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Free Radical Modulation of Insulin Release in INS-1 Cells Exposed to Alloxan

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ABSTRACT. Generation of free radicals is thought to mediate the cytotoxic action of alloxan on the pancreatic β-cell. In this investigation, the early effects of alloxan on cell function were studied. When INS-1D insulinoma cells were exposed to alloxan (1 mM) for 45 min followed by a 3-hr recovery period, the drug increased basal insulin release while abolishing the effect of glucose in static incubations. This was associated with impaired stimulation of cellular metabolism by glucose and reduced viability, both monitored colorimetrically with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). These alterations were largely counteracted by the antioxidant butylated hydroxyanisol (BHA). Similar changes occurred when glucose was added directly after 5 min of alloxan treatment, whereas KCl-induced secretion was only partially inhibited. In perifusion, alloxan caused transient insulin secretion to 50% of the rates obtained with glucose 30 min later. Under these conditions, epinephrine abolished the stimulation due to both agents. Membrane potential and cytosolic calcium concentrations ($[Ca^{2+}]_i$) were recorded to clarify the action of alloxan. Alloxan-induced insulin release correlated with depolarization of INS-1D cells and a rise in $[Ca^{2+}]_i$. Alloxan did not augment $[Ca^{2+}]_i$ in the presence of BHA or the absence of extracellular calcium. Nickel chloride blocked the effect of alloxan on $[Ca^{2+}]_i$, whereas verapamil was ineffective. This suggests that alloxan promotes Ca^{2+} influx through channels distinct from L-type channels, perhaps through non-selective cation channels. Thus, alloxan causes changes in INS-1D cells prevented by antioxidant treatment, suggesting that free radicals may modulate the ionic permeability leading to functional activation. BIOCHEM PHARMACOL **57**;6:639–648, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. alloxan; diabetes; INS-1D cells; free radicals; insulin secretion; calcium

The diabetogenic compound alloxan interacts specifically with the pancreatic β-cells, but its precise mode of action is still a matter of debate [1, 2]. Previous *in vivo* studies and later *in vitro* experiments using isolated rodent islets have prompted two hypotheses concerning the cytotoxic effect of the drug. First, due to its chemical instability in physiological solutions, alloxan is thought to generate both intra- and extracellular reactive oxygen species (free radicals) which are responsible for its toxic effect [3–5]. The deleterious actions of free radicals on the β-cells may be due either to direct damage to cellular structures such as nucleic acids, membrane lipids, and proteins, or indirectly, to depletion of essential nucleotides (NAD, ATP) as a consequence of free radical actions on key metabolic functions [6–9]. Second, alloxan, which shares structural similarities with glucose, has been suggested to compete for the sugar-binding site on glucokinase (EC 2.7.1.1). This latter hypothesis stems both from *in vivo* observations showing that glucose infusions protect animals against the diabetogenic action of alloxan, as well as pharmacological investigations using chemical analogs of alloxan on the enzymatic activity of purified glucokinase [10, 11]. Whereas alloxan-induced destruction of β-cells by free radicals is a progressive event occurring over a period of hours, it cannot entirely be ruled out that alloxan may bind rapidly to glucokinase and cause an early inhibition of glucose-induced insulin release. Thus, the explanations are not mutually exclusive, and each may adequately apply to the given experimental condition.

The aim of the present study was to develop a model for studying the mechanisms of action of alloxan by using insulin-secreting INS-1D cells. Compared to normal β-cells, INS-1 cells retain a differentiated β-cell phenotype with respect to glucose transport and metabolism, which are important functions for normal metabolism–secretion coupling [12, 13]. We have previously shown that INS-1 cells are sensitive to the effect of alloxan, which is in contrast to what has been reported for the less differentiated RINm5F

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cells [14]. Alloxan may cause irreversible breaks in DNA, and any effects observed within hours after exposure to the drug could theoretically be due to altered gene expression. The study was thus also oriented towards the exploration of early effects evoked by alloxan on INS-1 cells, as evaluated by the viability, metabolic rate, and secretory function of the cells. In addition, the mechanism of action of the diabetogenic drug was studied by recording the immediate changes in cell depolarization and variations in $[Ca^{2+}]_i$* induced by subtoxic doses of alloxan. Information on the latter parameter is crucial, as increases in $[Ca^{2+}]_i$ constitute the main triggering event in the exocytosis of insulin [15].

MATERIALS AND METHODS

Cell Culture and Materials

Novel sublines from parental INS-1 cells were established by high dilution of the cells in Petri dishes with cloning wells. Two clones, INS-1D and INS-1E, were selected based on their higher insulin secretion responses to glucose in the range 5–20 mM than the parental INS-1 cells [12] while displaying similar morphology. In the present study, the INS-1D cells (passages 46–70) were used and cultured in the CM composed of RPMI 1640 supplemented with 10 mM HEPES, 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol as originally described [12].

Reagents commonly used for the experiments were from Sigma Chemical Co., Fluka, or Merck. Alloxan and BHA were from Sigma and ethidium bromide, fura-2 acetoxymethylester, and bis-(1,3-dimethyl thiobarbiturate)trimethine-oxonol (bisoxonol) from Molecular Probes.

MTT Reduction Assay

Cell viability, or cellular metabolic rate, was examined by the MTT colorimetric assay as originally developed for INS-1 cells using short time incubations (30 min) [14].

Insulin Secretion: Static Incubation

Cells (2×10^4 cells/well in 96-well microtiter plates) were seeded and cultured for 3 days in CM. For the experiments, the cells were washed and incubated in modified KRBH composed of 140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 1.5 mM $CaCl_2$, 10 mM HEPES, 2 mM $NaHCO_3$, 0.1% BSA, and 2 mM D-glucose. Insulin secretion and cellular insulin content extracted with acid–ethanol [12] were determined by radioimmunoassay using rat insulin as a standard and an antiserum from Linco [12, 14]. Since alloxan is a highly reactive substance, the alloxan powder was dissolved in citrate buffer (pH 4.0) immediately prior to use in each experiment. The final concentration of citrate under either test or control conditions was 0.1 mM. INS-1D cells were exposed to alloxan by incubation in KRBH without BSA to minimize the interaction between alloxan and the sulfhydryl groups of albumin. BHA was freshly solubilized in ethanol and the final ethanol concentration in the culture medium or the KRBH never exceeded 0.1%. Ethanol at a concentration of 0.1% was present under all control conditions. BHA was not present during the insulin secretion test period in Figs. 1 and 2.

