Activation of a novel non-selective cation channel by alloxan and H_2O_2 in the rat insulin-secreting cell line CRI-G1

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1. Alloxan and its auto-oxidation product hydrogen peroxide (H_2O_2) irreversibly depolarize insulinoma cells by opening a non-selective cation channel. The channel opened is characterized by a linear current–voltage relation with a conductance of approximately 70 pS and very slow kinetics (of the order of seconds).

2. Cells are protected against the alloxan-induced channel opening and consequent cell depolarization by the presence of H_2O_2 and hydroxyl radical scavengers.

3. The free radical-activated non-selective cation channel is not operative in isolated patches but can be activated by the application of β-NAD^+ to the cytoplasmic aspect of the membrane.

Alloxan causes diabetes mellitus in experimental animals due to its destruction of pancreatic β-cells (Dunn, Sheehan & McLetchie, 1943). Therefore, an understanding of the mechanism of action of alloxan is important in elucidating the causes of diabetes. At present the mechanism of this toxicity is unclear, but there is strong evidence implicating the production of reactive oxygen species (ROS) such as the superoxide anion ($\cdot O_2^-$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($\cdot OH$). *In vivo*, alloxan is easily reduced to dialuric acid, which in the presence of oxygen undergoes autoxidation back to alloxan and generates H_2O_2 and $\cdot O_2^-$. The H_2O_2 reacts with either $\cdot O_2^-$ or Fe^{3+} in cells (Fenton reaction) to produce $\cdot OH$ and it is this highly reactive free radical species that is believed to cause the β-cell damage (Grankvist, Marklund, Sehlin & Täljedal, 1979; Fischer & Hamburger, 1980; Zhang, Öllinger & Brunk, 1995).

The critical sites of attack and molecular mechanisms of the free radical-induced cell damage have yet to be established. Mechanisms currently in favour include the possibility that oxygen free radicals cause DNA strand breaks leading to the activation of poly(ADP-ribose) synthetase, which causes critical depletion of cellular NAD^+ pools resulting in β-cell damage and ultimately death (Okamoto, 1985), or alternatively, ROS have been suggested to cause disruption of the plasma membrane through lipid peroxidation or to attack proteins directly (Gutteridge & Halliwell, 1996).

We now report that alloxan and H_2O_2 cause an irreversible depolarization of these cells by activating a novel non-selective cation channel. This depolarization can be prevented by agents known to protect islet β-cells against ROS and other diabetogenic agents.

METHODS

Cell culture

Cells from the insulin-secreting cell line CRI-G1 were grown in Dulbecco's modified Eagle's medium with sodium pyruvate and glucose, supplemented with 10% fetal calf serum and 1% penicillin–streptomycin at 37 °C in a humidified atmosphere of 95% air–5% CO_2. Cells were passaged at 2–5 day intervals as previously described (Carrington *et al.* 1986), plated onto 3·5 cm Petri dishes (Falcon 3001) and used 1–4 days after plating.

Electrophysiological recording and analysis

Experiments were performed in inside-out and cell-attached patch-clamp configurations for single-channel studies and the whole-cell current-clamp recording mode. Recording electrodes were pulled from borosilicate glass capillaries and had resistances of 8–12 MΩ for both inside-out patches and cell-attached recordings and 1–5 MΩ for whole-cell experiments when filled with electrolyte solution. Currents and voltages were measured using a List EPC-7 amplifier, data were recorded onto digital audiotape and replayed for illustration onto a Gould TA 240 chart recorder. The potential across the membrane is expressed using the usual sign convention, negative inside, and all inward currents, from extra- to intracellular, are shown as downward deflections. In current-clamp experiments, hyperpolarizing current pulses (20 pA and 0·2 s duration) were applied every 5 s to monitor input conductance changes. Typical values for the series resistance during whole-cell recording were 10–18 MΩ. All values in the text are expressed as means ± S.E.M. Statistical significance was tested using the Mann–Whitney *U* test.

For current-clamp experiments, the pipette solution contained (mM): 140 KCl, 0·6 $MgCl_2$, 3·95 $CaCl_2$, 5·0 EGTA, 10 Hepes, at pH 7·2 (free $[Ca^{2+}]$ of 1 μM) or an identical solution but with 2·73 $CaCl_2$ and 10 EGTA (free $[Ca^{2+}]$ of 100 nM). In some experiments 5 mM ATP was also present in the electrode solution. The bath solution consisted of normal saline (mM): 135 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 Hepes, at pH 7·4. Single-channel recordings were

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obtained with normal saline in the pipette and bath. All solution changes were achieved by superfusing the bath with a gravity feed system at a rate of 10 ml min^{-1}, which allowed complete bath exchange within 2 min. All experiments were performed at room temperature, 22–25 °C. All agents mentioned in the text were obtained from Sigma.

