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Mechanism of Inhibition by Alloxan of ATP-Driven Calcium Transport by Vascular Smooth Muscle Microsomes

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Abstract

The direct *in vitro* effects of alloxan on the Ca^{2+} handling by microsomal membranes isolated from dog mesenteric arteries were investigated. Preincubation of the vascular muscle microsomal membranes with alloxan showed a suppressive effect on both binding of Ca^{2+} (in the absence of ATP) and ATP-driven Ca^{2+} transport. Such an inhibition was time dependent, dose dependent, and temperature dependent. ATP-driven Ca^{2+} transport was much more susceptible to the inhibitory action of alloxan than Ca^{2+} binding under all experimental conditions examined. Alloxan inhibited ATP-driven Ca^{2+} transport at a comparable level over the entire period of Ca^{2+} uptake, but had no significant effect on the efflux of Ca^{2+} from preloaded microsomal membranes. This suggests that alloxan exerts its inhibitory effect on the ATP-driven Ca^{2+} transport via its action on the Ca-pump protein rather than the membrane permeability to Ca^{2+}. Catalase and mannitol but not superoxide dismutase partially protected against such as inhibition by alloxan. The possible involvement of H_2O_2 mediating the inhibitory action of alloxan was further supported by the finding of a similar *in vitro* inhibitory effect of H_2O_2 on the ATP-driven Ca^{2+} transport by the vascular smooth muscle microsomes.

Key Words: Alloxan; calcium transport; vascular smooth muscle; microsomes; hydrogen peroxide.

Introduction

Alloxan has been widely used to induce insulin-dependent diabetes mellitus in experimental animals (Webb, 1966). Usually a single bolus

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intravenous injection at a high dose (60–120 mg/kg) is effective in causing severe hyperglycemia within a few days. The diabetogenic action of alloxan has been attributed to its cytotoxic effect on the insulin releasing beta-cells of the pancreas (Malaisse, 1982). In addition to its diabetogenic action, the long-term *in vivo* effect of alloxan also includes the development of mild but sustained hypertension in rats (Igarashi *et al.*, 1979). However, it was not clear whether the hypertensive effect of alloxan was associated with the direct action of alloxan *per se* or alloxan-induced diabetes. It has long been noted (Webb, 1966, Macqueen, 1952) that *in vivo* administration of alloxan also caused transient vasoconstriction at the site of injection and slight elevation of blood pressure. At high doses, alloxan has also been observed to cause pulmonary vasoconstriction and capillary damage in dogs (Aviado and Schmidt, 1957). Since the cellular membranes of vascular smooth muscle play an important role in the finely integrated regulation of cytoplasmic level of Ca^{2+} to maintain the normal contractile function (Kuriyama *et al.*, 1982), it is conceivable that altered contractile function of blood vessels in cardiovascular diseases associated with hypertension and diabetes mellitus can be interpreted by a defect in the handling of Ca^{2+} by vascular muscle strips (Turlapaty *et al.*, 1982) or isolated microsomal membranes (Kwan, 1985). It is also possible that alloxan may exert a direct deleterious effect on the vascular muscle membranes leading to derangement of the regulation of cytoplasmic Ca^{2+}. In the present study, using microsomal membrane fractions isolated from dog mesenteric ateries, we directly demonstrated such an *in vitro* inhibitory effect of alloxan on the Ca^{2+} binding and transport properties and provided evidence that oxygen free radical intermediates form the basis for the mechanisms of auction of alloxan on the isolated vascular smooth muscle membranes. Preliminary results have previously been reported in an abstract form (Beazley and Kwan, 1987).

Materials and Methods

Animals and Tissue Trimming

Mesenteric arteries isolated from two to three mongrel dogs weighing 10–20 kg were used for each experiment. Dogs were killed by pentobarbital overdose and the mesenteric vasculatures were removed and placed in ice-cold 250 mM sucrose solution buffered with 10 mM imidazole at pH 7.2 (SUC/IM). The vasculature were trimmed to remove fat, mesenteries, nerve fibres and veins as previously described in detail (Kwan *et al.*, 1983).