Cell Permeabilization Assay

Cell permeabilization was tested by monitoring the changes in fluorescence of the DNA binding probe ethidium bromide [16]. Briefly, after a 2- to 3-hr spinner culture, 2×10^6 INS-1 cells were placed in a cuvette of an LS-50B fluorimeter with 2 mL KRBH containing 40 μg/mL ethidium bromide. Cells were then excited at 518 nm and emission was recorded at 605 nm. As a positive control of cell permeabilization, 20 μM digitonin was added at the end of each trace.

Insulin Secretion: Perifusion

Cells were kept in spinner culture for 3 hr in RPMI 1640 supplemented with 25 mM HEPES and 1% newborn bovine serum prior to being perifused in KRBH. Cells were placed in a small chamber (10^6 cells/chamber) and perifused at a flow rate of 1 mL/min [12].

Measurements of cytosolic Ca^{2+}

INS-1D cells (10^5) were seeded onto glass coverslips (21 × 26 mm) precoated with poly-L-ornithine and cultured in CM for 3 days. Cells were loaded with 1 μM fura-2 acetoxymethylester for 30–40 min in the CM. Single-cell $[Ca^{2+}]_i$ was measured in a microfluorimeter system at 37° as detailed elsewhere [17].

Measurement of Membrane Potential

Membrane potential was estimated by a fluorimetric method using bisoxonol as a probe [17]. After a 2- to 3-hr spinner culture, 10^6 INS-1D cells were placed in a cuvette of an LS-50B fluorimeter with 2 mL KRBH containing 100 nM bisoxonol. Membrane potential was recorded by monitoring the fluorescence at excitation and emission wavelengths of 535 nm and 570 nm, respectively.

Statistical Analysis

The results are means ± SEM of independent experiments. The differences between groups were analyzed by the

* *Abbreviations:* BHA, butylated hydroxyanisol; bisoxonol, bis-(1,3-dimethyl thiobarbiturate)trimethine-oxonol; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; CM, complete medium; KRBH, Krebs–Ringer bicarbonate HEPES buffer; and MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

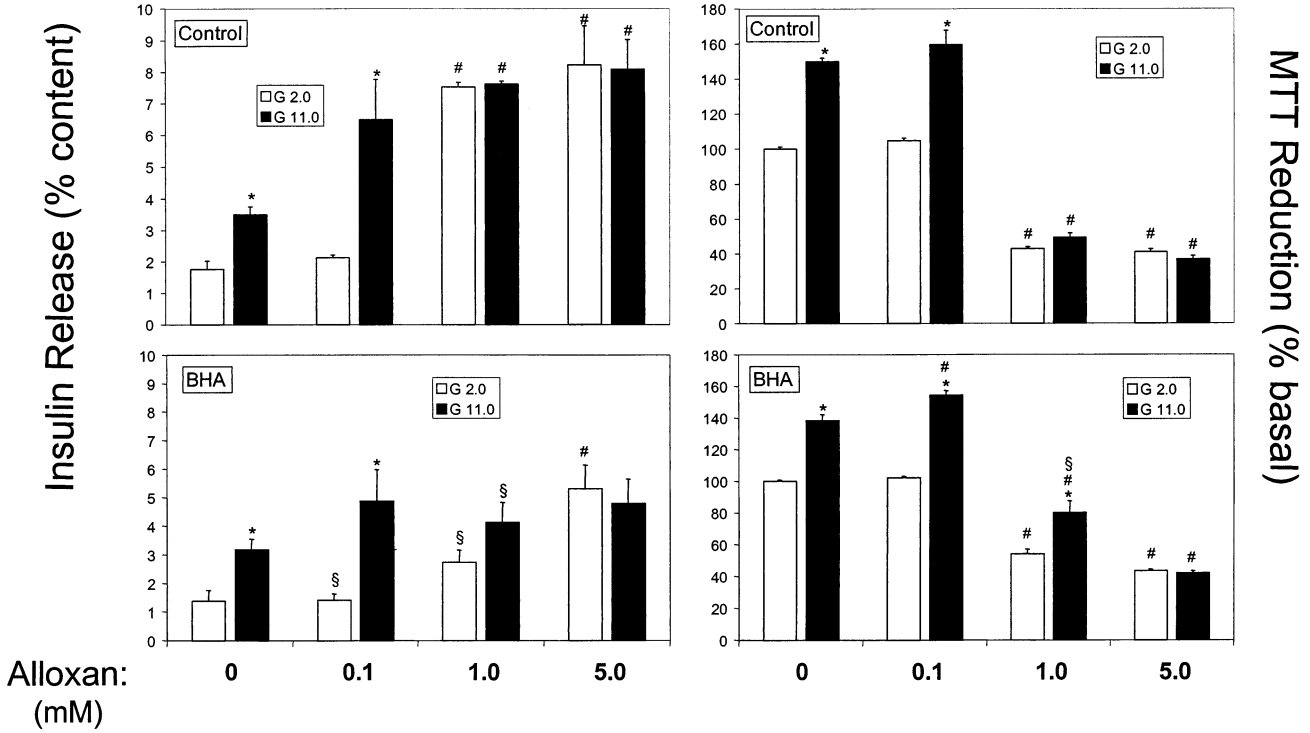

FIG. 1. Effects of alloxan (45-min exposure) and of the antioxidant BHA on insulin secretion and on cell viability/metabolism. INS-1D cells were cultured for 3 days in 96-well plates as described in Methods. The cells were incubated for 45 min in BSA-free KRBH at 2 mM glucose in the presence of different alloxan concentrations and in the absence (upper panels, control) or presence of 100 μM BHA (lower panels, BHA). The cells were returned to culture conditions for 3 hr in the absence or presence of BHA before being tested in KRBH for insulin secretion. The cells were preincubated for 30 min at 2 mM glucose and subsequently incubated for 30 min in the presence of basal 2 mM glucose (G 2.0) or 11 mM (G 11.0) before the incubations were continued in the presence of MTT for 30 min. Results are means ± SEM of 3–4 separate experiments: *$P < 0.05$ versus G 2.0; #$P < 0.05$ versus corresponding untreated group (0 mM alloxan); and §$P < 0.05$ versus corresponding control group (without BHA).