RESULTS

Current-clamp recordings from the rat insulinoma cell line CRI-G1 show that following formation of the whole-cell configuration the initial mean resting membrane potential was −42·0 ± 1·3 mV ($n = 10$). Dialysis of the cell with an ATP-free solution resulted in cell hyperpolarization with a concomitant decrease in input resistance which stabilized after 5–10 min at a membrane potential of −70 ± 1·2 mV ($n = 10$). This increase in conductance is due to the run-up of ATP-sensitive K^+ (K_{ATP}) channels as the cytoplasmic ATP is washed out from the cells (Trube, Rorsman & Ohno-Shosaku, 1986). The cells remain at this membrane potential for approximately 45 min before the K_{ATP} channel activity decreases due to channel run-down and the cells slowly depolarize towards a new stable membrane potential of approximately −40 mV (Fig. 1*A*). Application of alloxan (1 mM) and the reducing agent cysteine (1 mM) caused the appearance of an irreversible depolarization to −2·0 ± 0·8 mV ($n = 10$) in agreement with intracellular recordings from mouse pancreatic β-cells (Dean & Matthews, 1972). This effect was observed on every cell tested ($n = 15$). The onset of this depolarization began 10·4 ± 2·1 min following bath application of 1 mM alloxan + 1 mM cysteine, took

