel in skeletal muscle cells (20) and indirectly activate this channel via cellular metabolic inhibition in cardiomyocytes (21).

The K^+_{ATP} channel is characterized by substantial blockade by intracellular ATP (22). The channel is a major potassium conductance route determining the resting potential of pancreatic β-cells (23,24). The closure of the K^+_{ATP} channel induced by increasing the concentration of extracellular glucose has physiological importance as an initial change in electrical activity in β-cells. In the present study, we used the patch-clamp technique to study whether H_2O_2 acts on the activity of the K^+_{ATP} channel. Our results demonstrate that exogenous oxygen free radical hyperpolarizes the β-cell membrane by opening K^+_{ATP} channels. Some of these results have been reported (25).

RESEARCH DESIGN AND METHODS

Isolation of islet cells. Experiments were performed on single pancreatic β-cells isolated from the islets of Langerhans of Wistar rats weighing 200–300 g. The animals were anesthetized by intraperitoneal injection of pentobarbitone (80 mg/kg), and islets were isolated after digestion of pancreas by ductal injection of 3 mg/ml collagenase (type 4, Funakoshi, Japan) dissolved in Krebs-Ringer bicarbonate buffer (KRBB) solution as described elsewhere (26,27). Collected islets were dispersed mechanically into single cells in Ca^{2+}-free KRBB solution, and the cells were cultured for 1–4 days in Dulbecco's modified Eagle medium containing 5.6 mmol/l glucose with 100 μg/ml streptomycin, 100 IU/ml penicillin, and 10% fetal calf serum at 37°C in 95% air with 5% CO_2.

Electrophysiological measurements. Standard patch-clamp techniques (28) were used to record the K^+_{ATP} channel currents. Patch pipettes were pulled from hard glass tubing (Narishige Scientific Institute Laboratory, Tokyo, Japan), coated with silicon resin to reduce their electrical capacitance, and fire-polished just before use. The pipettes had resistances of between 5 and 10 MΩ when filled with the solution (140 mmol/l KCl) in either the cell-attached or inside-out patch configuration and resistances <3 MΩ when filled with the internal solution in the conventional or perforated whole-cell clamp configuration. The isolated islet cells were superfused with KRBB solution containing 2.8 mmol/l glucose at least 20 min before the experiments. We identified

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ALX, alloxan; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IS, internal-like solution; K^+_{ATP}, ATP-sensitive K^+; KRBB, Krebs-Ringer bicarbonate buffer; STZ, streptozotocin.

β-cells when two or three of the following criteria were fulfilled: openings of the K^+_{ATP} channel with a unit amplitude of ~3.5 pA at a pipette potential of 0 mV during superfusion of 2.8 mmol/l glucose; no observation of action current at this glucose concentration; and subsequent appearance of action currents when the glucose concentration was increased to 11.1 mmol/l in cell-attached membrane patches.

To make the dose-inhibition curve of the K^+_{ATP} channel current for concentrations of ATP, we calculate the mean K^+_{ATP} channel current (I) by constructing the amplitude histogram with a current level of 0.04 pA/bin on the abscissa from data obtained during superfusion with each test solution. The mean current was obtained by integrating open-channel distributions on the histogram and by averaging with total sampled points. The mean amplitude of the single-channel event was determined as the difference between the current level of the closed channel distribution and that of the first-step channel events. Relative channel current (I/Ic) was plotted as a function of ATP concentration, and each point was fitted with the Hill equation $I/Ic = 1/\{1+([ATP]/K_{1/2})^n\}$, where Ic is the control mean current recorded at 0 mmol/l ATP, $K_{1/2}$ is the ligand (ATP) concentration for the half-maximal inhibition of the channel activity, and n is a Hill coefficient.

The conventional whole-cell and perforated whole-cell clamp configurations were used to record the whole-cell K^+_{ATP} channel currents, voltage-dependent K^+ channel currents, voltage-dependent Ca^{2+} channel currents, or membrane potential. Nystatin at 100 μg/ml dissolved in 0.2% DMSO in the pipette was used to perforate the patch of membrane sealed to the tip of the pipette (29).