Student's *t*-test for unpaired data. For Fig. 4, the areas under the curve were calculated after subtraction of the baseline.

RESULTS

An initial series of experiments was performed to evaluate the sensitivity of INS-1D cells to the deleterious effect of alloxan after exposure to increasing concentrations of the drug. The cells were treated for 45 min with alloxan (0.1–5 mM) and then returned to cell culture for 3 hr prior to the assessment of glucose-stimulated insulin secretion. In addition, the metabolic rate of the cells was evaluated by the MTT method under parallel conditions. As shown in Fig. 1 (upper panels), exposure of INS-1D cells to alloxan induced a dose-related loss of viability and a parallel inhibition of glucose-induced insulin release. The impairment of cell metabolism was seen both as a reduction in the absolute levels of MTT and as an inhibition of the action of 11 mM glucose to cause the generation of reducing equivalents through its metabolism [13, 14]. At a low concentration (0.1 mM), alloxan had no detectable effect, while 1 and 5 mM alloxan markedly increased basal insulin secretion. Under these conditions, glucose (11 mM) failed to further stimulate insulin release above basal (2 mM glucose). Similar results were obtained using other nutrient secretagogues such as leucine or pyruvate, the end product of glycolysis (data not shown). To validate the hypothesis that alloxan exerts its effect by generating oxygen-free radicals, parallel experiments were performed where the cells were protected by the phenolic compound BHA. As shown in Fig. 1 (lower panels), BHA (100 μM) counteracted the deleterious actions of 1 mM alloxan on basal insulin secretion ($P < 0.001$) and glucose-induced MTT reduction ($P < 0.05$). It should be noted that BHA by itself had no effect on insulin release at basal or stimulatory glucose concentrations. These control experiments were reproduced repeatedly and are not included systematically in the following results.

Alterations caused by 5mM alloxan were not reversible by the antioxidant. Therefore, in all further experiments reported in the present study, alloxan was used at a concentration of 1 mM. Prolonged exposure of INS-1D cells to alloxan (45 min) followed by a 3-hr incubation period caused rounding up of the cells and detachment from the substratum (not shown). This was also reflected by the changes in insulin contents measured after the incubation periods. Insulin content (ng/well): control = 283.8 ± 63.2; alloxan = 149.8 ± 21.4; 1 mM alloxan + BHA = 159.4 ± 31.4 (means ± SEM of 6 separate experiments). Based on these observations, we investigated whether the effect of

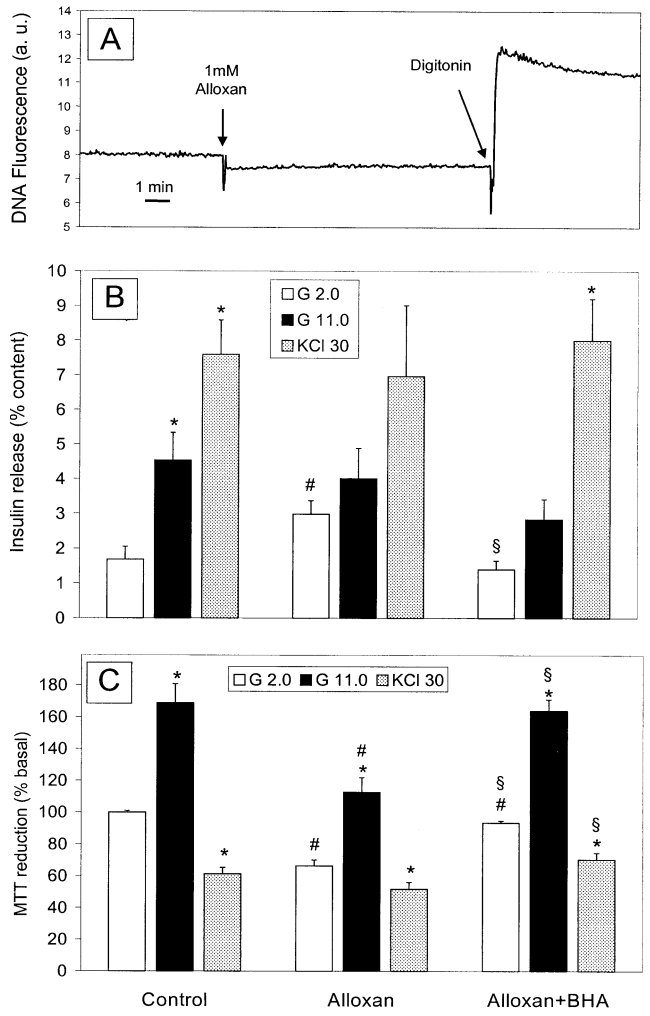

FIG. 2. Effects of short exposure (5 min) to alloxan and to the antioxidant BHA on insulin secretion and cellular metabolic rate (MTT reduction). Cell integrity following alloxan treatment was monitored using ethidium bromide fluorescence, and digitonin (20 μM) was used as a positive control for cell permeabilization (A). For insulin release (B) and MTT reduction assay (C), INS-1D cells were cultured for 3 days in 96-well plates as described in Methods. The cells were preincubated for 30 min in KRBH containing 2 mM glucose and then sequentially treated and incubated as follows: 5-min exposure to 1 mM alloxan in a BSA-free KRBH (2 mM glucose), washes, 45-min incubation in KRBH in the presence of 2 mM glucose (G 2.0), 11 mM (G 11.0), or 30 mM KCl (with basal 2 mM glucose), and sampling of an aliquot for insulin measurements (B) before continuing the incubations in the presence of MTT for 30 min (C). Numbers are means ± SEM of 3–5 separate experiments: *$P < 0.05$ versus G 2.0; #$P < 0.05$ versus corresponding control group (0 mM Alloxan); and §$P < 0.05$ versus corresponding alloxan group (without BHA).