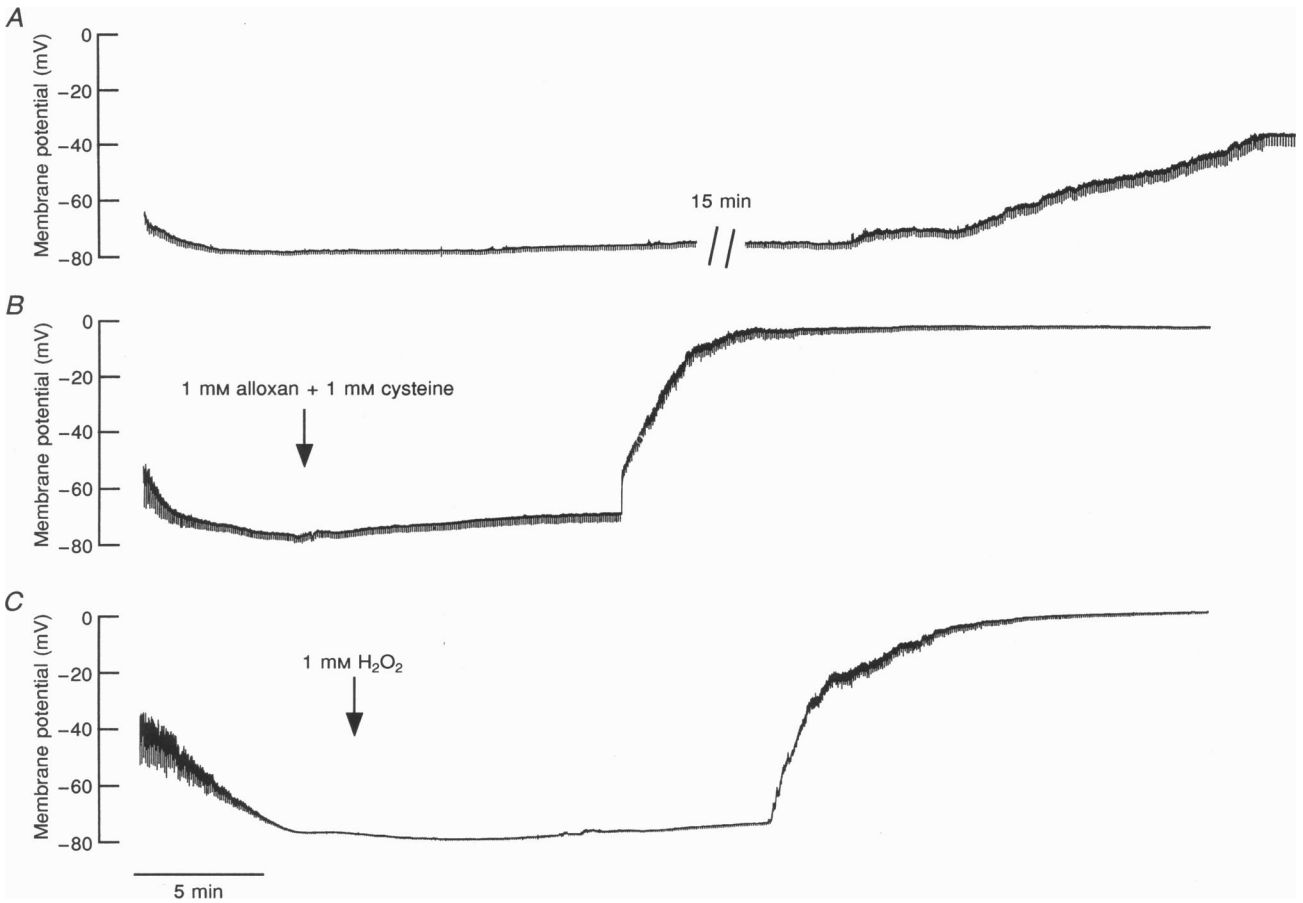

Figure 1. Effects of alloxan and H_2O_2 on CRI-G1 cell membrane potential

A, representative record of the time course of membrane potential change following formation of the whole-cell configuration in the absence of drugs. Note the rapid cell hyperpolarization and increase in conductance due to activation of K_{ATP} channels as ATP washed out from the cell which is well sustained over a 45 min time period, prior to the appearance of a slow depolarization associated with a decreased cellular conductance. The initial resting membrane potential of this cell was −42 mV. The trace begins 5 min following formation of the whole-cell configuration. *B* and *C*, representative recordings of CRI-G1 cell resting membrane potential changes following application of 1 mM alloxan + 1 mM cysteine (*B*) or 1 mM H_2O_2 (*C*) to the bathing solution. In this and subsequent figures the downward arrows indicate initiation of the drug challenge to cells and these agents are therefore present throughout the rest of the recording. Note the appearance of sudden, complete and irreversible depolarization for both treatments. The initial resting membrane potential was −46 mV (*B*) and −35 mV (*C*) and the traces begin 7 and 2 min after formation of the whole-cell configuration, respectively.

8·4 ± 0·9 min to complete and was associated with an increased cellular conductance. Cells remained completely depolarized over the remaining time course of recordings (up to 45 min), whether the 1 mM alloxan + 1 mM cysteine remained in contact with the cells or was washed out following depolarization. This effect was not seen in the absence of 1 mM alloxan + 1 mM cysteine ($n = 8$); i.e. complete depolarization did not occur over an identical time (45 min) period ($P < 0·001$). Longer times were not systematically examined due to the added complication of K_{ATP} channel run-down. Microscopic examination of the cells following 1 mM alloxan + 1 mM cysteine treatment established that cells underwent morphological changes such as membrane shrivelling and blebbing consistent with cell necrosis and ultimately cell death, detaching from the plate some 2–3 h later. These data are consistent with effects of alloxan on pancreatic β-cells and other cell types determined by cell viability tests (Grankvist *et al.* 1979; Zhang & Brunk, 1993; Zhang *et al.* 1995). The morphological changes and resultant cell death were not observed in untreated cells.

Application of H_2O_2 directly onto CRI-G1 cells reproduced the actions of 1 mM alloxan + 1 mM cysteine exactly; i.e induction of irreversible depolarization to approximately 0 mV with a concomitant increase in cellular conductance (Fig. 1*C*), the identical morphological changes and eventual cell death (as determined by detachment of cells from the plate). The delay before onset of irreversible depolarization by H_2O_2 was concentration dependent; 1 mM required 15·5 ± 4·0 min ($n = 4$); 10 mM, 8·7 ± 1·8 min ($n = 6$) and 44 mM,