The current was recorded by an amplifier (EPC7, List, Germany) and stored on a PCM digital data recorder (RD-101T, TEAC, Tokyo, Japan). Replayed data were then low-pass–filtered (24 dB/octave; E-3201A, NF, Tokyo, Japan) at a cutoff frequency indicated in the figure legends and digitized by a 12-bit analogue-to-digital converter for analysis by a computer (HP-9816, Hewlett Packard, Tokyo, Japan). All experiments were performed at room temperature (22–25°C).

Solutions. The composition of the pipette solution used in both the cell-attached and inside-out patch-clamp experiments was KCl, 140 mmol/l; $CaCl_2$, 2.0 mmol/l; and HEPES, 5.0 mmol/l (pH 7.2 adjusted with KOH). The standard internal solution for whole-cell clamp experiments contained: KCl, 50 mmol/l; K_2SO_4, 35 mmol/l; $MgCl_2$, 2.0 mmol/l; EGTA, 11 mmol/l; $CaCl_2$, 1.0 mmol/l; and HEPES, 11 mmol/l (pH 7.2 adjusted with KOH). The final K^+ concentration of this solution was 140 mmol/l. The pipette solution used in perforated whole-cell clamp contained: K_2SO_4, 40 mmol/l; KCl, 50 mmol/l; HEPES, 10 mmol/l; $MgCl_2$, 2.0 mmol/l; and EGTA, 0.5 mmol/l (pH 7.2 adjusted with KOH). The KRBB solution was: NaCl, 129 mmol/l; KCl, 4.7 mmol/l; $CaCl_2$, 2.0 mmol/l; $MgCl_2$, 1.2 mmol/l; KH_2PO_4, 1.2 mmol/l; and $NaHCO_3$, 5.0 mmol/l (pH of the KRBB solution was adjusted to 7.4 with 10 mmol/l HEPES-NaOH). Glyceraldehyde and 2-ketoisocaproic acid were purchased from Nakarai Tesque (Kyoto, Japan) and added to the KRBB solution as required. The internal solution used for the inside-out patch experiments contained: KCl, 113 mmol/l; $MgCl_2$, 2.0 mmol/l; EGTA, 11 mmol/l; $CaCl_2$, 1.0 mmol/l; and HEPES, 11 mmol/l (pH adjusted to 7.2 with KOH). ATP disodium salt (Boehringer Mannheim) was added to intracellular solutions as required.