alloxan could be detected shortly after exposure to the drug. INS-1D cells were briefly (5 min) treated with alloxan (1 mM) immediately before the static incubation for the measurement of insulin secretion. Under these experimental conditions, alloxan treatment did not result in cell permeabilization, as demonstrated by the absence of any increase in ethidium bromide fluorescence (Fig. 2A). On the contrary, and as a positive control of cell permeabilization, digitonin (20 μM) rapidly increased the DNA-dependent fluorescence. Glucose-stimulated insulin release and KCl-induced insulin release were selected to assess the function of INS-1D cells in parallel to the metabolic rate measured as MTT reduction for 30 min (Fig. 2B and C). This approach was felt to be more appropriate to study the mechanism of alloxan, since DNA damage and other long-term effects of the drug could complicate the interpretation of the results. In contrast to the preceding results for prolonged exposure periods, the insulin contents of INS-1D were as follows: (ng/well): control = 177 ± 32; 1 mM alloxan = 142 ± 52; alloxan + BHA = 170 ± 27 (means ± SEM of 3 separate experiments).

As shown in Fig. 2B, 1 mM alloxan altered glucose-induced insulin release mainly by increasing the insulin secretion at basal glucose concentrations (1.8-fold, $P < 0.05$), while the insulin release at stimulatory glucose concentrations remained unchanged by alloxan treatment. KCl induced a 4.5-fold increase in insulin release under control conditions ($P < 0.001$). When the INS-1 cells were protected by BHA, glucose-induced insulin release was ameliorated but not significantly restored (2-fold, $P = 0.08$), whereas KCl-induced insulin release was normal (5.7-fold, $P < 0.01$). The observed changes in insulin release evoked by glucose were paralleled by changes in MTT reduction (Fig. 2C). However, at basal glucose concentrations, alloxan decreased MTT reduction (−34%, $P < 0.001$) despite a preserved glucose (11 mM)-enhanced cell metabolism (+70%, $P < 0.01$). It is interesting to note that under control conditions, KCl-evoked insulin release was not accompanied by an increase in cell metabolism; rather, KCl provoked a decrease in MTT reduction which correlated with a reduction in cytosolic ATP levels.* It should be noted that under these conditions KCl acts primarily by membrane depolarization without a substantial increase in oxidative metabolism, and insulin secretion is the consequence of Ca^{2+} influx through voltage-gated calcium channels [15]. As for KCl-evoked insulin secretion, the cells were protected against the deleterious effect of alloxan as demonstrated by the results of the MTT assay (Fig. 2C).

The reproducible experimental model of short-term exposure (5 min) to intermediate concentrations of alloxan (1mM) allowed us to conduct other experiments aimed at understanding the mechanism of action of the drug. Using this model, different antioxidant compounds thought to act on lipid peroxidation were tested, and the results are given in Fig. 3 (for the mechanism of action of these antioxidants, see Ref. 18). Under similar experimental conditions as those in Fig. 2, alloxan at basal glucose concentrations induced a 50% stimulation ($P < 0.05$) of insulin secretion, which was accompanied by a proportional decrease in MTT reduction (−33%, $P < 0.02$). Whereas glucose-induced insulin secretion was no longer observed after alloxan

*Maechler P and Wollheim CB, unpublished observations.

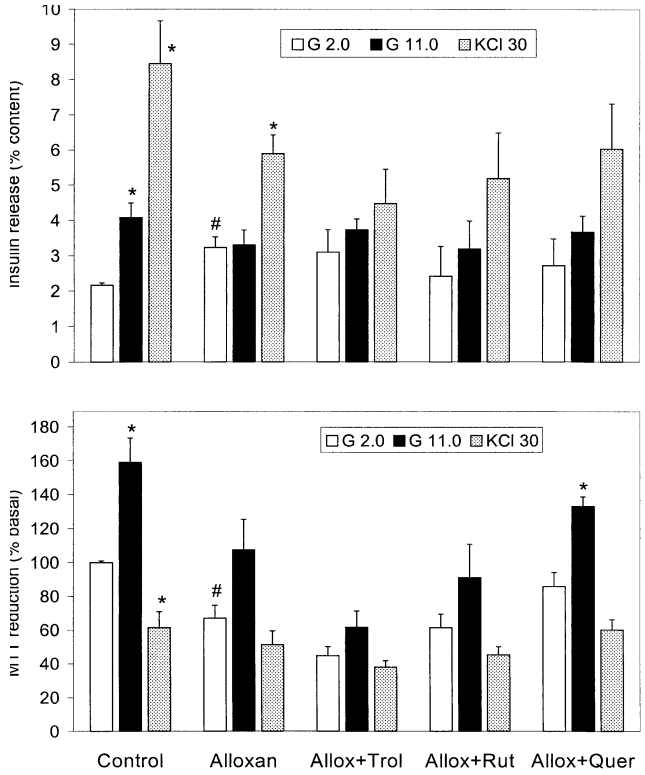

FIG. 3. Insulin release and viability of INS-1D cells after alloxan exposure: protection by antioxidants. INS-1D cells were cultured for 3 days in 96-well plates as described in Methods. The cells were incubated for 5 min in BSA-free KRBH at 2 mM glucose in the absence (control) or presence of 1 mM alloxan (alloxan). The cells were then incubated for 45 min under the following conditions: KRBH at basal (2 mM glucose; G 2.0) or stimulatory glucose concentrations (11 mM glucose; G 11.0), or stimulatory KCl concentrations (30 mM KCl) at basal 2 mM glucose. Where specified, the antioxidant compounds trolox (Trol; 0.5 μM), rutin (Rut; 50 μM), and quercitin (Quer; 5 μM) were present only during the initial 5-min exposure to alloxan. The results are means ± SEM of 3 separate experiments: *$P < 0.05$ versus G 2.0; #$P < 0.05$ versus corresponding control group.

treatment, KCl-induced insulin release was only partially affected by the drug (1.8-fold, $P < 0.02$). None of the tested antioxidant compounds was as effective as BHA in protecting against alloxan action (described above in Figs. 1 and 2). Trolox (a water-soluble derivative of vitamin E) had (at 0.5 μM) no protective effect on alloxan-mediated changes in insulin secretion and cell metabolism. Whereas the flavonoid products rutin (50 μM) and quercitin (5 μM) caused a limited but not significant protection against alloxan for insulin release, quercitin restored the glucose (11 mM)-induced MTT reduction (+33%, $P < 0.01$).