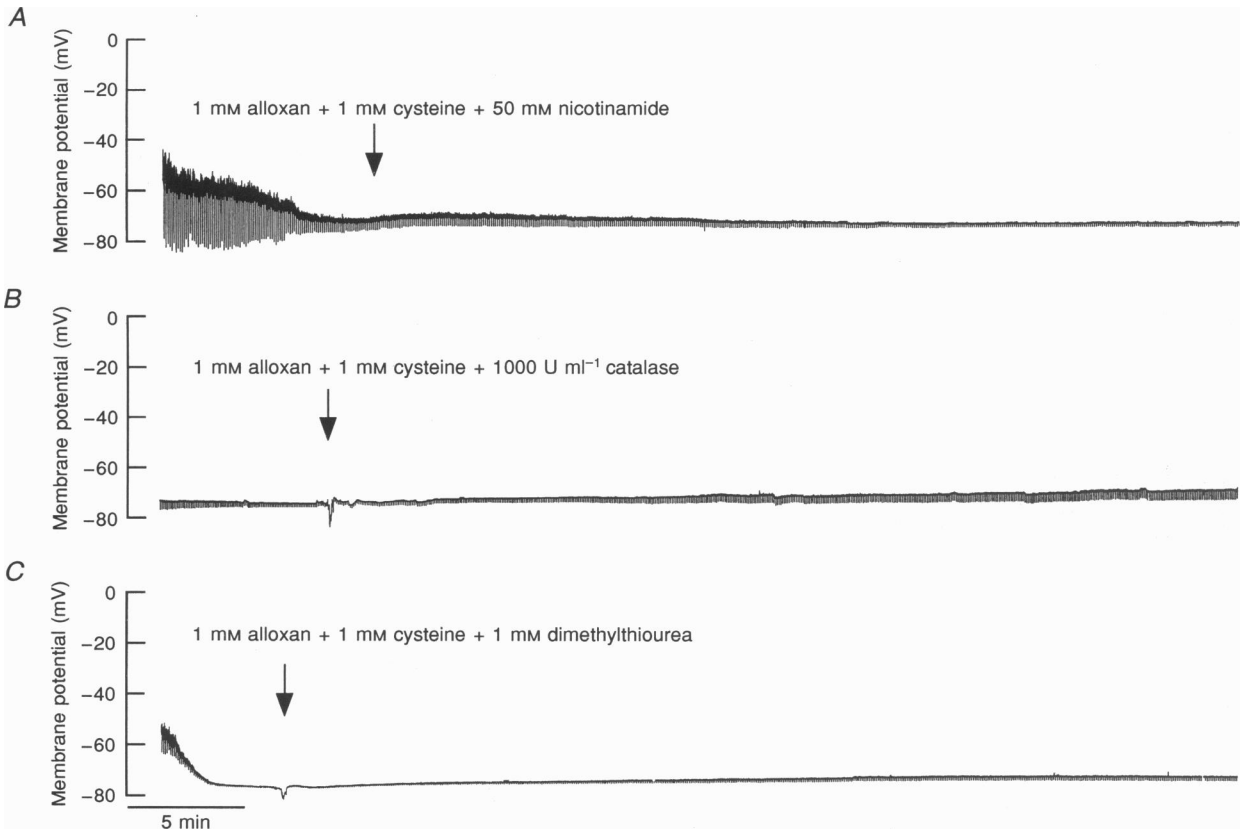

Figure 2. Protection against alloxan-induced cell depolarization

A, resting membrane potential change following formation of the whole-cell configuration and the subsequent application of 1 mM alloxan + 1 mM cysteine + 50 mM nicotinamide. The initial resting membrane potential of this cell was −39 mV. The trace begins 5 min following formation of the whole-cell configuration. Nicotinamide was added to the pipette and bath solutions 30 min prior to and concomitant with the 1 mM alloxan + 1 mM cysteine challenge. *B*, resting membrane potential change following formation of the whole-cell configuration and the subsequent application of 1 mM alloxan + 1 mM cysteine + 1000 U ml^{-1} catalase. The initial resting membrane potential of this cell was −43 mV. The trace begins 8 min following formation of the whole-cell configuration. Catalase was added to the bath solution only and applied at the same time as the 1 mM alloxan + 1 mM cysteine challenge. *C*, resting membrane potential change following formation of the whole-cell configuration and the subsequent application of 1 mM alloxan + 1 mM cysteine + 1 mM dimethylthiourea. The initial resting membrane potential of this cell was −39 mV. The trace begins 4 min following formation of the whole-cell configuration. Dimethylthiourea was added to the bath solution only, 15 min prior to and concomitant with the 1 mM alloxan + 1 mM cysteine challenge.