Preparation of Microsomal Membranes

Isolated arteries were homogenized in SUC/IM buffer solution (10 ml/g wet weight) by Polytron and separated into various fractions by differential centrifugation as previously described (Kwan *et al.*, 1983). Briefly, the vascular muscle homogenate was centrifuged at 900 *g* for 10 min to remove connective tissues and cell debris. The supernatant was centrifuged at 10,000 *g* for 10 min to remove the mitochondrial membranes and then at 100,000 *g* for 40 min to sediment the crude microsomal membranes. The pellet was resuspended and centrifuged again at 10,000 *g* for 10 min to further remove the contaminating mitochondrial fragments. The refined microsomal membrane fraction was used for studies of Ca^{2+} handling. This membrane fraction has been shown, as indicated by marker enzyme distribution, to be highly enriched in plasma membranes with small contamination by endoplasmic reticulum (Kwan *et al.*, 1983).

Preincubation of Alloxan and Other Chemicals with Membranes

Solutions of alloxan and other chemicals at desired concentrations were prepared in 250 mM sucrose solution buffered with 50 mM imidazole at pH 7.2. Equal volume of the drug solutions and microsomal membrane suspensions were mixed and placed in a water bath with constant shaking and temperature controlled at 37°C for a specific preincubation period. The control membrane fractions were similarly treated with the vehicle buffer solutions in the absence of alloxan. For different experiments, the preincubation conditions were specified in the corresponding figure legends.

Ca^{2+} Accumulation

Accumulation of Ca^{2+} was carried out by Millipore filtration technique (Kwan *et al.*, 1979, 1983). Accumulation of Ca^{2+} in the presence of ATP is hereafter operationally termed Ca^{2+} transport, whereas that in the absence of ATP is termed Ca^{2+} binding. Both Ca^{2+} binding and transport were always determined in the same runs under the same conditions. Ca^{2+} transport was performed in the presence of 5 mM ATP, 5 mM Mg^{2+} (free Mg^{2+}: 0.5 mM), 100 μM total Ca^{2+} with trace amount of $^{45}CaCl_2$ (free Ca^{2+}: 20 μM), and 0.5 mM azide in 250 mM sucrose solution buffered with 50 mM imidazole at pH 6.7, 37°C. In order to study the Ca^{2+} binding under similar conditions, the medium for Ca^{2+} binding contained 0.5 mM Mg^{2+}, 20 μM total Ca^{2+}, and 0.5 mM azide in the same buffer solution. Azide was always included in the Ca^{2+} accumulation media to eliminate the contribution to Ca^{2+} transport by any contaminating mitochondrial membranes. Ten minutes incubation time was routinely used because the optimal level of Ca^{2+} transport and binding

occurred about 5–10 min after the addition of microsomal membranes to the assay medium (Kwan *et al.*, 1979, 1983).

The Ca^{2+} concentration dependence of Ca^{2+} transport was performed by measuring the apparent initial rate of ATP-dependent Ca^{2+} transport (the ATP-supported Ca^{2+} transport corrected for the Ca^{2+} binding in the absence of ATP during the first minute) as a function of free Ca^{2+} concentrations using Ca-EGTA buffer system reported by Grover *et al.* (1982).

Ca^{2+} Efflux

The efflux of Ca^{2+} from vascular muscle microsomes was measured by the amount of the residual $^{45}Ca^{2+}$ remaining in the microsomal membrane vesicles. This efflux was measured at various time intervals after 10-fold dilution of the microsomal membranes which have been allowed to accumulate Ca^{2+} in the presence of ATP for 10 min. The solution for Ca^{2+} efflux was isotonic sucrose (250 mm) imidazole (50 mm) at pH 6.8 with or without alloxan (1 mg/ml).

Results

Figure 1 shows the effect of alloxan (2.5 mg/ml) on the binding and transport of Ca^{2+} as a function of preincubation time. It is clear that prolonged preincubation of control membranes at 37°C had no significant effect on the binding and transport of Ca^{2+}. However, upon preincubation of the membranes with 2.5 mg/ml alloxan, the vascular muscle microsomal membranes showed a substantial inhibition of ATP-supported Ca^{2+} transport which increases with increasing precincubation period. A complete inhibition by alloxan of Ca^{2+} transport occurred in 30 min. The inhibition of Ca^{2+} binding in the absence of ATP was much less pronounced. Ca^{2+} binding was not affected during the first minute of preincubation, but a ca. 40% inhibition was observed after 30 min preincubation.

Figure 2 illustrates the dose dependence of the inhibitory effect of alloxan, which was preincubated with the microsomal membranes at 37°C for 20 min. As low as 1.0 mg/ml alloxan caused $>60\%$ inhibition of Ca^{2+} transport, and a complete inhibition occurred at 5.0 mg/ml alloxan. It was also noted that addition of 0.1, 0.2, 0.5, and 1.0 mg/ml of alloxan to the Ca^{2+} accumulation media without preincubating it with the microsomal membranes had no significant effect on either binding or transport of Ca^{2+} (data not shown). Table I indicates that the does-dependent inhibition of Ca^{2+} binding and transport was also temperature dependent.