It is of interest that in these static incubation experiments alloxan was capable of stimulating insulin secretion from INS-1D cells after short exposure to subtoxic concentrations at low glucose. Perifusion experiments were performed to determine whether alloxan-induced insulin release could be modulated by epinephrine [15], a physiological inhibitor of insulin secretion (Fig. 4). INS-1D cells were exposed to alloxan for 5 min, which resulted in a rapid 2-fold increase in insulin release above basal. The response was reversible after withdrawal of alloxan. To assess the functional integrity of INS-1D cells after alloxan treatment, glucose-induced insulin release was monitored during a second stimulation period in the same experiment. Glucose (11 mM) evoked a 4-fold increase in insulin release which was reversible upon removal of the stimulus. Compared to alloxan, the glucose-induced insulin release was 2.8-fold greater according to the AUC (area under the curve) calculated for each of the responses (% of insulin content: 1.24 ± 0.33 and 3.47 ± 0.75 respectively, $P < 0.05$, n = 4 independent experiments). Control perifusions indicated that glucose-induced insulin release after alloxan (1 mM) exposure was only marginally inhibited (approximately 20%) as compared to the control glucose response without prior alloxan treatment (data not shown). When epinephrine (10^{-5}M) was perifused throughout, the secretory response to alloxan and glucose were inhibited by 86% ($P < 0.05$) and 94% ($P < 0.02$), respectively, according to the AUC. Basal insulin secretion was also decreased by epinephrine exposure.

To gain further insight into the mechanisms by which alloxan stimulates insulin release, experiments were performed to record changes in membrane potential (Fig. 5). Using the bisoxonol fluorescent probe to estimate the membrane potential changes, it was found that 1 mM alloxan caused a small depolarization corresponding to approximately 10% of the maximal response evoked by 30 mM KCl at the end of the recording. After alloxan exposure, 11 mM glucose induced a cell depolarization of a magnitude similar to that induced by alloxan. As expected, the cell membrane potential could be subsequently repolarized by adding diazoxide (100 μM), which induced a rapid and marked hyperpolarization by opening ATP-sensitive K^+ channels [12, 15]. The effect of alloxan was dose-dependent and no change was observed at concentrations below 1 mM. The following results are derived from one representative experiment and the values are expressed as a percentage of the depolarization induced by 30 mM KCl: 0.1 mM alloxan = 0%; 1 mM alloxan = 6.8%; 2 mM alloxan = 10.2%; 5 mM alloxan = 18.8%; and 10 mM alloxan = 46.2%. When INS-1D cells were first hyperpolarized by diazoxide, a subsequent challenge with alloxan did not induce depolarization (not shown).

As in β-cells, the depolarization of INS-1D cells triggers the gating of L-type voltage-sensitive Ca^{2+} channels, which leads to an increase in cytosolic Ca^{2+} and subsequent insulin release [15]. Experiments were performed in single fura-2 loaded cells to elucidate whether the cellular depolarization caused by alloxan was accompanied by changes in $[Ca^{2+}]_i$ and the results are shown in Fig. 6. At basal glucose concentration, 1 mM alloxan augmented $[Ca^{2+}]_i$, an increase which was not sustained but appeared as a series of peaks of large amplitude (250–320 nM). This pattern was different from control increments seen with stimulatory concentrations of glucose or depolarizing con-

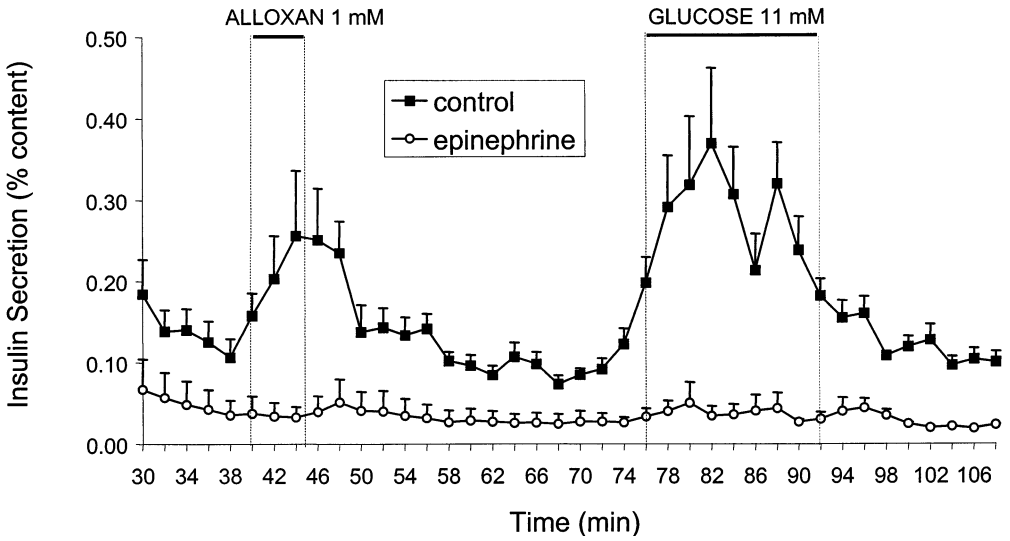

FIG. 4. Effects of alloxan and epinephrine on insulin secretion from perifused INS-1D cells. Cells were perifused for 45 min for equilibration and then exposed to 1 mM alloxan for 5 min. A second stimulation was imposed by perifusing the cells at stimulatory glucose concentration (11 mM) for 15 min (■). When present, 10 μM epinephrine was applied throughout the perifusion (○). Results are means ± SEM of 3–4 separate experiments. For statistics, see Results section.