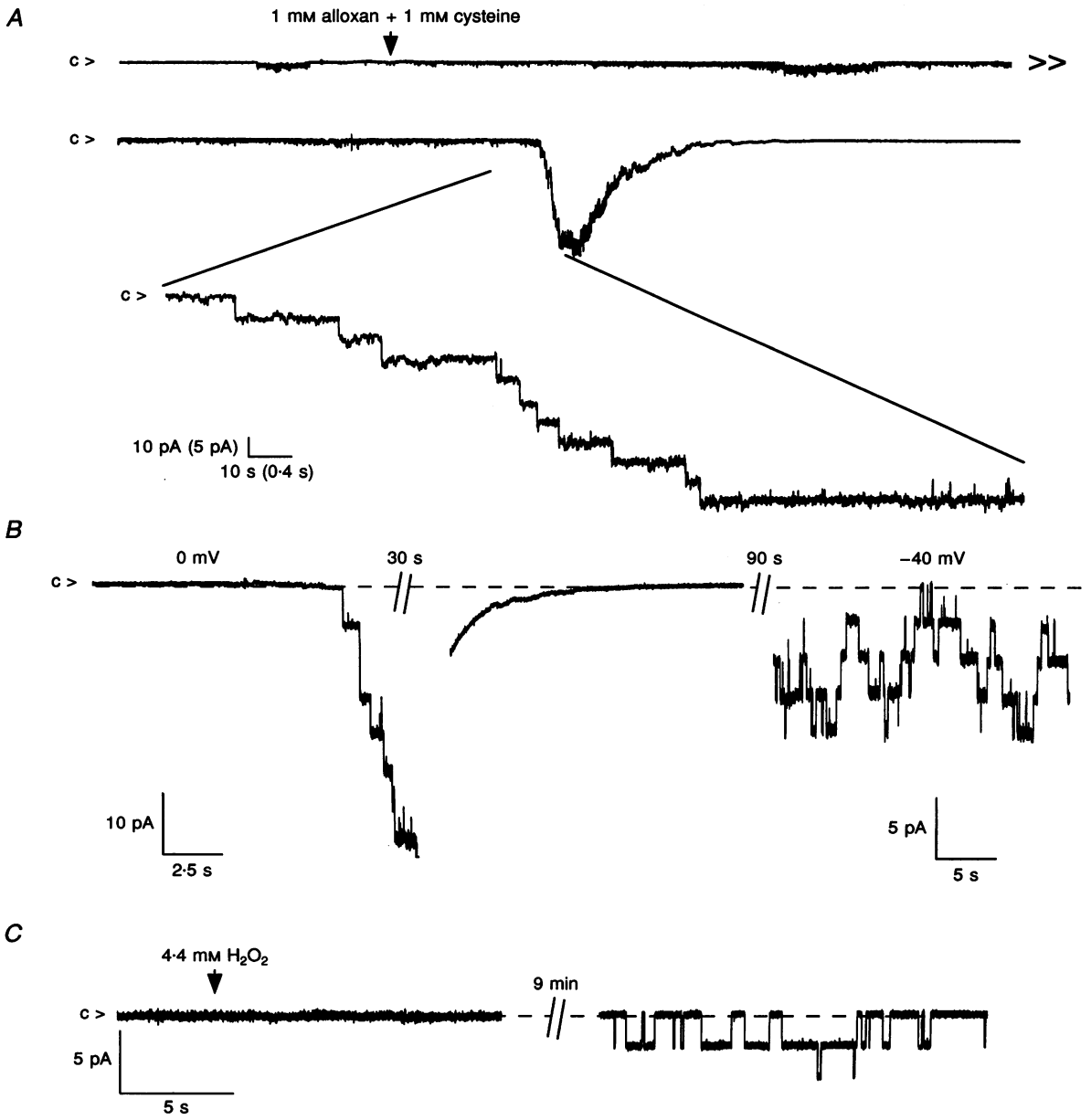

Figure 3. Induction of single-channel currents by alloxan and H_2O_2

A, representative continuous cell-attached recording (no applied pipette potential) illustrating the spontaneous, sudden appearance of an inward current approximately 4 min following exposure of the cell to 1 mM alloxan + 1 mM cysteine. The current peaks within seconds and rapidly declines until no current is again observed. Inset is an expanded trace at higher gain illustrating the rapid recruitment of channels and the nature of the channel currents responsible for the depolarization. The >> denotes the continuity of the trace. *B*, a similar trace, under identical recording conditions, from a separate experiment clearly shows that the channel recruited by the 1 mM alloxan + 1 mM cysteine treatment is still active following depolarization. Following depolarization and loss of single-channel events, application of 40 mV to the pipette results in the re-appearance of single-channel currents indicating that the loss of the inward current was due to the collapse of the cell membrane potential. Note the breaks in the traces. *C*, representative cell-attached recording, with no applied pipette potential, illustrating the appearance of single-channel activity 9–10 min following exposure of the cell to 4·4 mM H_2O_2. Note the similarity (amplitude of current and the long open durations) to the channel opened by 1 mM alloxan + 1 mM cysteine as illustrated in Fig. 3*A* and *B*. Later in the recording, single-channel current amplitude declined until no current activity could be discerned. As was the case for 1 mM alloxan + 1 mM cysteine this was due to the collapsed membrane potential as application of voltage to the pipette interior resulted in the immediate appearance of channel currents indistinguishable from those previously recorded (data not shown). Here, and also in Fig. 4, the 'c' on the left-hand side of the trace indicates the closed state.

3·8 ± 0·7 min ($n = 6$). In time-matched control experiments (membrane potential monitored for 60 min) no spontaneous depolarizations of this nature were observed and for all three concentrations of H_2O_2 the depolarization event was significant ($P < 0·01$). Furthermore, H_2O_2-induced depolarization of CRI-G1 cells was observed at low (100 nM) internal Ca^{2+} concentration ($n = 3$) and also if 5 mM ATP was present ($n = 4$) in the electrode solution (thus preventing K_{ATP} channel run-up and cell hyperpolarization).