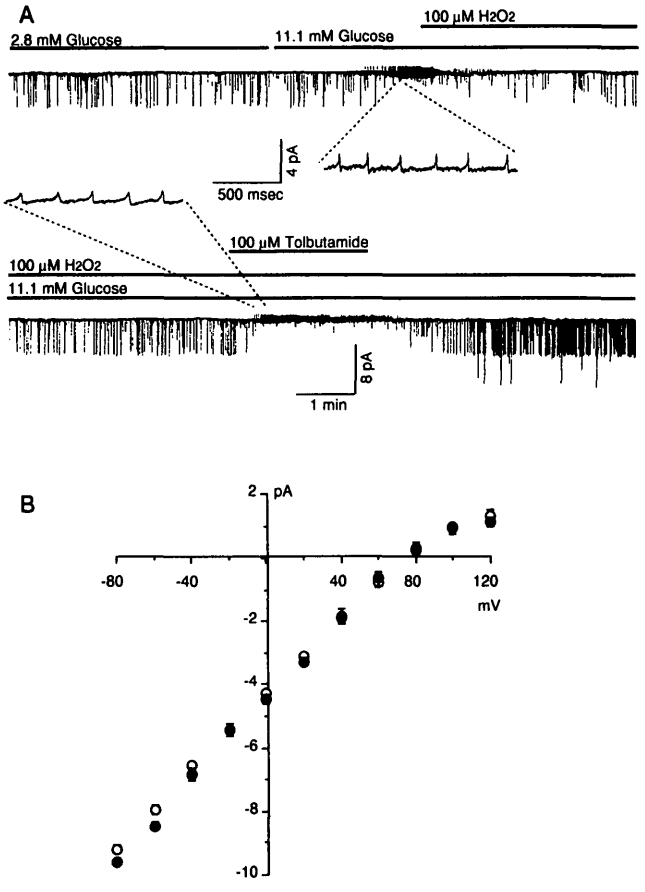

FIG. 1. Effect of H_2O_2 on K^+_{ATP} channel currents in cell-attached membrane patches. ***A:*** **The β-cell was superfused with KRBB solution containing 2.8 or 11 mmol/l glucose and 100 μmol/l H_2O_2 for the period indicated by the bar. Since the pipette potential was 0 mV, the K^+_{ATP} channel current is driven by the resting membrane potential (−70 mV), resulting in inward current as downward deflections of channel openings. Insets in the time-expanded scale are the replayed data at the period indicated. The data were low-pass–filtered at 800 Hz. The action currents are shown by biphasic deflections of the baseline current level reflecting to the action potentials induced by 11.1 mmol/l glucose or 100 μmol/l tolbutamide.** ***B:*** **Single-channel current-voltage relationship in the presence (●) and absence (○) of H_2O_2. The plotted data were expressed as means ± SE. Abscissa, membrane potentials deviating from the resting potential of β-cells.**

RESULTS

Effects of hydrogen peroxide on K^+_{ATP} channels. The effects of H_2O_2 on the K^+_{ATP} channel current of a single pancreatic β-cell superfused with a KRBB solution recorded in a cell-attached membrane patch are shown in Fig. 1*A*. Increasing the glucose concentration of the KRBB from 2.8 to 11.1 mmol/l decreased the activity of K^+_{ATP} channels and evoked biphasic deflections of baseline current level. The latter current fluctuations or action currents (Fig. 1*A*, inset of upper trace) are known to reflect to action potentials of β-cells (23,24). Unit amplitudes of channel events were 3.6 pA at the pipette potential of 0 mV, identical with those of the K^+_{ATP} channels (23). Subsequent exposure to 100 μmol/l H_2O_2 abolished the action currents and increased the open-state probability of the channel without changing the unit amplitude of the channel (Fig. 1*A*, lower trace). The H_2O_2-induced activation of the channel was reversibly inhibited by 100 μmol/l tolbutamide; this was in association temporally with the reappearance of action currents. The single-channel slope conductance measured at the potentials negative to the reversal potential of the current-voltage relationships plotted from data of six separate cell-attached membrane patches were 59 ps at 2.8 mmol/l glucose and 60 ps in the presence of both 11.1 mmol/l glucose and 100 μmol/l H_2O_2 (Fig. 1*B*). These observations indicate that the H_2O_2-induced channel current is due to the opening of K^+_{ATP} channels. We observed K^+_{ATP} channel activation by H_2O_2 at concentrations >30 μmol/l and suppression of action currents induced by superfusion of 11.1 mmol/l glucose in 19 of 20 cell-attached membrane patches. However, there was no activation of the channel by H_2O_2 at concentrations <10 μmol/l during exposure of β-cells to 11.1 mmol/l glucose (n = 5).

Indirect activation of K^+_{ATP} channels by H_2O_2. We then examined whether the activation of the K^+_{ATP} channel induced by H_2O_2 is caused by direct effects on the channel. Fig. 2*A* shows the activation of the K^+_{ATP} channel and inhibition of the action currents by 100 μmol/l H_2O_2 in the presence of 11.1 mmol/l glucose (upper trace). The bathing solution was changed from KRBB solution to the internal-