centrations of KCl (Fig. 6A). When extracellular calcium was chelated with 2.5 mM EGTA, the alloxan-induced increase in $[Ca^{2+}]_i$ was abolished (Fig. 6B). To determine whether alloxan promoted the entry of Ca^{2+} through L-type voltage-sensitive Ca^{2+} channels or another type of Ca^{2+} channel, INS-1D cells were exposed to either 20 μM verapamil or 1 mM nickel chloride (Ni), respectively. In the presence of Ni, alloxan did not increase $[Ca^{2+}]_i$ (Fig. 6D). Surprisingly, in the presence of verapamil, alloxan was still able to increase $[Ca^{2+}]_i$, whereas, as expected, a glucose-induced increase in $[Ca^{2+}]_i$ was inhibited by both Ca^{2+} channel-blocking agents (Fig. 6C). Similar observa-

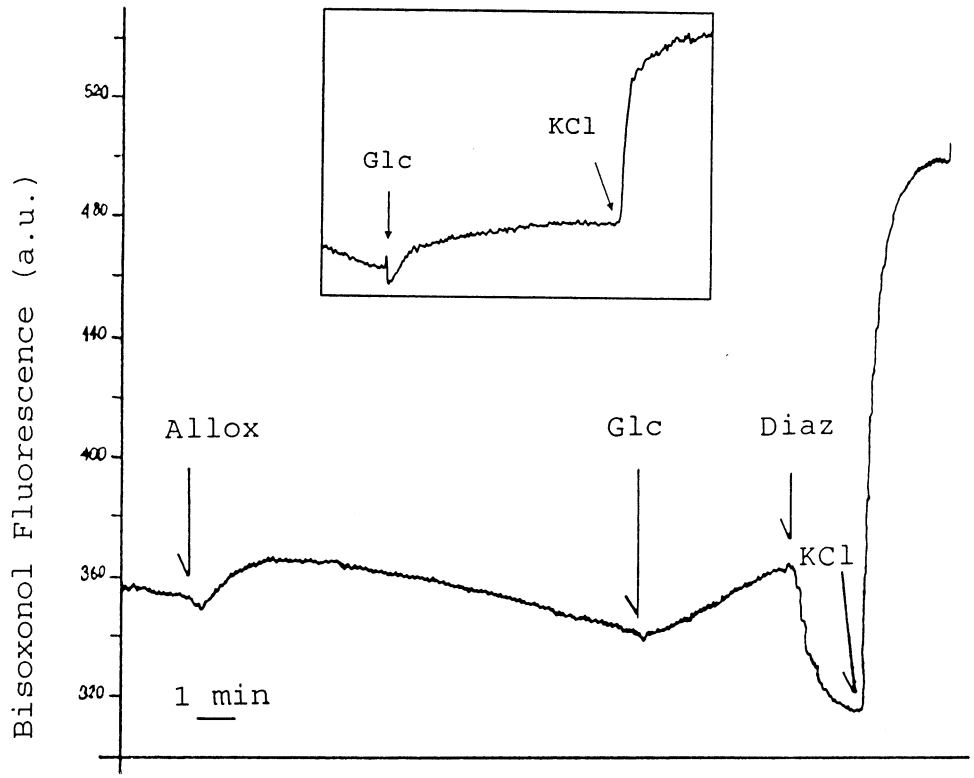

FIG. 5. Effect of alloxan on membrane potential in INS-1D cells. A representative trace, out of 4 independent experiments, of fluorometric recordings with 100 nM bisoxonol is shown. The cells, in KRBH with 2 mM glucose, were stimulated consecutively with 1 mM alloxan, 11 mM glucose, 200 μM diazoxide, and 30 mM KCl. A control trace is shown in the inset. The fluorescence was monitored at excitation and emission wavelengths of 535 nm and 570 nm, respectively.

