Hydrogen Peroxide Induces Intracellular Calcium Overload by Activation of a Non-selective Cation Channel in an Insulin-secreting Cell Line*  

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Fura-2 fluorescence was used to investigate the effects of H2O2 on [Ca2+]i, in the insulin-secreting cell line CRI-G1. H2O2 (1–10 mM) caused a biphasic increase in free [Ca2+]i, an initial rise observed within 3 min and a second, much larger rise following a 30-min exposure. Extracellular calcium removal blocked the late, but not the initial, rise in [Ca2+]i. Thapsigargin did not affect either response to H2O2, but activated capacitative calcium entry, an action abolished by 10 μM La3+. Simultaneous recordings of membrane potential and [Ca2+]i demonstrated the same biphasic [Ca2+]i response to H2O2 and showed that the late increase in [Ca2+]i coincided temporally with cell membrane potential collapse. Buffering Ca2+ to low nanomolar levels prevented both phases of increased [Ca2+]i, and the H2O2-induced depolarization. The H2O2-induced late rise in [Ca2+]i was prevented by extracellular application of 100 μM La3+. La3+ (100 μM) inhibited the H2O2-induced cation current and NAD-activated cation (NSNAD) channel activity in these cells. H2O2 increased the NAD/NADH ratio in intact CRI-G1 cells, consistent with increased cellular [NAD]. These data suggest that H2O2 increases [NAD], which, coupled with increased [Ca2+]i, activates NSNAD channels, causing unregulated Ca2+ entry and consequent cell death.

Oxidative stress, through the production of oxygen metabolites, particularly H2O2, and other reactive oxygen species (free radicals), results in destruction of many cell types through putative necrotic/apoptotic processes (1, 2). Furthermore, excessive production of reactive oxygen species (e.g. via mitochondrial oxidation) has been causally related in the etiology of numerous degenerative disorders, including many age-related neurodegenerative diseases such as Parkinson’s disease, Huntington’s disease, and Alzheimer’s disease (3–5). Although reactive oxygen species have been implicated in cell death, the exact mechanism(s) are, as yet, unclear. A favored hypothesis is that H2O2 causes DNA strand breaks, leading to the activation of nuclear poly(ADP-ribose) polymerase, which critically depletes the cell of NAD, leading to eventual cell death (6). It has also been postulated that H2O2 disrupts the cell membrane integrity in a nonspecific manner through lipid peroxidation (7). However, there is also a good correlation between oxidative stress (H2O2 toxicity), induction of reactive oxygen species, and an increase in intracellular Ca2+ levels immediately preceding the final destructive events (8).

Pancreatic beta cells have long been known to be particularly susceptible to oxidative stress-induced destruction (9), making these cells useful models for mechanistic studies. Indeed, alloxan, which is toxic to pancreatic beta cells through the production of H2O2 and ultimately the highly reactive hydroxyl radical (‘OH), (10–12), was observed to cause diabetes mellitus in experimental animals over 50 years ago (13). This susceptibility has been correlated with a reduced capacity to withstand free radical attack through a limited cellular defense mechanism, as pancreatic beta cells have been reported to be deficient in glutathione peroxidase, catalase, and superoxide dismutase (14, 15) relative to other tissues. Therefore, we have combined Ca2+ imaging and electrophysiological recordings of an insulin-secreting cell line (CRI-G1) to enable an investigation of the cellular consequences and mechanisms underlying mammalian cell responses to oxidative stress. Previously, it had been demonstrated, by intracellular recordings, that exposure to alloxan causes irreversible depolarization of mouse pancreatic beta cells (16). These initial observations have more recently been substantiated using whole-cell recordings, which show that alloxan and H2O2, through the production of reactive oxygen species, cause complete and irreversible depolarization of CRI-G1 insulin-secreting cells (17). This study also demonstrated that the H2O2-driven collapse of the membrane potential is mediated by the opening of a previously quiescent novel non-selective cation (NSNAD) channel. Although activated by oxidative stress in intact cells, this non-selective cation channel requires the presence of both Ca2+ and NAD on the cytoplasmic aspect of excised patches for channel activity to be observed (18, 19). Permeation studies indicate that this channel has a significant conductance for divalent cations, most notably Ca2+ (18); and therefore, oxidative stress-induced activation of this channel would be expected to allow a significant Ca2+ influx associated with the collapse of the membrane potential.

We now report that exposure of CRI-G1 cells to concentrations of H2O2 that activate NSNAD channels causes a biphasic rise in [Ca2+]i. The second, late rise in [Ca2+]i, induced by H2O2 reaches micromolar concentrations, indicating unregulated calcium influx. It is proposed that the second rise in [Ca2+]i, caused by H2O2-induced activation of NSNAD channels, leading to concurrent depolarization and eventual cell death through calcium overload. Some of these data have been reported previously in preliminary form (20).