Figure 3 shows the effect of alloxan on the binding and transport of Ca^{2+} in two types of experiments. In one set of experiments, we compared the

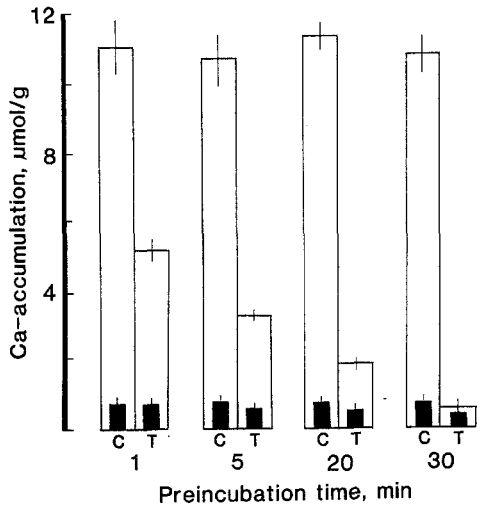

Fig. 1. Inhibition of Ca^{2+} transport (□) and binding (■) by alloxan as a function of preincubation time. The preincubation medium contained 50–80 mg/ml microsomal membrane protein and 2.5 mg/ml alloxan in 250 mM sucrose buffered with 50 mM imidazole at pH 7.2, 37° C. At the end of the desired preincubation time aliquots of the membrane/alloxan mixture were added to the corresponding Ca^{2+} accumulation media to start the Ca^{2+} transport and binding. Vertical bars represent the standard deviation from the mean obtained from four replicates. C = no alloxan, T = 2.5 mg/ml alloxan.

Ca^{2+} binding and transport by alloxan (1.0 mg/ml) treated microsomes and those by untreated microsomes over a 10-min Ca^{2+} accumulation period. It is clear that the Ca^{2+} transport, but not Ca^{2+} binding, was prominantly suppressed over the entire period in alloxan-treated microsomes. In another set of experiments, the microsomal membrane fractions loaded with Ca^{2+} for 10 min in the presence of ATP was diluted in isotonic SUC/IM solutions with and without 1.0 mg/ml alloxan. It was found that addition of alloxan caused little inhibition of binding or transport of Ca^{2+} for the subsequent 5 min compared to the control (addition of vehicle buffer solution).

Figure 4 shows the apparent initial rate determined during the first minute of Ca^{2+} accumulation for ATP-dependent Ca^{2+} transport (corrected for Ca^{2+} binding in the absence of ATP) as a function of free Ca^{2+} concentrations. It is evident that the microsomal membranes pretreated with 1.0 mg/ml alloxan for 20 min at 37°C showed substantially inhibited Ca^{2+} transport over the entire range of free Ca^{2+} concentrations studied. It is obvious from Fig. 4 that the apparent affinity for Ca^{2+} of the Ca^{2+} transport, approximated by the Ca^{2+} concentration at half-maximal apparent initial rate of transport, for control and alloxan-treated microsomes is in the range of 0.2–0.5 μM.

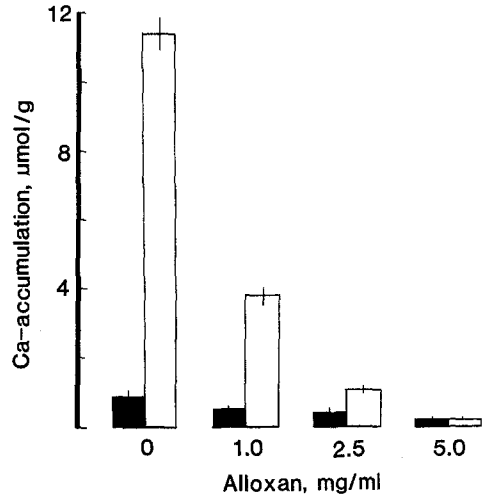

Fig. 2. Inhibition of Ca^{2+} transport (□) and binding (■) of vascular muscle microsomes by 20 min preincubation at pH 7.2 with increasing concentrations of alloxan at 37°C. Since alloxan at concentrations higher than 1 mg/ml tended to lower the pH of the buffer solution, the pH of the alloxan solutions were first reajusted to 7.2 before mixing with the microsomal fraction. Data are expressed as mean ± SD from four replicates.