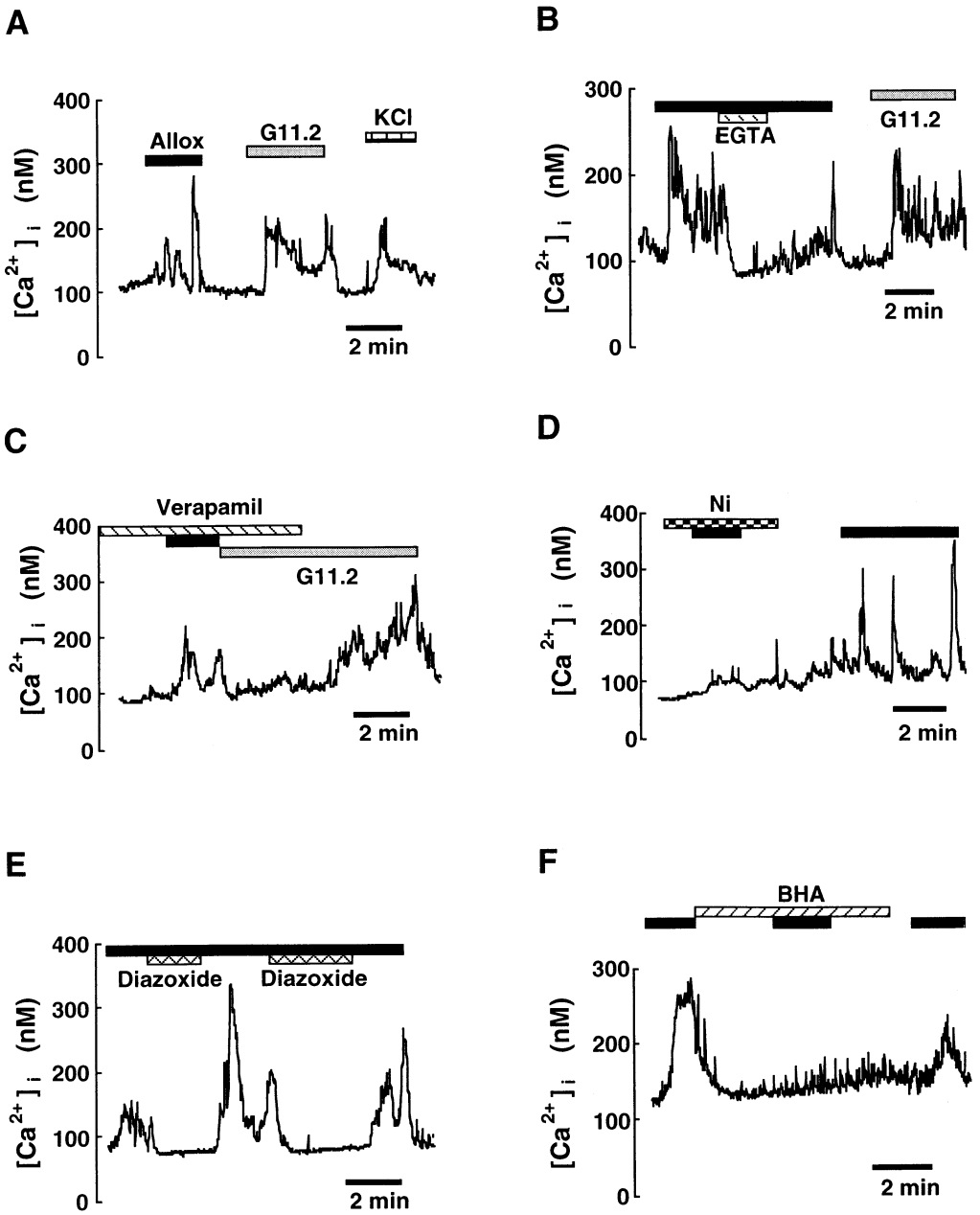

FIG. 6. Effect of alloxan on cytosolic Ca^{2+} ($[Ca^{2+}]_i$) in single INS-1D cells. Changes in $[Ca^{2+}]_i$ were investigated in fura-2-loaded cells stimulated with 1 mM alloxan at 2 mM glucose. (A) Cells were stimulated consecutively with 1mM alloxan, 11.2 mM glucose, and 30 mM KCl. The alloxan stimulation periods are indicated throughout by the black bars. (B) 2.5 mM EGTA was introduced in the middle of the alloxan stimulation. (C) Cells were exposed to 20 μM verapamil, and alloxan or glucose were added as stimuli. (D) Cells were exposed to 1 mM nickel chloride and alloxan. (E) Cells were exposed to the continuous presence of alloxan and 200 μM diazoxide was introduced twice. (F) Cells were exposed to 1 mM alloxan and 100 μM BHA. Cells were attached to glass coverslips and loaded with 1 μM fura-2/AM for 30 min. $[Ca^{2+}]_i$ was monitored by the measurement of the 340 nm/380 nm excitation ratio during continuous perifusion with KRBH containing 2 mM glucose as basal condition. Representative traces of at least 3–5 separate recordings are shown.

tions were made when the compound SR-7037, another selective inhibitor of L-type Ca^{2+} channels, was used (not shown). The alloxan-induced increase in $[Ca^{2+}]_i$ was no longer present when INS-1D cells were hyperpolarized with 100 μM diazoxide (Fig. 6E). The effect of alloxan on Ca^{2+} permeability could also be inhibited by using the antioxidant BHA. In the presence of BHA, alloxan did not increase $[Ca^{2+}]_i$ (Fig. 6F). In control experiments, we observed that BHA did not inhibit glucose-induced $[Ca^{2+}]_i$ increase (not shown).

DISCUSSION

More than 50 years after the first published report that alloxan induces diabetes in rabbits [19], the mechanisms of action of the drug on β-cells are still poorly understood.

Pioneering *in vivo* experiments have shown that both glucose administration and reducing agents protect against the diabetogenic effect of alloxan. It has further been postulated that alloxan binds to the glucose-binding site of glucokinase and oxidizes the sulfhydryl reactive groups [1, 10, 11]. However, alloxan is thought to generate oxygen-free radicals which are toxic to β-cells in several ways. Alloxan produces free radicals in a two-step reaction: schematically, the initial conversion of alloxan to dialuric acid by interaction with any available sulfhydryl groups (i.e. the extracellular plasma membrane sites, cytosolic glutathione, or thioredoxin) is followed by the auto-oxidation of dialuric acid generating $O_2^{\bullet}$ [20–23]. These radicals can in turn form H_2O_2, which ultimately will react with trace amounts of metal ions (e.g. Fe^{3+}) to produce the highly toxic $OH^{\cdot}$ radical species [18]. When intact cells are exposed to alloxan, damage could occur initially at the outer part of the plasma membrane [24]. Since alloxan is transported across the plasma membrane, alloxan redox reactions occurring inside the β-cells will allow the targeting of free radicals directly to the nucleic acids and various organelles [7, 25, 26]. In particular, alterations of mitochondrial function have been described after direct exposure of isolated liver mitochondria to alloxan [25, 27–29].

The deleterious action of alloxan is time- and dose-dependent, which reflects, to a certain extent, the balance between the antioxidant resources of the cells and the toxic potential of the drug [5, 30–32]. In addition, there is a species-dependent variation in the sensitivity of islets to alloxan cytotoxicity, probably due to differences in antioxidant defense mechanisms [33, 34].