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Cells from the insulin-secreting cell line CRI-G1 were grown in Dulbecco’s modified Eagle’s medium containing sodium pyruvate (0.01%) and glucose (0.1%) and supplemented with 10% fetal calf serum and 1% (v/v) penicillin/streptomycin at 37 °C in a humidified...
The bathing medium consisted of 140 mM NaCl, 1 mM MgCl₂, 1 mM KCl, 5.02 mM CaCl₂, 10 mM HEPES (pH 7.4), resulting in a free Ca²⁺ concentration of 50 μM, and the bathing medium consisted of 140 mM NaCl, 1 mM MgCl₂, 10 mM CaCl₂, and 10 mM HEPES (pH 7.2). The pipette solution for whole-cell recordings contained 140 mM KCl, 0.6 mM MgCl₂, 10 mM EGTA, and 10 mM HEPES (pH 7.2), resulting in a free Ca²⁺ concentration in the low nanomolar range, before stimulation with H₂O₂, and allowing an increase in free Ca²⁺ concentration during stimulation. Some whole-cell recordings were performed with a pipette solution containing 140 mM KCl, 0.6 mM MgCl₂, 10 mM EGTA, and 10 mM HEPES in order to clamp intracellular Ca²⁺ in the low nanomolar range. The bath solution consisted of normal saline (135 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, and 10 mM HEPES (pH 7.4)). Additionally, when investigating voltage-dependent Ca²⁺ channel currents, the pipette contained 140 mM CaCl₂, 10 mM EGTA, 2.73 mM CaCl₂, and 10 mM HEPES (pH 7.2), resulting in a free Ca²⁺ concentration of 100 μM; and the bath solution consisted of normal saline supplemented with Ba²⁺ (135 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 10 mM BaCl₂, and 10 mM HEPES (pH 7.4)).

Imaging Intracellular Calcium of Single Cells—After washing with normal saline supplemented with 3 mM glucose, the plated cells were incubated with 2 μM fura-2/AM for 45–120 min at room temperature (22°C) prior to experiments in which [Ca²⁺]i was measured in intact cells. Single coverslips were mounted in a chamber on top of an inverted fluorescence microscope and perfused with normal saline plus 3 mM glucose. Measurements of [Ca²⁺]i, in individual cells were made from the fluorescence ratio (excitation at 340/380 nm and emission at >510 nm for fura-2) using a specially designed filter wheel assembly, incorporating a CCD camera, a photomultiplier, and a suite of software (MAGICAL, Applied Imaging, Sunderland, United Kingdom) that samples emission following excitation at 340- and 380-nm wavelengths at 15-s intervals. [Ca²⁺]i was calculated from a calibration curve using the equation \[ \frac{[Ca^{2+}]_{i}}{[Ca^{2+}]_{o}} = R_{i}/R_{o} = (R_{o} - R_{min})/(R_{max} - R_{o}) \], where Rmax, Rmin, and R are the maximum ratio, minimum ratio, and measured ratio, respectively; β is minimum 380/maximum 380; and Kᵣ represents the Ca²⁺ binding affinity of fura-2. Rmax, Rmin, and β were determined from free standing solutions of 2 and 0 mM CaCl₂ (+5 mM EGTA) in HEPES buffer solution as described previously (22, 23); these values did not vary significantly from day to day. Zero extracellular Ca²⁺ was obtained by the removal of CaCl₂ from the normal saline as well as by the addition of 5 mM EGTA. All intact cell imaging was performed on at least three separate cultures, and numbers quoted (n) represent the number of individual cells analyzed. The intrinsic autofluorescence of cells, induced by the presence of reduced pyridine nucleotides, was examined without loading with fura-2/AM. Significant changes in autofluorescence were observed using excitation at 360 nm and emission at >510 nm.

Combined Ca²⁺ Imaging and Electrophysiology—Methods for detecting [Ca²⁺]i in cells while recording in the whole-cell configuration were essentially the same as those used for intact cells, except that the pipette solution contained 140 mM KCl, 0.6 mM MgCl₂, 10 mM HEPES, and 50 μM fura-2 (pH 7.2), and in some experiments, 10 mM EGTA was added to buffer intracellular Ca²⁺ to low nanomolar levels. The bath solution consisted of normal saline (135 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM HEPES, and 3 mM glucose (pH 7.4)). Under these conditions in the cell-attached configuration, the fluorescence ratio indicated <10 nM free Ca²⁺, contamination of the pipette solution from stock reagents. In these experiments, the cells were viewed on an upright Zeiss Axioskop microscope with the appropriate dichroic mirror (405 nm) and trinocular mounting to send the emitted light to the photomultiplier tube (PHOCAL, Life Sciences Resources, Basingstoke, United Kingdom).