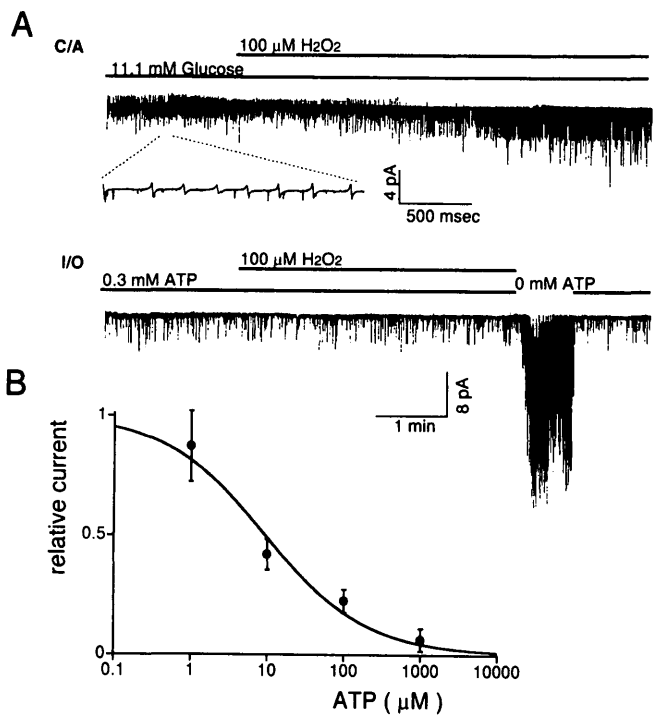

FIG. 2. Effects of H_2O_2 on single K^+_{ATP} channel currents in the inside-out membrane patches. *A:* The K^+_{ATP} channel currents were recorded in the cell-attached patch (C/A) on a β-cell exposed to 11.1 mmol/l glucose (upper trace; pipette potential, 0 mV). Subsequently, the membrane patch was excised to form the inside-out patch (I/O) configuration (lower trace; pipette potential, 60 mV). Both current traces were recorded from the same membrane patch. The IS in I/O mode contained 140 mmol/l K^+ plus 0.3 mmol/l ATP. Inset, replayed and time-expanded action current trace of the indicated place. The data were low-pass–filtered at 800 Hz and plotted on the chart recorder. *B:* Dose-inhibition relationship of the relative current (*I/Ic*) for ATP concentrations in the IS was constructed from the data of four separate inside-out membrane patches. The curve, obtained by the least-squares method, shows the Hill coefficient of 0.7 and the $K_{1/2}$ of 9 μmol/l ATP.

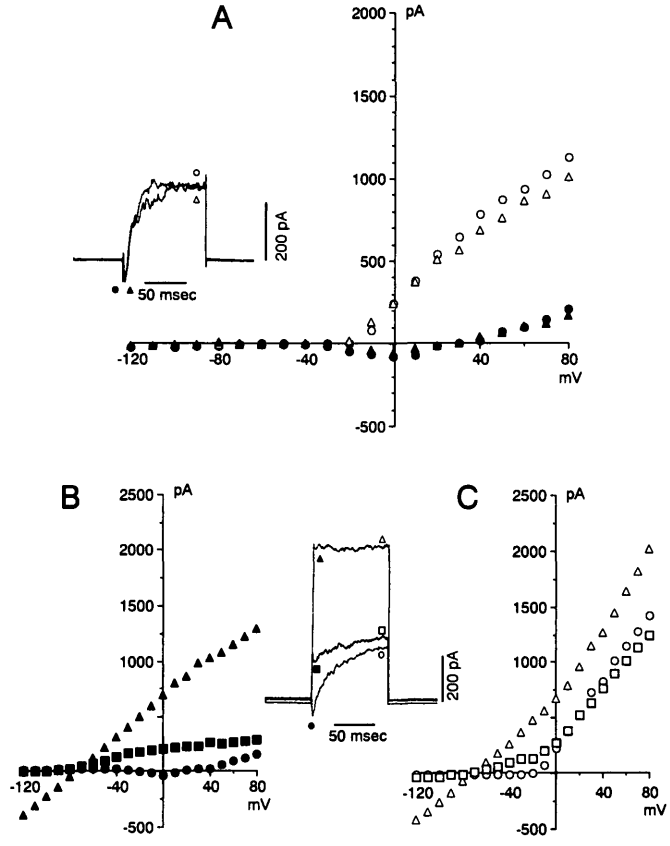

FIG. 3. Comparison of the current-voltage relationships of the membrane currents between conventional (*A*) and the perforated whole-cell clamp experiments (*B* and *C*). In both experiments, the holding potential was held at −70 mV. During depolarized and hyperpolarized pulses of 100 ms duration, the initial current levels (●, ▲, ■) and steady-state levels (○, △, □) were measured at each voltage and plotted as a function of the membrane potential. The glucose concentration in a bath solution was 2.8 mmol/l. ●, ○, control; ▲, △, data at 1 mmol/l H_2O_2; ■, □, 11.1 mmol/l 2-ketoisocaproic acid in the presence of 1 mmol/l H_2O_2 after observations of H_2O_2-induced increase in the background conductance. Insets, original current traces evoked by a voltage step to 0 mV. In *A*, the pipette solution contained 3 mmol/l ATP.

