Alloxan Inhibition of a Ca\textsuperscript{2+}- and Calmodulin-dependent Protein Kinase Activity in Pancreatic Islets*

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Alloxan was found to inhibit a Ca\textsuperscript{2+}- and calmodulin-dependent protein kinase recently identified in pancreatic islets. This effect of alloxan may be specifically related to the inhibitory action of alloxan on insulin secretion from islets since: 1) In islet-cell subcellular fractions, alloxan at micromolar concentrations irreversibly inhibits the Ca\textsuperscript{2+}- and calmodulin-dependent protein kinase activity; 2) Pretreatment of intact islets with alloxan at concentrations that inhibit insulin secretion similarly inhibits the protein kinase activity; and 3) Alloxan inhibition of both insulin secretion and protein kinase activity in intact islets can be prevented by d-glucose. This inhibition by alloxan appears to be a direct effect on the enzyme since alloxan treatment of either the islet homogenate or the microsomal fraction enriched in protein kinase activity inhibited the kinase activity with similar concentration dependence. These results suggest that alloxan-induced inhibition of a Ca\textsuperscript{2+}- and calmodulin-dependent protein kinase may represent a critical inhibitory site which mediates alloxan-induced inhibition of insulin secretion.

Although the initial observation that alloxan produced diabetes was made some 40 years ago (1), the mechanism by which alloxan exerts its inhibitory effect on insulin secretion from the β-cell remains to be elucidated. Recent studies using isolated islets and the perfused pancreas (2, 3) have demonstrated that brief exposure to alloxan directly inhibits subsequent glucose-stimulated insulin secretion. Interestingly, this effect of alloxan can be prevented by exposure of the islets to d-glucose (3, 4). It also has been noted that the structure of alloxan is similar to that of d-glucose (5) and that, in fact, prior to the inhibition of insulin secretion, alloxan itself stimulates the release of insulin (6). Other compounds (e.g. 3-O-methyl-D-glucose, methylxanthines, cytochalasin B) that have been proposed to affect the rate of entry of alloxan into islets (4, 6, 7) also protect against alloxan-induced inhibition of insulin secretion. Alloxan is also known to be highly reactive and once reduced to diatomic acid may generate generally toxic free radicals (8). Thus, the inhibitory action of alloxan may occur as a direct effect of alloxan or indirectly following the generation of free radicals (9–11).

Earlier investigations have examined the possible effects of alloxan on specific enzymes. However, effects of alloxan in cell-free systems generally were observed only at concentrations that were too high to be of biological importance (12). In order for an effect of alloxan on a specific enzymatic system to be related to the inhibition of insulin secretion by the β-cell, it would seem that the following criteria should be met. 1) A direct effect of alloxan should be observed at concentrations of the drug that inhibit insulin secretion i.e. less than 1 mM (2). 2) This effect should be irreversible. 3) The inhibition should occur also upon pretreatment of intact islets with alloxan. 4) This effect of alloxan produced in intact islets should be prevented by treatments that are known to protect against inhibition of insulin secretion by alloxan in vitro.

We have recently developed techniques to obtain subcellular fractions from pancreatic islets (13) and have described a Ca\textsuperscript{2+}- and calmodulin-dependent protein kinase activity that is localized in the islet-cell microsomal fraction (14). This enzyme activity phosphorylates two endogenous substrates of 57 kDa and 54 kDa. During the course of these studies, we have determined that this protein kinase is inhibited by micromolar concentrations of alloxan. Furthermore, the present studies suggest that inhibition of the Ca\textsuperscript{2+}- and calmodulin-dependent protein kinase by alloxan may be directly related to the ability of this agent to inhibit insulin secretion.

EXPERIMENTAL PROCEDURES

Materials—Sprague-Dawley rats (180–200 g, body weight) were obtained from Sasco (O’Fallon, MO). Collagenase type IV was obtained from Worthington, calmodulin from Calbiochem (La Jolla, CA), [γ-\textsuperscript{32}P]ATP from Amersham, bovine brain tubulin was a gift of Dr. L. A. Brown (St. Louis University, St. Louis, MO), phosphorylase b and phosphorylase kinase (rabbit muscle) and all other chemicals were purchased from Sigma.