Hydrogen Peroxide-induced Calcium Overload

FIG. 1. H₂O₂ causes a biphasic rise in intracellular calcium levels. Shown are fluorescence measurements of fura-2/AM-loaded CRI-G1 cells. A, representative imaging experiment, from three cells (in this and subsequent figures), illustrating the biphasic response caused by the addition of 10 mM H₂O₂ to the bathing medium. The initial rise occurred within 5 min, and a second, massive rise (which reached near-dye saturation (>1 μM)) could be seen after ~30 min. B, a separate experiment illustrating the effect of the addition of 10 mM H₂O₂ to the bathing medium in the absence of extracellular calcium. Only the initial small sustained rise in intracellular calcium levels occurred in the absence of extracellular calcium. The re-addition of extracellular calcium resulted in an immediate massive calcium increase (near-dye saturation (>1 μM)). Note that exposure to H₂O₂ in the absence of extracellular Ca²⁺ was longer in duration than the time taken to reach a maximal response in A.
Figure 2. Cd\textsuperscript{2+} inhibits voltage-dependent Ba\textsuperscript{2+} currents and abolishes depolarization-induced calcium influx. A, mean current-voltage relation, from a cell at a holding potential of -70 mV, obtained over the voltage range -40 to +40 mV with 140 mM Cs\textsuperscript{+} in the pipette (●) and the 1 mM extracellular Ca\textsuperscript{2+} replaced with 10 mM Ba\textsuperscript{2+}. This current had a threshold for activation of near -40 mV and was maximal at 0 mV. The addition of 100 μM Cd\textsuperscript{2+} (○) to the bathing medium completely abolished this current. Note the presence of a small residual current at both hyperpolarized and depolarized potentials, probably due to contamination by non-selective cation or anion currents. Representative barium currents were observed at -10 mV; the initial inward spike is due to activation of voltage-gated sodium currents. The application of 100 μM Cd\textsuperscript{2+} resulted in complete and reversible inhibition of the barium current, but had no effect on the sodium current. B, fluorescence measurements of a single fura-2AM-loaded CRI-G1 cell illustrating the rise in [Ca\textsuperscript{2+}], caused by depolarization elicited by 40 mM extracellular K\textsuperscript{+}. The additional presence of 100 μM Cd\textsuperscript{2+} completely abolished the 40 mM K\textsuperscript{+}, depolarization-induced calcium increase. Inhibition produced by Ca\textsuperscript{2+} was reversed upon removal (data not shown).

The [Ca\textsuperscript{2+}]\textsubscript{i} response to 1 and 10 mM H\textsubscript{2}O\textsubscript{2} differed temporally (data not shown), the magnitude of both the early and late phases of increased [Ca\textsuperscript{2+}]\textsubscript{i}, was indistinguishable (p > 0.05). The initial rise occurred within 3 min, and the fura-2 ratio increased from an initial resting level of 0.44 ± 0.01 (48 nM) to 0.93 ± 0.02 (190 nM; n = 72). The second, late response occurred -30 min later (-40 min for 1 mM H\textsubscript{2}O\textsubscript{2}) and induced a rise to near-dye saturation (fura ratio > 2; >1 μM Ca\textsuperscript{2+}). Removal of extracellular calcium by the addition of 5 mM EGTA decreased the initial rise in [Ca\textsuperscript{2+}], (ratio rising from 0.40 ± 0.01 (48 nM) to 0.73 ± 0.02 (130 nM); n = 52; p < 0.01) and completely abolished the second rise induced by 10 mM H\textsubscript{2}O\textsubscript{2} (Fig. 1B). The re-addition of extracellular calcium resulted in an immediate increase in [Ca\textsuperscript{2+}], again rising to near-dye saturation, indicating that a calcium permeation pathway had been activated by the H\textsubscript{2}O\textsubscript{2} exposure. These results indicate that the H\textsubscript{2}O\textsubscript{2}-induced early increase in intracellular calcium levels results predominantly from mobilization of Ca\textsuperscript{2+}, from an intracellular source and that the second, late increase in intracellular calcium levels is a result of extracellular calcium influx.