like solution (IS), and the patch membrane was then excised in the IS containing 0.3 mmol/l ATP (lower trace). The channel activity was immediately inhibited after the membrane patch was exposed to ATP in the IS. Subsequent superfusion with an IS to which 100 μmol/l H_2O_2 was added did not increase the channel activity. Channel activity was increased by eliminating ATP in the IS, suggesting that the channels retain sensitivity to ATP and do not display run-down in their activity after patch excision. The mean patch current with 0.3 mmol/l ATP in the IS was 0.1% of that recorded during exposure of the patch membrane to an ATP-free IS. We observed consistent results in 14 other membrane patches at H_2O_2 concentration ranging from 10 to 1,000 μmol/l. Furthermore, when we tested the sensitivity of the K^+_{ATP} channel to intracellular ATP in inside-out membrane patches excised after obtaining a significant increase in K^+_{ATP} channel activity by incubation of β-cells in KRBB solution containing 300 μmol/l H_2O_2 for at least 30 min, the half-maximal inhibition of the channel activity for ATP concentration was 9 μmol/l (Fig. 2*B*), which was similar to that in intact β-cells reported elsewhere (22,24,30). These observations suggest that H_2O_2-induced K^+_{ATP} channel activation is not due to a change in the sensitivity of the channel to ATP during exposure to H_2O_2 in the cell-attached membrane patches or to direct activation of the channel by these oxidants. Rather, it seemed to be due to indirect activation.

Effects of H_2O_2 on membrane currents of β-cells. We further explored the effects of H_2O_2 on the membrane currents other than the K^+_{ATP} channel current in conventional whole-cell clamp configurations. The transient inward current presumed to be a voltage-dependent Ca^{2+}- and sustained outward current presumed to be a voltage-dependent K^+-channel evoked by depolarization of membrane from the holding potential of −70 to 0 mV in the presence and absence of H_2O_2 are shown in Fig. 3*A* (inset). The pipette solution contained 3 mmol/l MgATP, which inhibits K^+_{ATP} channels by 99%, assuming that the half-maximal inhibition of the channel is 15 μmol/l ATP (30). In current-voltage relationships, neither transient inward nor sustained outward currents were influenced by H_2O_2. The K^+_{ATP} channel current was not activated. However, with perforated whole-cell clamp configuration, 1 mmol/l H_2O_2 increased the outward current and decreased the inward current levels (Fig. 3*B* and *C*). A kinetic property of this H_2O_2-induced outward current during a depolarized pulse was time-independent (Fig. 3, triangles in the inset). The reversal potential of the outward current was −70 mV, consistent with the conductance properties of K^+_{ATP} channels (27). Since the K^+_{ATP} channel current is poorly dependent on the membrane potential (23,24), the outward current increase during expo-

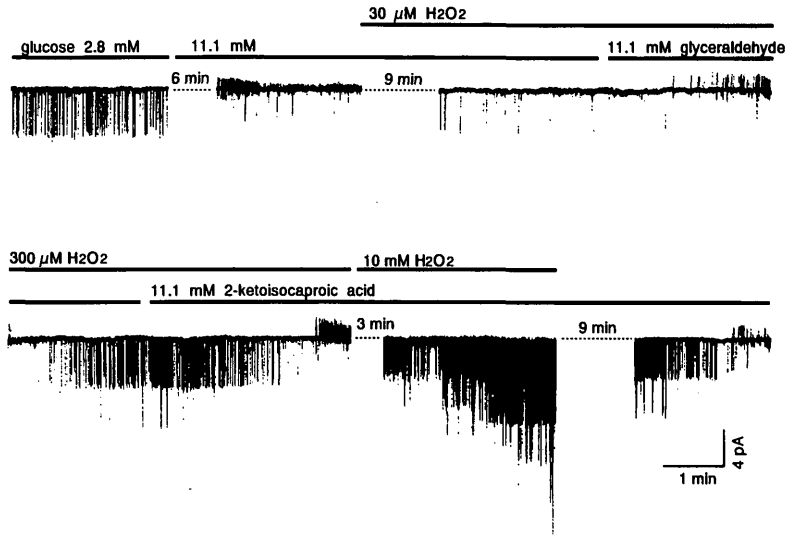

FIG. 4. Effects of various concentrations of H_2O_2 on the activity of the K^+_{ATP} channel during superfusion with intermediates of the glucose-metabolic pathway. The experiment was performed in cell-attached patch-clamp mode, and the data were recorded from the same membrane patch. Upper and lower traces were continuous. The cell was superfused with KRBB solution containing various doses of glucose, glyceraldehyde, 2-ketoisocaproic acid, and H_2O_2 during the period indicated by the bar. The pipette potential was held at 0 mV and the data were low-pass–filtered at 800 Hz.

sure to 1 mmol/l H_2O_2 should be through the K^+_{ATP} channels. At the membrane potentials positive to 0 mV, the steady-state current-voltage relationship showed slight outward rectification. This observation seems to be due to activation of the voltage-dependent outward current (delayed rectifying K^+ current).