The common feature of both alloxan and H_2O_2 action in cells is the production of oxygen free radical species (Cohen & Heikkila, 1974; Halliwell & Gutteridge, 1990). Therefore, we examined the actions of nicotinamide, catalase and dimethylthiourea (DMTU), agents known to protects islet β-cells from the deleterious effects of a variety of cytotoxic factors including alloxan (Fox, 1984; Pipeleers & Van de Winkel, 1986; Zhang *et al.* 1995). Nicotinamide delayed the onset of depolarization in response to 1 mM alloxan + 1 mM cysteine (Fig. 2*A*); using 5 and 50 mM nicotinamide depolarization occurred after 29·2 ± 5·3 min ($n = 8$; $P < 0·01$ compared with 1 mM alloxan + 1 mM cysteine alone) and 41·9 ± 2·1 min ($n = 8$; $P < 0·001$), respectively. Complete protection of the effects of 1 mM alloxan + 1 mM cysteine were not observed in these experiments. We also examined whether alloxan action is mediated through the production of H_2O_2 as an intermediate in the alloxan-induced generation of free radicals (Grankvist *et al.* 1979; Zhang & Brunk, 1993) by adding catalase (1000 U ml^{-1}) in the presence of alloxan. Catalase was shown to afford complete protection (Fig. 2*B*) over the time course (> 45 min) of the electrophysiological experiments ($n = 5$; $P < 0·001$). Addition of the hydroxyl radical scavenger DMTU (1 mM) also afforded complete protection ($n = 5$; $P < 0·001$) to the deleterious effects of

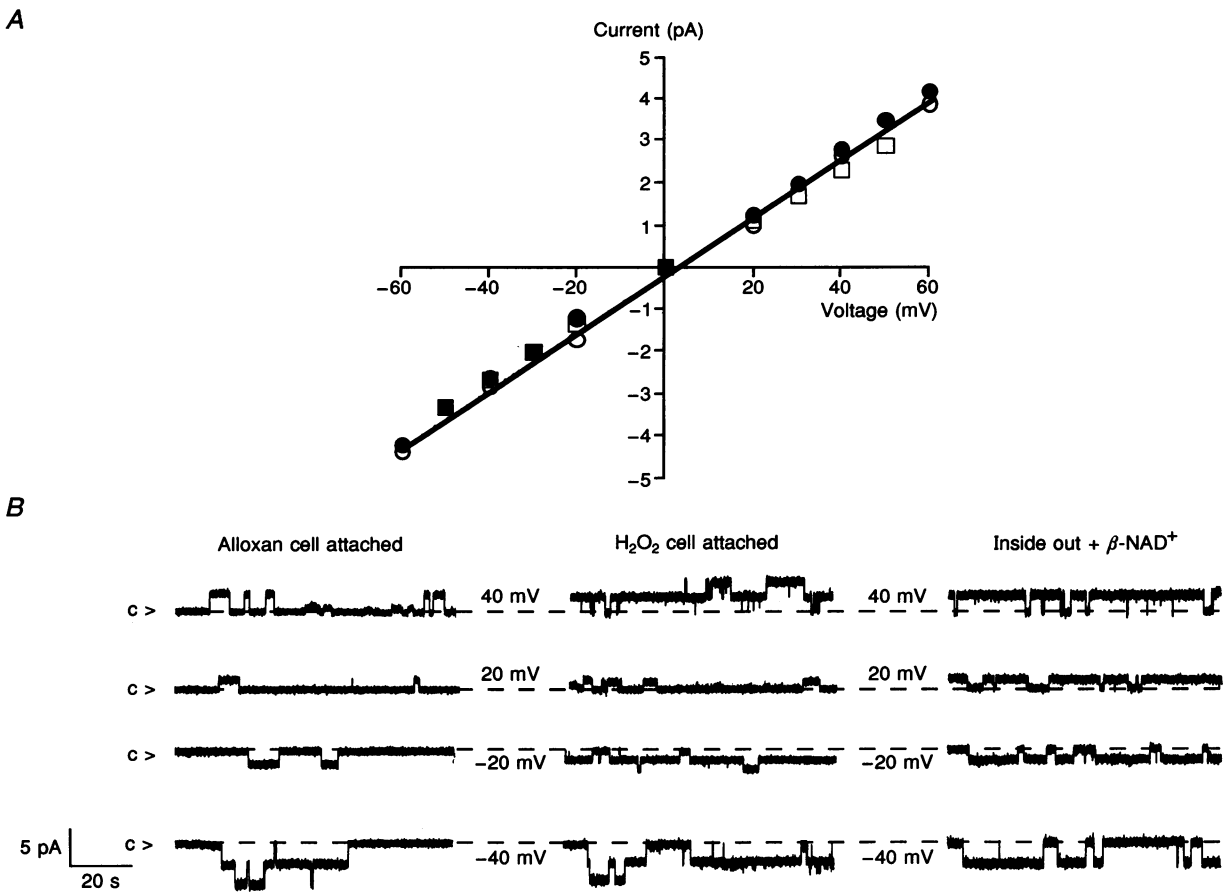

Figure 4. Characteristics of single-channel currents recorded in cell-attached and inside-out mode