Previous studies examining the consequences of mammalian cell exposure to reactive oxygen species have generally been inconclusive with respect to the identity of the permeation pathway for the oxidative stress-induced rise in [Ca\textsuperscript{2+}], consequent to Ca\textsuperscript{2+} entry from the extracellular medium. There are reports that implicate voltage-gated calcium channels (through the use of organic Ca\textsuperscript{2+} channel blockers) as the source of calcium influx (24, 25), whereas others indicate that such a pathway is unlikely (26, 27). Even if voltage-gated calcium channels were not the primary path, reactive oxygen species-derived depolarization of cells could activate these channels, which could then secondarily contribute to the increased intracellular calcium levels. To examine whether voltage-gated calcium channels contribute in any way to the H\textsubscript{2}O\textsubscript{2}-induced increase in [Ca\textsuperscript{2+}], in CRI-G1 cells, 100 μM Cd\textsuperscript{2+} was applied to the extracellular environment of the cells. This concentration of Cd\textsuperscript{2+} completely inhibits voltage-gated calcium currents (n = 5) in this cell line, an action partially reversible on washout of the Cd\textsuperscript{2+} (Fig. 2A). As further verification that Cd\textsuperscript{2+} inhibits the function of voltage-gated calcium channels in these cells, the increased [Ca\textsuperscript{2+}], response associated with a depolarization-induced (40 mM K\textsuperscript{+}) calcium influx was shown to be completely prevented by 100 mM Cd\textsuperscript{2+} (n = 12) (Fig. 2B). However, in the presence of extracellular calcium, co-application of 100 μM Cd\textsuperscript{2+} and 10 mM H\textsubscript{2}O\textsubscript{2} failed to inhibit the H\textsubscript{2}O\textsubscript{2}-induced rise in [Ca\textsuperscript{2+}], with the fura-2 ratio increasing to dye saturation (n = 13) (Fig. 3A). The presence of Cd\textsuperscript{2+} did produce a striking change in the time course of the [Ca\textsuperscript{2+}], response in that the second phase of the H\textsubscript{2}O\textsubscript{2}-induced increase in [Ca\textsuperscript{2+}], occurred almost immediately on application of the oxidative stress, with no obvious evidence of the initial [Ca\textsuperscript{2+}], response (n = 20). This acceleration of the [Ca\textsuperscript{2+}], response is possibly consistent with Cd\textsuperscript{2+} catalyzing the decomposition of H\textsubscript{2}O\textsubscript{2} to
form reactive oxygen species (9). The effects of Cd²⁺ on [Ca²⁺]ᵢ were also examined in the absence of extracellular calcium, and Cd²⁺ was demonstrated to have no effect on [Ca²⁺]ᵢ, per se and did not alter the initial H₂O₂-induced rise in [Ca²⁺]ᵢ (ratio rising from 0.37 ± 0.01 (42 ns) to 0.83 ± 0.06 (155 ns); p > 0.05 compared with data in the absence of Cd²⁺). However, re-addition of 1 mM Ca²⁺-containing extracellular solution in the continued presence of Cd²⁺ induced a rapid, large increase in [Ca²⁺]ᵢ (n = 13) (Fig. 2B). Consequently, it appears highly unlikely that activation of voltage-dependent calcium channels is responsible for, or contributes significantly to, the H₂O₂-induced second, late phase of increased [Ca²⁺]ᵢ, in CRI-G1 cells.

Another possible contributor to this second, large rise in [Ca²⁺]ᵢ, is by means of an intracellular Ca²⁺-triggered capacitive entry system for Ca²⁺ (CRAC) that has somehow become unregulated by the presence of H₂O₂. The data described above indicate that the initial phase of increased [Ca²⁺]ᵢ, in response to H₂O₂ challenge is due to mobilization of calcium from intracellular stores. Consequently, the possibility that the calcium originates from microsomal stores was investigated using thapsigargin, an endoplasmic reticulum Ca²⁺-ATPase inhibitor that depletes intracellular Ca²⁺ stores by blocking the uptake of calcium (28). The addition of 1 μM thapsigargin to intact cells resulted in a rapid rise in [Ca²⁺]ᵢ, which peaked and then declined, but did not return to initial pre-thapsigargin levels (Fig. 4A), with the fura-2 ratio increasing from 0.41 ± 0.01 (50 ns) to 0.83 ± 0.03 (155 ns) and then plateauing to a new level of 0.59 ± 0.02 (90 ns) (n = 32). Following thapsigargin treatment, removal of extracellular calcium decreased [Ca²⁺]ᵢ to a level below that seen in control experiments, 0.32 ± 0.01 (32 ns; p < 0.05). The subsequent addition of 10 mM H₂O₂ resulted in a response indistinguishable from that of cells not exposed to thapsigargin (Fig. 1A), i.e. an initial sustained increase in [Ca²⁺]ᵢ, with the fura-2 ratio rising from 0.32 ± 0.01 (32 ns) to 0.76 ± 0.02 (137 ns) (p > 0.05), followed by a second, larger rise following re-addition of extracellular Ca²⁺, reaching near-dye saturation. It is therefore clear that H₂O₂ mobilizes intracellular calcium from a thapsigargin-insensitive source. Previous studies have demonstrated that emptying of intracellular Ca²⁺ stores in pancreatic β cells triggers capacitive calcium entry.

**Fig. 3.** Cd²⁺ does not inhibit H₂O₂-induced Ca²⁺ influx. A, fluorescence measurements of fura-2-AM-loaded CRI-G1 cells showing a rapid and maximal increase in intracellular Ca²⁺ levels caused by the application of 10 mM H₂O₂ in the presence of 100 μM Cd²⁺. Note the highly accelerated rate of the [Ca²⁺]ᵢ rise compared with Fig. 1A, B, separate experiment illustrating the lack of effect of 100 μM Cd²⁺ on Ca²⁺, per se and the effect of subsequent addition of 10 mM H₂O₂ plus Cd²⁺ to the bathing medium in the absence of extracellular calcium, which caused a small sustained rise in intracellular calcium levels. The re-addition of extracellular calcium in the continued presence of Cd²⁺ resulted in an immediate massive rise in Ca²⁺ levels, which reached near-dye saturation (>1 μM).