Islets and Membrane Preparations—Pancreatic islets were obtained by collagenase digestion (15) and separated on ficoll gradients (16). Isolated islets were then washed in Hanks’ solution and selected under a dissecting microscope. Islet-cell homogenates were prepared in 50 mM MES, 0.25 mM sucrose, 1 mM EDTA, pH 7.2 (fractionation buffer). Subcellular fractionation of islet cells to obtain the microsomal fraction was by modification of the technique of Naher et al. (13) as described previously (17). This fraction is devoid of mitochondria and secretory granules (17).

Protein Kinase Assays—Protein kinase assays were performed as previously described (14). Briefly, assays employed the cell homogenate (5–20 μg/tube) or the microsomal fraction (4–6 μg/tube) as a source of both protein kinase and protein substrates. The assays (50 μl) were conducted at pH 7.0 in 50 mM Pipes, 10 mM MgCl\textsubscript{2}, 0.2 mM EGTA, 10 μM [γ-\textsuperscript{32}P]ATP with and without 1 μM calmodulin and 1 mM CaCl\textsubscript{2}. Dithiothreitol was omitted from the reaction mixture since it altered the rate of spontaneous decay of alloxan to alloxanic acid. Following preincubation of the tissue with the remainder of the reaction mixture, the reactions were initiated by the addition of [γ-\textsuperscript{32}P]ATP and terminated after 5 s by the addition of 1% sodium dodecyl sulfate, 3% β-mercaptoethanol, and 5% glycerol. The phosphoproteins were then separated on 10% polyacrylamide slab gels (14). A 5-s assay was used so that the endogenous substrates would not become rate limiting. Under these conditions, this time period

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\[1\] The abbreviations used are: MES, 2-(N-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethoxy)ether)-N,N,N',N'-tetraacetic acid; Pipz, 1,4-piperazinediethanesulfonic acid; Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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approximates the initial rate; the rate of kinase activity was not linear at incubation times in excess of 15 s. Ca""- and calmodulin-dependent protein kinase activity was determined by quantitation of 32P incorporated into the endogenous 57-kDa and 54-kDa protein substrates. The Ca""- and calmodulin-dependent activity was determined by subtracting the 32P incorporation that occurred in the absence of Ca""- and calmodulin in parallel assays.

**Effects of Alloxan on Cell-free Protein Kinase Activity**—Protein kinase assays employing the islet-cell homogenate or microsomal fraction were carried out as described above following pretreatment of the islet-cell membranes with various concentrations of alloxan for 5 min in the assay reaction mixture at 37 °C. Alloxan concentrations were standardized spectrophotometrically by absorption at 270 nm (f = 980 M-1 cm-1) (18). In control experiments, alloxan was allowed to decompose to alloxanic acid in the reaction buffer (10 min at pH 7.0, 37 °C) before addition of tissue.

**Intact Islet Experiments**—Isolated islets were washed in Krebs’ bicarbonate buffer containing 115 mM NaCl, 24 mM NaHCO3, 5 mM KCl, 1 mM MgCl2, 2.5 mM CaCl2, 0.1% bovine serum albumin, 5 mM Hepes, pH 7.4 (incubation medium). Two hundred islets were then selected at random into Kontes 18 AAA homogenizing tubes. Excess medium was removed with a Pasteur pipette. The islets were then incubated in 200 μl of incubation medium containing various concentrations of alloxan, alloxanic acid, and/or added hexoses. After incubation at 37 °C for 10 min, the islets were washed three times with fractionation buffer and homogenized with a Teflon pestle. Protein kinase assays with the individual homogenates were then carried out as described above. The length of the incubation and three washes with fractionation buffer together with the short half-life of alloxan under these conditions (<1 min) assured that alloxan was not present at the time of homogenization. Immunoreactive insulin was measured by the method of Wright et al. (19). Protein was measured with fluorescamine (20).