Reclosable phenomenon of K^+_{ATP} channels by glycolytic intermediates. Subsequent superfusion with 11.1 mmol/l 2-ketoisocaproic acid partially reduced the H_2O_2-induced outward current (Fig. 3, squares in inset) to the control level. Time-dependent increase in the relaxation current during the depolarized pulse again appeared. The membrane conductance measured at −70 mV was 10.3 nS at 1 mmol/l H_2O_2 and 1.9 nS at 11.1 mmol/l 2-ketoisocaproic acid added to 1 mmol/l H_2O_2 in contrast to 0.1 nS in the control. In conventional whole-cell clamp configuration, the intracellular ATP concentration must be maintained at higher levels by equilibration of cytoplasm with IS pipette solution containing 3 mmol/l ATP; while in the perforated whole-cell clamp configuration, the intracellular ATP level may vary more physiologically under conditions of metabolic stress. It is thought that 2-ketoisocaproic acid increases production of ATP via generation of 2-ketoglutarate in mitochondria, closes the K^+_{ATP} channel, and eventually stimulates the insulin secretion (31). Thus, these observations suggest that H_2O_2 indirectly activates K^+_{ATP} channels presumably with depleting cytoplasmic ATP levels by an inhibition of glucose metabolism. We observed consistent results in three other cells.

Glucose metabolism is required for the inhibition of K^+_{ATP} channel activity and stimulation of insulin release (32). We used glyceraldehyde and 2-ketoisocaproic acid as intermediate products of glycolysis and the Krebs cycle as well as activators of glutamate dehydrogenase (33,34). In Fig. 4, 30 μmol/l H_2O_2 activated K^+_{ATP} channels in association with suppression of action currents evoked by 11.1 mmol/l glucose. Subsequent exposure to 11.1 mmol/l glyceraldehyde inhibited activity of the channel, followed by the appearance of action currents. The activity of the K^+_{ATP} channels was increased with increasing the H_2O_2 concentration to 300 μmol/l. Further, 11.1 mmol/l 2-ketoisocaproic acid counteracted the stimulatory effects on the channel activity of H_2O_2 and again produced action current. The activating effect of 10 mmol/l H_2O_2 on the channel was stronger than the inhibitory effect of 11.1 mmol/l 2-ketoisocaproic acid and was reversible on washout. Consistent results were obtained in two other cells. The relative action of 11.1 mmol/l 2-ketoisocaproic acid in inhibiting the channel was stronger than the effect of H_2O_2 at concentrations up to 3 mmol/l (n = 5). In fact, an elevation of glucose concentration to 44.4 mmol/l did not restore the increased activity of the K^+_{ATP} channel evoked by 100 μmol/l H_2O_2 (n = 4). Thus, we postulated that H_2O_2 at about 100–300 μmol/l may mainly inhibit enzymes of the glycolytic pathway at several steps. If this is the case, acceleration of glucose metabolism may be induced after removal of enzyme suppression of the glycolytic pathway by a relatively high dose of H_2O_2.

Transient inhibition of K^+_{ATP} channels after washout of H_2O_2. Figure 5 demonstrates a rebound phenomenon in the electrical activity of β-cells (arrows) after washout of 100 μmol/l H_2O_2 in the presence of 11.1 mmol/l glucose. A transient appearance of action potentials was seen even when the glucose concentration was lowered to 2.8 mmol/l with the washout of H_2O_2. The increased activity of the K^+_{ATP} channel remained after the rebound phenomenon. When 100–1,000 μmol/l H_2O_2 were applied for 5–10 min in the presence of 11.1 mmol/l glucose, irreversible activation of the K^+_{ATP} channel after the washout was observed with the rebound phenomenon in 12 of 17 cells. On the other hand, activation of the K^+_{ATP} channel by 30 μmol/l H_2O_2 applied during superfusion of 11.1 mmol/l glucose was reversible in 8 of 12 cells. The rebound phenomenon was also observed in 7 of 8 cells when H_2O_2 at concentrations between 100 and 1,000 μmol/l was applied during exposure of β-cells to 2.8 mmol/l glucose.