**Fig. 4.** Thapsigargin does not affect the H₂O₂-induced Ca²⁺ response. A, shown are fluorescence measurements of fura-2-AM-loaded CRI-G1 cells illustrating the biphasic rise in intracellular Ca²⁺ levels initiated by 1 μM thapsigargin (Thaps). Following thapsigargin treatment, removal of extracellular Ca²⁺ decreased [Ca²⁺]ᵢ, to a level below control values. The re-addition of extracellular Ca²⁺ caused a biphasic Ca²⁺ response essentially identical to that caused by thapsigargin. This is due to capacitive calcium entry. The subsequent addition of 10 mM H₂O₂ in the absence of extracellular Ca²⁺ caused a sustained rise in [Ca²⁺]ᵢ, indistinguishable from controls. The re-addition of extracellular calcium resulted in an immediate massive rise in Ca²⁺ levels, which reached near-dye saturation (>1 μM). B, shown is a separate experiment illustrating the complete and reversible inhibition of thapsigargin-induced capacitive calcium entry by 10 μM La³⁺. In contrast, 10 μM La³⁺ did not prevent the H₂O₂-induced Ca²⁺ influx seen upon re-addition of extracellular Ca²⁺. C, in cells not treated with thapsigargin, H₂O₂ induced the initial rise in Ca²⁺ levels in the absence of extracellular Ca²⁺, but re-addition of extracellular Ca²⁺ in the presence of 100 μM La³⁺ prevented the immediate second, large [Ca²⁺]ᵢ increase. Removal of the La³⁺ in the presence of extracellular calcium allowed the late phase of calcium entry to be re-established.
through $I_{\text{CRAC}}$ (29, 30) and that low ($10$–$100 \mu M$) concentrations of La$^{3+}$ inhibit this process (31). In the CRI-G1 cells, emptying of intracellular Ca$^{2+}$ stores with thapsigargin resulted in the activation of $I_{\text{CRAC}}$. This can be seen (Fig. 4, A and B) by removal and re-addition of extracellular calcium, which resulted in [Ca$^{2+}$], transients not observed without exposure to thapsigargin ($n = 32$ and 24 with and without thapsigargin, respectively). The induction of [Ca$^{2+}$], transients indicative of $I_{\text{CRAC}}$ activation in these cells was maximally and reversibly inhibited by the presence of $10 \mu M$ La$^{3+}$ (Fig. 4B). In contrast, the presence of $10 \mu M$ La$^{3+}$ did not abolish the influx of extracellular Ca$^{2+}$ ($n = 13$) induced by $10 \mu M$ H$_2$O$_2$, although there was an $\sim 30\%$ inhibition (Fig. 4B). However, application of $100 \mu M$ La$^{3+}$ did erode the second [Ca$^{2+}$], transient due to H$_2$O$_2$-induced Ca$^{2+}$ influx ($n = 24$) (Fig. 4C), an action that was reversible, as washout of the La$^{3+}$ in the continued presence of extracellular Ca$^{2+}$ resulted in eventual massive influx of Ca$^{2+}$. Consequently, these data indicate that the H$_2$O$_2$-induced Ca$^{2+}$ influx is pharmacologically distinct from the $I_{\text{CRAC}}$ pathway in these cells and also exclude a nonspecific membrane breakdown as mediator of Ca$^{2+}$ entry.

The identity of the Ca$^{2+}$ entry pathway responsible for the second, late increase in [Ca$^{2+}$], induced by H$_2$O$_2$ in CRI-G1 cells was investigated using whole-cell current clamp recordings with $50 \mu M$ fura-2 in the intracellular solution in order to monitor simultaneously membrane potential and the levels of free intracellular calcium. After initiation of the whole-cell recording mode, the cell interior was diazoxed with an ATP-free solution, which resulted in cell hyperpolarization (Fig. 5A, upper trace). The membrane potential changed from an initial hyperpolarization and decreased input resistance are due to the opening of ATP-sensitive K$^+$ (K$\text{ATP}$) channels as internal ATP is washed out of the cell (17, 32). Following stabilization of the membrane potential and input resistance, application of $10 \mu M$ H$_2$O$_2$ caused a sustained increase in [Ca$^{2+}$], (Fig. 5A, lower trace), which preceded any effect on membrane potential. This increase occurred within $3$ min ($2.6 \pm 0.2$ min; $n = 6$), and the fura-2 ratio rose from $0.45 \pm 0.02$ (46 nM) to $0.87 \pm 0.08$ (150 nM). There was then a further delay of $\sim 10$–$12$ min before a second phase of increased [Ca$^{2+}$], was observed (fura-2 ratio rising from $0.87 \pm 0.08$ to $1.94 \pm 0.16$ (>600 nM)) (Fig. 5A, lower trace). These [Ca$^{2+}$], responses to $10 \mu M$ H$_2$O$_2$ almost exactly recapitulated those obtained in intact cells. The decreased delay for the second phase of increased [Ca$^{2+}$], during whole-cell recordings in comparison with intact cells is likely due to dialysis of the cells’ natural antioxidant protective mechanisms (e.g. glutathione). The second, late phase of increased [Ca$^{2+}$], coincided with the appearance of a slow, irreversible depolarization to $\sim 0$ mV. This H$_2$O$_2$-induced depolarization is consistent with our previous report on the action of H$_2$O$_2$ in this cell line (17).