In our studies, INS-1D cells were sensitive to alloxan in the same concentration range as reported for rat islets (Fig. 1 and [33]). It was also found that a 45-min exposure to alloxan concentrations above 2–5 mM leads to rapid (within hours) irreversible changes in cell viability, which underscores the difficulties in establishing a reproducible model for alloxan cytotoxicity. Using the differentiated insulin-secreting INS-1D cells, we studied functional characteristics of the cells over an early period (up to 45 min) immediately after short exposure to alloxan (usually 5 min) by monitoring four parameters: insulin secretion, MTT reduction, membrane potential, and $[Ca^{2+}]_i$. This experimental approach eliminates indirect effects caused by alterations in gene expression due to nucleic acid damage in the cells after several hours in culture following initial exposure to the drug. Relatively few studies have been published on the action of alloxan on insulin-secreting cell lines. RINm5F cells and parental rat transplantable tumor cells are thought to show resistance to alloxan toxicity, which has been attributed either to poor uptake of alloxan or to low levels of glucokinase [11, 35, 36]. However, in other cell lines, alloxan cytotoxicity has indeed been documented. Alloxan caused mitochondrial DNA damage in the insulin-secreting RINr 38 cells [37] and lysosomal alterations in the lymphoma cell line J-774 [26]. In addition, in a previous paper, we showed that INS-1 cells are sensitive to alloxan by using the MTT reduction test [14]. In the present study, we have confirmed that alloxan causes a dose-dependent decrease in MTT reduction (Fig. 1). Alloxan has already been reported to affect islet metabolism [38]. In islets and INS-1 cells, the MTT test gives a quantitative as well as qualitative assessment of cell viability, since it reflects both the absolute number of metabolically active cells and the variation in glucose metabolism between basal and stimulatory glucose concentrations [13, 14]. We have demonstrated that the glucose sensitivity for MTT reduction paralleled that for insulin secretion (1.5- to 2.0-fold increase), and alloxan uniformly lowered INS-1D cell viability for every experimental group (Figs. 1–3), an effect prevented by the antioxidant BHA. Exposure of INS-1D cells to stimulatory concentrations of glucose or to other metabolic stimuli after a 45-min treatment with alloxan did not reverse alloxan toxicity, as was already observed in islets [38].

To qualify as a model system for studies on the mode of action of alloxan, the cell line must display a sensitivity similar to that of native β-cells. We have focused our study on a specific effect of alloxan, i.e. an early and transient stimulation of insulin release at low glucose concentration (Figs. 1–4). This is reminiscent of previous observations where alloxan caused a dose-dependent transient stimulation of insulin secretion in perifused isolated islets and in perfused pancreas [39, 40]. Most of these early studies were undertaken to investigate the protective effect of glucose on alloxan toxicity rather than the characterization of the stimulation of insulin secretion by alloxan.

In INS-1D cells, alloxan caused membrane depolarization and an increase in $[Ca^{2+}]_i$ (Figs. 5 and 6). Alloxan has previously been shown to depolarize mouse islets and increase the cytosolic Ca^{2+} of isolated *ob/ob*-mouse β-cells [41, 42]. To our knowledge, the present study is the first to document these closely time-related functional changes with the parallel stimulation of insulin release. In contrast to glucose [15], alloxan-induced membrane depolarization may not be explained by a decrease in K^+ permeability. On the contrary, it has been shown that alloxan increases $^{86}Rb^+$ efflux from perifused islets [38]. Recently, it has been shown that alloxan and its auto-oxidation product H_2O_2 open a non-selective cation channel in the rat insulin-secreting cell line CRI-G1 [43]. As this channel is permeable to both Na^+ and Ca^{2+}, its opening could explain not only the depolarization but might also contribute to the increase in $[Ca^{2+}]_i$. It should be noted, however, that H_2O_2 has been reported to activate the ATP-sensitive K^+ channels in rat pancreatic β-cells [44], which should lead to membrane hyperpolarization.

In contrast to the modest and steady elevation in cytosolic $[Ca^{2+}]$ reported in *ob/ob*-mouse islet cells, alloxan-induced $[Ca^{2+}]_i$ increases in INS-1D cells were of greater magnitude and also occurred in a series of transient peaks, more like the glucose effect observed in control traces. The effect of alloxan was dependent on the presence of extracellular Ca^{2+}, as it did not occur after chelation of

extracellular Ca^{2+} with EGTA. However, the rise in $[Ca^{2+}]_i$ could not simply be attributed to the gating of voltage-sensitive L-type Ca^{2+} channels by alloxan, since verapamil was ineffective (Fig. 6). The inhibition of the $[Ca^{2+}]_i$ rise by nickel chloride does not allow identification of the ion channel in question, and the non-selective cation channel [43] could well be inhibited by 1 mM nickel. The hyperpolarizing agent diazoxide blocked both the depolarization and $[Ca^{2+}]_i$ rise evoked by alloxan. As the opening of the non-selective cation channel by alloxan was suggested to be mediated by H_2O_2 and since the antioxidant BHA counteracted the effects of the drug, this channel could be the target for alloxan in INS-1D cells. In this case, diazoxide appears to inhibit the opening of the non-selective cation channel. The reversibility of alloxan effects after treatment with the antioxidant BHA indicates that alloxan exerts a modulation of cell function via oxidation of redox-sensitive sites, either by direct interaction or indirectly via the generation of free radicals.

In conclusion, experimental evidence demonstrating that the antioxidant BHA prevented the effect of alloxan on insulin secretion, MTT reduction, and $[Ca^{2+}]_i$ supports the theory that alloxan mediates its action, at least in part, by the production of free radicals. KCl-stimulated insulin release after alloxan treatment is less affected than that evoked by glucose (Figs. 2 and 3). This situation is reminiscent of insulin secretion from INS-1 cells in which mitochondrial DNA was depleted [45]. Indeed, as the mitochondria play a central role in metabolism–secretion coupling [15, 46], it is possible that they are targets for alloxan action, resulting in decreased oxidative phosphorylation [38]. We propose that the differentiated INS-1 cells can be used to study the mechanism of action of alloxan on β-cells. The characterization of the transient insulin secretion observed after alloxan exposure of the cells provides a valid model, since most of its features have also been reported in rodent islets. The oxidative modulation of hormonal secretion may be useful in understanding the putative links between the biology of free radicals and their role in the physiological signaling pathways of insulin secretion and in physiopathology of the β-cell.

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