A, single-channel current–voltage relations for the channels activated by 1 mM alloxan + 1 mM cysteine (○) from the experiment in Fig. 3*A*; 4·4 mM H_2O_2 (□) from the experiment in Fig. 3*C* in cell-attached recordings following cell depolarization, and for the channel activated by 0·5 mM β-NAD^+ (●) in inside-out patches from CRI-G1 cells. Straight lines show the best fits having slope conductances of 74, 68 and 72 pS, respectively. *B*, representative traces of single-channel currents recorded from CRI-G1 cells at different membrane potentials (given to the side of the traces) showing an example of currents recorded cell attached following cell depolarization by 1 mM alloxan + 1 mM cysteine (same cell as Fig. 3*A*), an example of currents recorded cell attached following cell depolarization by 4·4 mM H_2O_2 (same cell as Fig. 3*C*) and an example of single-channel currents recorded from an excised inside-out patch following the addition of 0·5 mM β-NAD^+ to the bath solution.

1 mM alloxan + 1 mM cysteine (Fig. 2C). Furthermore, the morphological changes and resultant cell death did not occur or were substantially delayed in cells treated with 1 mM alloxan + 1 mM cysteine in the presence of nicotinamide, catalase or DMTU in agreement with published data (Fox, 1984; Pipeleers & Van de Winkel, 1986; Zhang *et al.* 1995).

In order to establish the nature of the ionic conductance underlying the irreverible depolarization caused by both alloxan and H_2O_2, cell-attached single-channel recordings were performed. The electrode solution contained normal saline to reduce contamination of the recordings by K_{ATP} channel currents. Consistent with the whole-cell membrane potential data, there was a delay of approximately $4{\cdot}3 \pm 0{\cdot}8$ min ($n = 8$) following the addition of 1 mM alloxan + 1 mM cysteine before the development of an inward current (Fig. 3A). The emergence of this current was often sudden and on occasion many channels appeared to be activated over a very short time period (< 30 s). This current reached a peak and declined again until the patch was effectively silent once more. However, this was simply due to the complete collapse of the cell resting membrane potential (Fig. 1B) and the consequent decrease in driving force for current flow. This was shown by application of voltage to the recording pipette whereupon discrete channel events were observed again (Fig. 3B). Under these conditions channel activity was detected for as long as recordings were made thereafter (approximately 20 min). Application of 1 mM alloxan + 1 mM cysteine induced the appearance of this inward current in fifteen out of twenty cell-attached patches examined. Similar data were obtained following application of H_2O_2 (4–44 mM), which activated an inward current in sixteen out of twenty-three separate experiments (Fig. 3C). The channel activated by application of H_2O_2 was essentially indistinguishable in amplitude and time course to that opened by 1 mM alloxan + 1 mM cysteine (Figs 3A–C and 4B).

Construction of current–voltage relations, following the collapse of the cell membrane, indicate that the channel is characterized by a linear relation with a reversal potential of 0 mV under both conditions for activation (Fig. 4A). The single-channel conductance was $68{\cdot}8 \pm 1{\cdot}1$ pS ($n = 3$) and $72{\cdot}0 \pm 2{\cdot}7$ pS ($n = 5$) for 1 mM alloxan + 1 mM cysteine and H_2O_2 activation, respectively. Excision of the patch from the cell following activation of this channel resulted in the complete and immediate cessation of channel activity (data not shown). Direct application of 1 mM alloxan + 1 mM cysteine ($n = 5$) or 44 mM H_2O_2 ($n = 10$) to the cytoplasmic aspect of inside-out membrane patches did not cause restoration of channel activity. However, application of β-NAD^+ (0·1–1 mM) to the bathing solution resulted in the appearance of a channel with properties indistinguishable from those of the channel described above (Fig. 4B). This β-NAD^+-activated (NS_{NAD}) channel was also characterized by long open durations (seconds), a linear current–voltage relationship with a slope conductance of $73{\cdot}8 \pm 0{\cdot}5$ pS ($n = 13$) and a reversal potential of 0 mV (Fig. 4A and B). The channel described in both recording configurations is a non-selective cation channel for the following reasons. In cell-attached recordings the electrode contained normal saline (NaCl) and the predominant cation intracellularly is potassium and in inside-out patches the membrane was exposed to normal saline on both sides. Further experiments with cation and anion substitutions using inside-out patches clearly indicate that the NS_{NAD} channel is permeable to monovalent cations such as Na^+, K^+ and Cs^+ and has an appreciable permeability to Ca^{2+} but is impermeable to Cl^- (Reale, Hales & Ashford, 1994; P. S. Herson, K. A. Dulock & M. L. J. Ashford, unpublished observations). Therefore, we conclude that the channel activated by alloxan and H_2O_2 in intact cells is the same as that opened by β-NAD^+ in inside-out patches. Indeed, the frequency that the NS_{NAD} channel is detected in inside-out patches (71%) is virtually identical to that observed for the alloxan- or H_2O_2-induced channel in cell-attached recordings.