**RESULTS**

Initial experiments demonstrated that pre-exposure of islet-cell homogenates (5 min, 37 °C) to alloxan produced a concentration-dependent inhibition of the subsequently assayed Ca""- and calmodulin-dependent protein kinase activity (Fig. 1). Alloxan at concentrations from 5 to 100 μM did not inhibit Ca""-independent phosphorylation of the 57-kDa and 54-kDa protein bands. Thus, in 7 experiments, the Ca""-independent phosphorylation in the absence of alloxan or at 100 μM alloxan was 0.72 ± 0.08 versus 0.60 ± 0.06 pmol of P_i/mg/5 s and 0.56 ± 0.15 versus 0.54 ± 0.11 pmol of P_i/mg/5 s for the 57-kDa and 54-kDa protein bands, respectively. Half-maximal inhibition of the Ca""- and calmodulin-dependent protein kinase activity occurred at approximately 10 μM alloxan whether the activity was measured by incorporation of 32P into either the 57-kDa or 54-kDa endogenous substrates (Fig. 1). This effect did not occur if alloxan was allowed to decompose to alloxanic acid in the presence of the incubation buffer containing Ca""- and calmodulin before initiation of the assay (results not shown). To determine whether the effect of alloxan was a reversible oxidation, islet-cell homogenates were pretreated with varying concentrations of alloxan as shown in Fig. 1 and then exposed to 0.1 mM dithiothreitol for 2 min before assay of protein kinase activity. The effect of alloxan was not reversed by dithiothreitol nor was the effect of these concentrations of alloxan prevented by a 2-min preincubation of the homogenate with 20–50 mM D-glucose (results not shown).

As the effect of alloxan on the islet-cell homogenate may not necessarily be a direct interaction of alloxan with the protein kinase, experiments were performed with the islet-cell microsomal fraction that is enriched in the protein kinase activity (14). Pretreatment of the microsomal fraction with alloxan produced the same concentration-dependent inhibition of protein kinase activity as observed in the homogenate (Fig. 1). This inhibition by alloxan may theoretically result from an effect on either the protein kinase or on the endogenous substrates. Previous studies have shown that α and β subunits of tubulin may serve as substrates for this islet-cell Ca""- and calmodulin-dependent kinase. Thus, to eliminate the possibility that alloxan interfered with the protein substrates, bovine brain tubulin (1 μg/tube) was added to the reaction buffer following 5-min exposure of the microsomal fraction to 5–100 μM alloxan, and before the initiation of the protein kinase assay by γ[32P]ATP. Alloxan (100 μM) inhibited the Ca""- and calmodulin-dependent protein kinase activity by 82 ± 3%. No further phosphorylation was obtained by the subsequent addition of tubulin, indicating that the...
kinase was no longer active. Hence, the inhibition by alloxan would appear to be a direct effect of alloxan on the protein kinase.

To determine if exposure of intact islets to alloxan would have a similar effect on the protein kinase activity, intact islets were exposed briefly (10 min) to alloxan and then homogenized for subsequent determination of protein kinase activity. Exposure of intact islets to alloxan produced a concentration-dependent inhibition of the subsequently assayed protein kinase activity. However, higher concentrations of alloxan were needed to inhibit the protein kinase activity than required when alloxan was present in cell-free experiments (Fig. 2). The simultaneous presence of 25 mM \( \beta \)-glucose and alloxan prevented the inhibitory effects of alloxan on both protein kinase activity and insulin secretion (Fig. 3). \( \beta \)-glucose offered complete protection of protein kinase activity against the effect of 500 \( \mu \)M alloxan; 3-O-methyl-\( \beta \)-glucose produced partial protection, while L-glucose was without effect (Table I).

**DISCUSSION**

The inhibition of the Ca\(^{2+}\)- and calmodulin-dependent protein kinase activity by alloxan reflects a direct interaction of alloxan with the kinase as: 1) alloxan did not interact with calmodulin; 2) the inhibition was not reversed by the addition of exogenous protein substrate; and 3) alloxan was an equally potent inhibitor of protein kinase activity in the islet-cell homogenate and in the microsomal fraction that is enriched in protein kinase activity. At concentrations of alloxan from 5 to 100 \( \mu \)M the Ca\(^{2+}\)- and calmodulin-dependent phosphorylation of the 57-kDa and 54-kDa protein bands was the only significant alteration noted in the autoradiograms of kinase assays employing the islet-cell homogenate or microsomal fraction. As Ca\(^{2+}\)-independent phosphorylation of these proteins also was not affected, the effect of these concentrations of alloxan may be rather selective. Indeed, in three experiments conducted under the same conditions, alloxan at concentrations up to 100 \( \mu \)M did not detectably inhibit phosphorylase \( \beta \) kinase; 500 \( \mu \)M alloxan produced only 40 ± 5% inhibition of this calmodulin-dependent kinase.