DISCUSSION

In this study, we have examined the effects of H_2O_2 on the activity of K^+_{ATP} channel current in rat β-cells. H_2O_2 (≥30 μmol/l) markedly activated K^+_{ATP} channel current in both the cell-attached membrane patches and the perforated whole-cell clamp experiments, two recording conditions in which cellular metabolic activity may be regulated in a near-physiological manner. This effect was not observed in inside-out membrane patches, when the internal surface of

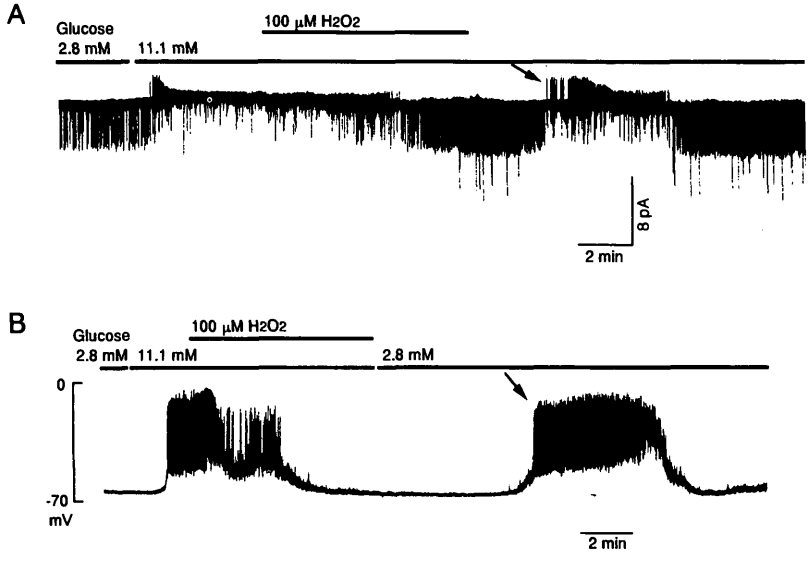

FIG. 5. Rebound phenomenon of electrical activity of β-cells after washout of H_2O_2. The experiments were performed in the cell-attached membrane patch (*A*) or the perforated current clamp mode (*B*). The cells were superfused with KRBB solution containing glucose or H_2O_2 during the period indicated by the bar. Arrows, transient overshoot (rebound phenomenon) of electrical activity after application of 100 μmol/l H_2O_2 for 5–10 min. The pipette potential in *A* was held at 0 mV and the data were low-pass-filtered at 800 Hz.

the patch was directly exposed to H_2O_2, or in standard whole-cell experiments, when the cytoplasmic surface of the membrane is exposed to fixed chemical conditions. Channels activated by H_2O_2 in the cell-attached mode retained normal sensitivity to intracellular ATP after patch excision. These results suggest that the K^+_{ATP} channel activation by H_2O_2 was not due to a direct effect such as oxidation of the channel protein by H_2O_2 or a change in the sensitivity of the channel to intracellular ATP but rather to possible inhibition of the ability of the cell to metabolize nutrients. Failure of K^+_{ATP} channels to close in response to nutrient metabolites should reduce nutrient-induced electrical activity and insulin secretion.

We speculate that H_2O_2 may activate the channel indirectly via inhibition of glycolysis and/or oxidative phosphorylation and, as a consequence, presumably via a decrease in the concentration of intracellular ATP. These results are in good agreement with the activation of K^+_{ATP} channels by H_2O_2 in isolated guinea-pig ventricular cells (21). Weik and Neumcke (20), however, demonstrated that 50 mmol/l H_2O_2 directly inhibits the K^+_{ATP} channel of mouse skeletal muscle cells. Thus, higher concentrations of H_2O_2 seem to inhibit the channel by a direct effect, due to oxidation of the channel (20).