DISCUSSION

In agreement with previous intracellular recordings from mouse pancreatic β-cells (Dean & Matthews, 1972) we have shown that alloxan causes complete and irreversible collapse of the membrane potential of CRI-G1 insulin-secreting cells. However we now demonstrate that this membrane depolarization is caused by the opening of a previously quiescent novel non-selective cation channel.

Alloxan cytotoxicity is considered to be mediated by the production of oxygen free radicals (Cohen & Heikkila, 1974; Okamoto, 1985). Alloxan is reduced extracellularly to dialuric acid in the presence of a reducing agent (e.g. cysteine) and oxidation of dialuric acid in the presence of oxygen results in the production of both the superoxide anion and hydrogen peroxide, the latter of which can diffuse across the plasma membrane and into the cell interior (Zhang *et al.* 1995). Neither of these molecules are overly reactive, but they, in turn, lead to the production of the highly reactive hydroxyl radical (Halliwell & Gutteridge, 1990; Gutteridge & Halliwell, 1996). In order to demonstrate the link between alloxan-induced channel activation and oxygen radical production the effects of hydrogen peroxide were also examined. Application of H_2O_2 to CRI-G1 cells mimicked the effects of alloxan exactly, causing activation of a channel with identical characteristics to that induced by alloxan, as well as cellular depolarization. The enzyme catalase, which catalyses the removal of H_2O_2 extracellularly, has been shown to protect against the deleterious effects of alloxan on pancreatic β- and other cells (Zhang & Brunk, 1993; Zhang *et al.* 1995). Catalase completely protected CRI-G1 cells against alloxan-induced depolarization further substantiating the premise that alloxan action in these cells is through the production of H_2O_2. Furthermore, dimethylthiourea, which has been shown to protect cells via effective scavenging of hydroxyl radicals (Fox, 1984), also prevented alloxan-

induced depolarization, implicating the hydroxyl radical as the final insulting molecule. Nicotinamide has been used to prevent β-cell damage mediated by alloxan (Pociot, Reimers & Andersen, 1993) and is purported to prevent the onset of diabetes in non-obese diabetic mice (Nomikos, Prowse, Carotenuto & Lafferty, 1986) by scavenging oxygen free radicals (Wilson, Patton, McCord, Mullins & Mossman, 1984). This agent, although not conferring complete protection against the oxidative insult presented by alloxan, did significantly delay the onset of irreversible depolarization. Thus the data presented herein are consistent with the notion that alloxan, through the creation of H_2O_2 and consequent intracellular production of hydroxyl free radicals, causes the complete and irreversible depolarization of CRI-G1 cells which ultimately results in cell death (Zhang & Brunk, 1993; Zhang *et al.* 1995).

The spontaneous depolarization of the CRI-G1 cells elicited by alloxan is mediated through the activation of a previously quiescent cation-selective channel. The channel observed in cell-attached recordings appears to be identical to a channel selectively activated by β-NAD^+ in inside-out patches from this cell line. Both channels present a number of unique features being characterized by a fairly large single-channel conductance and very long stable open events having a duration of seconds with few closures during an opening burst. Reale *et al.* (1994) have shown, using inside-out patches from CRI-G1 cells, that the latter channel (NS_{NAD}) is impermeable to Cl^- and permeable to Na^+ and K^+ and more recently we have demonstrated significant permeability to Cs^+ and Ca^{2+} ions (P. S. Herson, K. A. Dulock & M. L. J. Ashford, unpublished observations). Activation of such a channel would, therefore, result in massive sodium and calcium influx resulting in collapse of the membrane potential. Interestingly, oxidant stress has also been shown to cause depolarization of calf pulmonary artery endothelial cells by activation of a non-selective cation channel. However, this channel is not identical to the one described herein as it has a lower conductance (30 pS), much faster kinetics, is active when excised from the cell and is also activated by application of oxidized glutathione to the cytoplasmic aspect of excised patches (Koliwad, Elliot & Kunze, 1996), an effect not observed for the channel we have desribed in CRI-G1 cells (P. S. Herson & M. L. J. Ashford, unpublished observations).

Oxidative stress, the production of H_2O_2, and the generation of hydroxyl radicals in cells has been implicated in numerous reports as a mechanism of cell death in a number of disease states; for example ischaemic reperfusion injury and some neurodegenerative diseases (Gutteridge, 1993; Gutteridge & Halliwell, 1996). Previously lipid peroxidation has been a favoured theory by which cellular integrity has been breached. We now propose another possible explanation, which is that the action of oxidative stress is much more selective and subtle via the activation of specific ion channels. The novel free radical-activated cation channel we report represents a new pathway of free radical toxicity. Therefore, such a channel is potentially a valuable pharmacological target not only for the treatment and/or prevention of non-insulin-dependent diabetes mellitus, but perhaps other oxidative stress-induced pathologies.

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