To determine whether the later large increase in [Ca$^{2+}$], induced by H$_2$O$_2$ is dependent upon the initial increase in [Ca$^{2+}$], simultaneous recordings of membrane potential and [Ca$^{2+}$], were made with $10 \mu M$ EGTA included in the intracellular pipette solution to clamp the [Ca$^{2+}$], to low nanomolar levels. As illustrated in Fig. 5B, the inclusion of $10 \mu M$ EGTA in the pipette clamped the [Ca$^{2+}$], below the resting level ($0.26 \pm 0.01$; <1 nM), and this did not change after application of $10 \mu M$ H$_2$O$_2$. Furthermore, although the reduction in intracellular calcium had no effect on the hyperpolarization induced by the washout of the internal ATP (the mean cell hyperpolarization was to $-75 \pm 2.4$ mV; $n = 4$), it completely prevented the H$_2$O$_2$-induced depolarization (Fig. 5B, lower trace).

Clearly, activation of the late phase of increased [Ca$^{2+}$], by
H$_2$O$_2$ is dependent upon the initial rise in calcium levels, is through a specific permeation pathway, and is consequent to the depolarization of the cells. Previous studies have demonstrated that the H$_2$O$_2$-induced depolarization of CRI-G1 cells is due to the opening of a novel non-selective cation conductance (NSNAD channel), which also allows permeation of Ca$^{2+}$ (17, 18). Activation of this channel by H$_2$O$_2$ is shown in Fig. 6, where cell-attached single channel recordings from CRI-G1 cells were performed. Following application of H$_2$O$_2$ (4.4 mM), there was a delay of ~9–10 min before the appearance of single channel currents (Fig. 6A). This current was characterized by a linear current-voltage relation with a conductance of 70 pS and a reversal potential of 0 mV (Fig. 6, B and C). Excision of the patch into the inside-out configuration resulted in loss of channel activity, which could only be recovered by application of NAD to the cytoplasmic aspect of the membrane patch (Fig. 6C), identifying the channel as NSNAD (17). We also examined the likelihood that this channel is responsible for the late phase of Ca$^{2+}$ entry induced by H$_2$O$_2$ by determining the sensitivity of this conductive pathway to inhibition by La$^{3+}$. Following attainment of the whole-cell recording configuration (Fig. 7A), the conductance of the cell was small (1.46 ± 0.54 nS; n = 3), with a reversal potential of −42.3 ± 4.0 mV (n = 3), which is close to the cell resting membrane potential of approximately −40 mV. After dialysis with the ATP-free pipette solution, the conductance increased to 18.2 ± 2.80 nS, with a reversal potential of −70 ± 3.6 mV, which is attributed to activation of K$_{\text{ATP}}$ channels. In contrast, subsequent to the H$_2$O$_2$-induced depolarization of CRI-G1 cells, voltage clamp recordings demonstrated the activation of a non-selective cation current characteristic by a linear slope conductance (Fig. 7A) of 4.6 ± 1.1 nS with a concurrent loss of K$_{\text{ATP}}$ current, indicated by a shift in the reversal potential to 5.7 ± 3.6 mV (n = 3). Application of 100 μM La$^{3+}$ caused a 50–80% inhibition of the H$_2$O$_2$-activated current, decreasing the slope conductance to 2.1 ± 0.1 nS with only a slight shift in the reversal potential (to −1 mV). The inhibition of this current by La$^{3+}$ was partially reversed after a >5-min wash, with the slope conductance increasing to a value of 3.7 ± 3.6 mV (n = 3) (Fig. 7A). The inhibitory effect of La$^{3+}$ was also determined on single channel currents activated by 1 mM NAD$_3$ in the presence of 50 μM Ca$^{2+}$ in isolated outside-out membrane patches. In all outside-out patches examined, the addition of 100 μM La$^{3+}$ to the extracellular membrane aspect caused complete cessation of channel activity (Fig. 7B), an action not reversible even with prolonged washing (n = 6).