Our observations account for inhibition of glucose-induced insulin secretion of β-cells by H_2O_2, which is generated during exposure of islets to ALX or STZ (1,2). DNA in rat pancreatic islets is reported to be fragmented at H_2O_2 concentrations of 100–1,000 μmol/l (2). This effect is followed by a decrease in the intracellular levels of NAD, a cofactor of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of the glycolytic pathway, by activating poly(ADP-ribose) polymerase in association with DNA strand breakage (13,14). Furthermore, H_2O_2 causes inhibition of GAPDH activity (9–11) and mitochondrial electron transport chain components (10,12). These effects result in a rapid decline in intracellular ATP levels (10,15,16) and eventually lead to reduction of cell viability. Henquin et al. (35) demonstrated that ALX increases ^{86}Rb efflux at 2.8 mmol/l glucose and abolishes the secretory response to 15 mmol/l glucose. Their observations can be explained by H_2O_2-induced K^+_{ATP} channel activation demonstrated in the present study if ALX produces H_2O_2 in the cytoplasm of β-cells. Thus, H_2O_2-induced K^+_{ATP} channel activation may be an important mechanism involved in the drug-induced impairment of insulin secretion in response to glucose metabolism (1,35,36).

The relative potency of secretagogues to antagonize the K^+_{ATP} channel activation produced by exogenous H_2O_2 is in the order 2-ketoisocaproic acid > glyceraldehyde > glucose (Fig. 4). Thus, the enzymes in mitochondria might be less sensitive to H_2O_2 than are glycolytic enzymes. The relative potencies of secretagogues on the insulinotropic effect during exposure to ALX are similar (35). From the data in Figs. 4 and 5, we speculate that H_2O_2 may inhibit several steps in glucose metabolism with different sensitivities. The transient enhancement of action potentials (rebound phenomenon) after washout of H_2O_2 was accompanied by inhibition of K^+_{ATP} channels and subsequent reactivation of the channels. The rebound phenomenon was observed when 100 μmol/l H_2O_2 was removed from a solution containing either 11.1 or 2.8 mmol/l glucose. Similar observations were also made during exposure to 11.1 mmol/l glyceraldehyde (n = 3; data not shown). Because 30 μmol/l H_2O_2 seems mainly to inhibit the glycolytic pathway before GAPDH and 300 μmol/l H_2O_2 (possibly >100 μmol/l) inhibits activity of GAPDH (Fig. 4), the inhibition of sites proximal to GAPDH may also be involved in the sustained increase in activity of the channel after the rebound phenomenon.

A similar phenomenon has been also demonstrated by Misler et al. (37). Using sodium azide, a reversible inhibitor of mitochondrial respiration, they observed transient overshoot of electrical activity at substimulatory glucose levels after brief application of this agent. They suggested that reduction of intracellular ATP during application of sodium azide might disinhibit glycolysis, and the increased metabolic intermediates might be able to enter the next steps after removal of the agent. Inhibition of several steps of glycolysis and accumulation of substrates of these enzymes probably occurs upon stimulation of the enzymes by lowered ATP levels during exposure to H_2O_2. Sustained activation of the K^+_{ATP} channel after the rebound phenomenon agrees with the hypothesis of multiple inhibitory actions on the glucose-metabolic pathway by H_2O_2. The rebound phenomenon of action potentials after removal of H_2O_2 has also been reported (38).

The transient inhibition and subsequent sustained activation of the K^+_{ATP} channel that we demonstrated suggest that enzyme inhibition by H_2O_2 is reversible at distal sites but is irreversible at proximal sites in the glucose metabolic pathway. We observed that exposure to H_2O_2 for more than 5–10 min at high concentrations (≥0.1 mmol/l) resulted in irreversible K^+_{ATP} channel activation unless intermediates of the glucose-metabolic pathway are superfused instead of glucose. Thus, our observations may explain in part the mechanism of the impairment of the glucose responsiveness of insulin secretion in β-cells produced by administration of ALX or STZ (1,35,36). Our results also account for observations that β-cells from rats with non-insulin-dependent diabetes mellitus induced by treatment with injection of low-dose STZ maintain the ability to secrete insulin in response to 2-ketoisocaproic acid (36). In such a model, we speculate that H_2O_2-induced K^+_{ATP} channel activation may protect β-cells from severe cellular damage by suppression of electrical excitability when the cytoplasmic ATP concentration does not fall to irreversible levels, even though the cells continue to show an impaired responsiveness to glucose.

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