The sensitivity of the Ca\(^{2+}\)- and calmodulin-dependent protein kinase to low concentrations of alloxan raises the question as to whether this is a primary inhibitory site for the effect of alloxan on insulin secretion. Similar to the effect of alloxan on insulin secretion, the inhibition by alloxan of protein kinase activity was irreversible. Thus, the alloxan-induced inhibition of the protein kinase activity was not reversed by subsequent treatment with dithiothreitol and was also retained in subcellular fractions following pretreatment of intact islets with alloxan. Importantly, the inhibition of protein kinase activity caused by alloxan treatment of intact islets was attenuated by concentrations of \( \beta \)-glucose and 3-O-methyl-\( \beta \)-glucose that are known to protect the \( \beta \)-cell against the inhibitory actions of alloxan on insulin secretion in vitro (2, 3).

The protection by \( \beta \)-glucose against the inhibitory effect of alloxan on protein kinase activity and insulin secretion may occur as the result of competition at a specific site within the \( \beta \)-cell, or \( \beta \)-glucose may protect against alloxan by preventing active alloxan from reaching the inhibitory site. Weaver et al. (4) have shown that \( \beta \)-glucose actually increases rather than decreases \( ^{14}\)C]alloxan uptake by isolated islets. As \( \beta \)-glucose did not directly prevent the inhibition by alloxan in cell-free experiments in the present studies, the mechanism by which \( \beta \)-glucose protects against the effects of alloxan remains an

![Fig. 3. Glucose protection against the inhibitory effects of alloxan on insulin secretion and protein kinase activity in intact islets](image-url)

**Table I**

| Treatment                  | Protein kinase activity pmol P\(_1\)/mg protein/5 s |
|----------------------------|-----------------------------------------------------|
| Control                    | 2.63 ± 0.38 (14)                                     |
| Hexose alone               | 2.87 ± 0.33 (14)                                     |
| 500 \( \mu \)M Alloxan     | 1.76 ± 0.22 (14)                                     |
| 500 \( \mu \)M Alloxan plus | 3.71 ± 0.75 (7)                                     |
| \( \beta \)-glucose        | 2.17 ± 0.32 (7)                                     |
| 3-O-Methyl-\( \beta \)-glucose | 1.63 ± 0.40 (7)                                   |

\* Statistically significant hexose protection (\( p < 0.05 \), paired \( t \) test).

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**TABLE 1**

*Effect of hexoses on alloxan inhibition of Ca\(^{2+}\)- and calmodulin-dependent protein kinase activity in intact islets*

Islets were pretreated for 10 min with 500 \( \mu \)M alloxan with or without 25 mM of the indicated hexoses as shown. Ca\(^{2+}\)- and calmodulin-dependent protein kinase activity was then measured in the islet-cell homogenate as described under "Experimental Procedures." Data represent the mean ± S.E. Ca\(^{2+}\)- and calmodulin-dependent phosphorylation of the 57-kDa protein substrate in \( n \) experiments.

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**Protein Kinase Activity**

- Insulin Secretion
- Glucose-Stimulated Insulin Secretion (\( \mu U/\text{Hil/Min} \))
- Protein Kinase Activity
- Ca\(^{2+}\)-Stimulated Protein Kinase Activity (pmol P\(_1\)/mg protein/5 sec)
Effect of Alloxan on Islet-Cell Protein Kinase

The effective concentrations for inhibition of islet-cell Ca²⁺- and calmodulin-dependent protein kinase activity by alloxan varied depending on whether alloxan was presented to intact islets or to cell-free membranes. The effective inhibitory concentrations of alloxan in the intact islet experiments agrees with the concentrations necessary to produce alterations in insulin secretion in vitro (2). The higher extracellular concentrations of alloxan required in the intact cell in comparison with the cell-free system are undoubtedly needed to achieve an active concentration of alloxan at the intracellular site.

The function of the Ca²⁺- and calmodulin-dependent protein kinase in the β-cell has not yet been elucidated. However, the correlation of the inhibitory effects of alloxan on insulin secretion and on the Ca²⁺- and calmodulin-dependent protein kinase activity indicates that this enzyme may play an essential role in the insulin secretory process. Further study of the consequence of alloxan inhibition of the Ca²⁺- and calmodulin-dependent protein kinase activity in the β-cell offers unique advantages for evaluation of critical steps in alloxan-induced impairment of insulin secretory function and the complex interplay of agents that protect against these effects of alloxan.

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