The data presented above are consistent with the notion that H$_2$O$_2$ induces the release of Ca$^{2+}$ from an as yet undefined intracellular Ca$^{2+}$ store, which yields the first phase of increased [Ca$^{2+}$]$_i$, and this is obligatory for the subsequent activation of a substantial Ca$^{2+}$ influx through NSNAD channels, producing the second phase of increased [Ca$^{2+}$]$_i$. The activity of the NSNAD channel in isolated patches is Ca$^{2+}$-dependent, but the presence of intracellular Ca$^{2+}$ per se is not sufficient to sustain channel activity; there is also an absolute requirement for intracellular NAD. Therefore, the effect of H$_2$O$_2$ exposure on pyridine nucleotide levels was examined in intact cells utilizing the autofluorescence properties of these nucleotides (30, 33). Application of 10 mM H$_2$O$_2$ caused a rapid (within 2–3 min) but transient decrease in NADPH autofluorescence both in the absence and presence of extracellular calcium (n = 24; p >
FIG. 7. La3+ inhibits H2O2-activated whole-cell current and single NSNAD channel activity. A, current-voltage relations obtained from a voltage-clamped cell, over the voltage range −130 to −50 mV, from a holding potential of −70 mV prior to and following H2O2-induced (10 mM) cell depolarization. Initially, the current was linear with a reversal potential near −40 mV (●), and following dialysis of the cell with an ATP-free solution (○), the current dramatically increased, and the reversal potential shifted more negative, indicating activation of the KATP current. In the presence of H2O2, the KATP current was abolished, and there was an inward current characterized by a linear current-voltage relation (□), which, on application of 100 μM external La3+, was significantly reduced (△), an action only partially reversed on washout of La3+ (▲). Representative families of currents were generated in the same experiment. B, representative continuous outside-out recording illustrating the irreversible inhibition of NAD-activated (NSNAD) single channel activity by 100 μM external La3+. This experiment was performed on an outside-out patch with 1 mM internal NAD and 50 μM free Ca2+ in the pipette at a membrane potential of −40 mV.

DISCUSSION

Previous studies have demonstrated that H2O2, most probably through the generation of reactive oxygen species, causes an increase in intracellular calcium levels that precedes, if not causes, cell death in a wide variety of cell types, including cardiac (27) and smooth muscle cells (26), pancreatic acinar cells (34), alveolar macrophages (35), and central nervous system neurons (36). Here, we describe the effects of H2O2 on [Ca2+]i in the insulin-secreting cell line CRI-G1 and clearly demonstrate that H2O2 also disrupts calcium homeostasis in these cells. There is no clear consensus in the literature indicating the likely mechanism by which H2O2 causes an increase in [Ca2+]i; suggestions that have been promulgated include influx through voltage-dependent calcium channels (25, 35), nonspecific changes in membrane calcium permeability (37, 38), alteration in Na+-Ca2+ exchange (39), or changes in calcium release from intracellular stores (40, 41).

The data presented herein indicate that both release of calcium from an intracellular source and influx of extracellular calcium contribute to the rise in free [Ca2+]i, induced by H2O2 in CRI-G1 cells. In the absence of extracellular calcium, H2O2 caused a significant increase in [Ca2+]i, which began within 2–3 min, reached a plateau quickly, and was sustained at the elevated level for as long as the extracellular calcium was absent. The subsequent re-addition of extracellular Ca2+ resulted in a rapid increase in [Ca2+]i. On the basis of these data, it is concluded that H2O2 initially stimulated release of calcium from an intracellular source and a later influx of extracellular Ca2+. Additional studies indicated that depletion of the intracellular calcium stores with thapsigargin had no effect on the magnitude of the [Ca2+]i increase elicited by H2O2 in the absence of extracellular calcium, indicating that H2O2 induced release of calcium from a thapsigargin-insensitive source. This effectively negates the possibility that H2O2 stimulated calcium release from either inositol 1,4,5-trisphosphate-sensitive or calcium-induced endoplasmic reticulum stores. The possibility that other intracellular organelles, in particular the nucleus (41) or mitochondria (42, 43), are responsible for the initial calcium response requires investigation.

Simultaneous measurements of membrane potential and [Ca2+]i were made on single CRI-G1 cells, and regardless of the intracellular dialysis caused by the whole-cell recording configuration, H2O2 caused a biphasic increase in [Ca2+]i, with the second, larger rise coinciding temporally with cellular depolar-
stores (30, 46, 47). Depletion of intracellular Ca\textsuperscript{2+} stores in CRI-G1 cells with thapsigargin resulted in the activation of a calcium influx pathway, indicative of the presence of I\textsubscript{CRAC} in these cells. I\textsubscript{CRAC} is known to be inhibited by low concentrations (<100 \(\mu\)M) of extracellular metal ions, particularly La\textsuperscript{3+} (31, 48). In CRI-G1 cells, the capacitive calcium entry pathway was completely blocked by the application of low (10 \(\mu\)M) concentrations of La\textsuperscript{3+}. However, this concentration of La\textsuperscript{3+} had only a small inhibitory effect on the H\textsubscript{2}O\textsubscript{2}-induced late increase in [Ca\textsuperscript{2+}], indicating that the calcium influx activated by H\textsubscript{2}O\textsubscript{2} is distinct from that activated by emptying of intracellular Ca\textsuperscript{2+} stores. Although low concentrations of La\textsuperscript{3+} failed to block the H\textsubscript{2}O\textsubscript{2}-induced calcium influx, a higher concentration of La\textsuperscript{3+} (100 \(\mu\)M) completely abolished the calcium influx. This also indicates that although the calcium influx is independent of both I\textsubscript{CRAC} and voltage-dependent calcium channels, it cannot be explained by nonspecific membrane degradation.

Recently, it has been demonstrated that application of H\textsubscript{2}O\textsubscript{2} to CRI-G1 cells evokes the activation of a calcium-dependent non-selective cation (NSNAD) channel, which results in the complete and irreversible collapse of the cell membrane potential (17). The NSNAD channel is permeable to calcium (18), and therefore, the possibility that the second, larger rise in [Ca\textsuperscript{2+}], resulted from influx of calcium through the NSNAD channel was examined. Dual calcium imaging and whole-cell current clamp recordings support the contention that the activation of the NSNAD channel by H\textsubscript{2}O\textsubscript{2} is causally related to the late increase in [Ca\textsuperscript{2+}]. There is an excellent temporal coincidence between the cellular depolarization and the late phase of Ca\textsuperscript{2+} influx caused by H\textsubscript{2}O\textsubscript{2}, and removal of extracellular Ca\textsuperscript{2+} prevents the depolarization. Although H\textsubscript{2}O\textsubscript{2} also inhibits K\textsubscript{ATP} currents (most likely due to Ca\textsuperscript{2+} entry initiating K\textsubscript{ATP} current rundown/inactivation) concomitant with the appearance of the non-selective cation current (see Fig. 7A), the overriding influence for cell depolarization is the activation of the NSNAD channel as evidenced by the cell-attached recordings. Furthermore, strong evidence for the NSNAD channel providing the depolarizing driving force is provided by the inhibition of the H\textsubscript{2}O\textsubscript{2}-induced Ca\textsuperscript{2+} influx by 100 \(\mu\)M La\textsuperscript{3+}, a concentration of La\textsuperscript{3+} that was also observed to inhibit the H\textsubscript{2}O\textsubscript{2}-activated macroscopic current as well as the NAD-activated single channel currents. In the absence of a specific inhibitor of the NSNAD channel, we cannot exclude other explanations for the above results, but activation of this permeation pathway appears to be the most parsimonious interpretation. It should be noted that the NSNAD channel appears to be a separate conductance pathway from that activated by incretin hormones and maito-toxin, which has the characteristics of a small conductance (30 pS), calcium-activated, non-selective cation channel (49).

The diabetogenic compound alloxan and H\textsubscript{2}O\textsubscript{2} have been postulated to destroy pancreatic beta cells through DNA damage, which consequently activates nuclear poly(ADP-ribose) synthase, leading to critical depletion of cellular NAD pools (6). This appears to contradict the mechanism proposed above, as cellular NAD is presumably required to activate the NSNAD channel. However, the autofluorescence results indicate that H\textsubscript{2}O\textsubscript{2} causes an immediate oxidation of NAD/PDH, resulting in an increased NAD/NADH ratio, which then declines again within 1 h. Therefore, although the final outcome of H\textsubscript{2}O\textsubscript{2} exposure may well be NAD depletion, the acute effect is an increase in NAD levels, which, coupled with a rise in [Ca\textsuperscript{2+}], may well be sufficient to activate the NSNAD channel, causing the cell to depolarize, to flood with calcium, and ultimately to die.

Consequently, we propose that H\textsubscript{2}O\textsubscript{2} causes a loss of intracellular calcium homeostasis in CRI-G1 cells by inducing a
biphasic rise in [Ca$^{2+}$]i, with the first phase caused by mobilization of intracellular calcium and the second, larger phase due to influx from the extracellular medium. The source of intracellular calcium is not the inositol 1,4,5-trisphosphate- or cyclic ADP-ribose-sensitive endoplasmic reticulum stores, and its identity is as yet elusive. However, the second phase of increased [Ca$^{2+}$]i is consistent with the influx of extracellular calcium permeating through the NS NAD channel. This represents a novel calcium influx pathway activated by oxidative stress. The wider implications of this are unclear as yet, but there have been many studies in which a large influx in calcium has been observed following oxidative stress, but in which the influx pathway has not been identified. For example, Bielefeldt et al. (26) have recently shown that intestinal smooth muscle cells exposed to H$_2$O$_2$ produce a biphasic rise in [Ca$^{2+}$]i, reminiscent of the data presented within. Additionally, their response is insensitive to both depletion of intracellular Ca$^{2+}$ stores and inhibition of voltage-dependent calcium channels. Similarly, the human astrocytoma cell line UC11MG has been shown to respond with a biphasic rise in [Ca$^{2+}$]i to exposure to H$_2$O$_2$, which was also insensitive to calcium channel blockers (50). Therefore, it is possible that these and other unattributed Ca$^{2+}$ influxes resulting from cell death-inducing stimuli are caused by the activation of the NS NAD channel or by a similar non-selective cation channel.

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