Table I. Effect of Temperature on the Inhibition by Alloxan of Ca^{2+}-Binding in the Absence of ATP and Ca^{2+} Transport in the Presence of ATP[a]

	No ATP		5 mM ATP	
Alloxan	4°C	37°C	4°C	37°C
Control	1.28 ± 0.24	1.24 ± 0.06	12.53 ± 1.62	13.19 ± 2.90
0.5 mg/ml	1.13 ± 0.19	1.07 ± 0.14	10.64 ± 1.06	6.73 ± 0.47[b,c]
1.0 mg/ml	1.23 ± 0.17	1.01 ± 0.08[c]	9.67 ± 0.23[b]	5.79 ± 0.35[b,c]
2.5 mg/ml	1.03 ± 0.03[b]	0.55 ± 0.10[b,c]	7.72 ± 0.62[b]	1.98 ± 0.16[b,c]

[a]Data are expressed as mean ± SD from four replicates. The microsomal membranes were preincubated with alloxan at desired concentrations for 20 min at 4 and 37°C. The control membranes were similarly preincubated in the vehicle buffer solution in the absence of alloxan.
[b]Significantly different from the control values.
[c]Significantly different from values obtained at 4°C.

To gain some insight into the action of mechanisms underlying the inhibitory effect of alloxan on the Ca^{2+} transport, we first examined the possible protective effect of D-glucose, which is structurally similar to alloxan and has been shown to elicit protective effects against the deleterious *in vitro* or *in vivo* action of alloxan (Harman and Fischer, 1982). Figure 5 shows that over a wide range of levels of inhibition (5–50%) of ATP-supported Ca^{2+} transport by alloxan, no protection by D-glucose (10–50 mM) was observed.

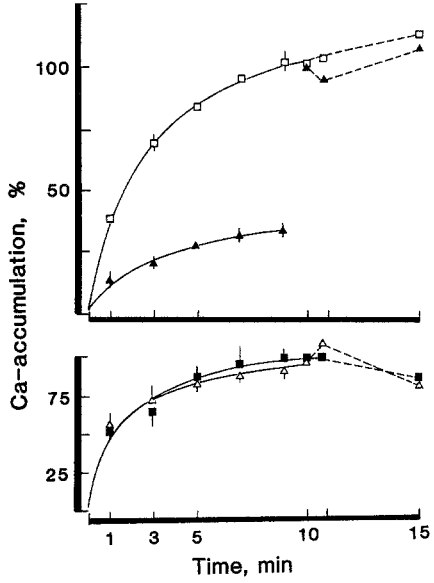

Fig. 3. Uptake and release of Ca^{2+} by vascular muscle microsomes as a function of time in the presence (top) and absence (bottom) of ATP. Triangles stand for the uptake and release of Ca^{2+} in the presence of 1 mg/ml alloxan. Squares stand for the corresponding controls in the absence of alloxan. For the Ca-release experiment, the microsomal fractions preloaded with Ca^{2+} in the presence of ATP for 10 min were first diluted 12-fold with isotonic sucrose (250 mM) containing 50 mM imidazole (pH 7.2 at 37°C) and then filtered at desired intervals. The Ca accumulation was expressed relative to the plateau level obtained at 10 min of Ca uptake. Data are expressed as mean ± SD from triplicates, and a similar result was also obtained in a separate experiment.

We then examined the possible protective effects of other reagents including superoxide dismutase (SOD, a superoxide anion radical scavenger), catalase (CAT, a hydrogen peroxide, H_2O_2, scavanger), fatty acid-free bovine serum albumin (BSA, which binds free fatty acids), ascorbate (ASC, an anti-oxidant), and dithiothreitol (DTT, a sulfhydryl group reducing reagent). Figure 6 clearly shows that among these reagents, CAT (200 units) is the only one partially protecting the membranes from the inhibition of Ca^{2+} transport by alloxan without having any significant effect on the control membranes not treated with alloxan. SOD (200–2000 units) and BSA (0.2%) had no significant protective effect, whereas ASC (2 mM) and DTT (2 mM) further enhanced the inhibition of Ca^{2+} in the presence of alloxan. We have also investigated the effect of mannitol, a commonly used scavenger for hydroxyl radicals which are frequently generated from the H_2O_2 intermediate. Figure 7 shows that the 10–100 mM mannitol had no apparent effect on the ATP-driven Ca^{2+} transport by the control membranes. In alloxan (at both 0.5 and 1.0 mg/ml concentrations) treated membranes, however, inclusion of

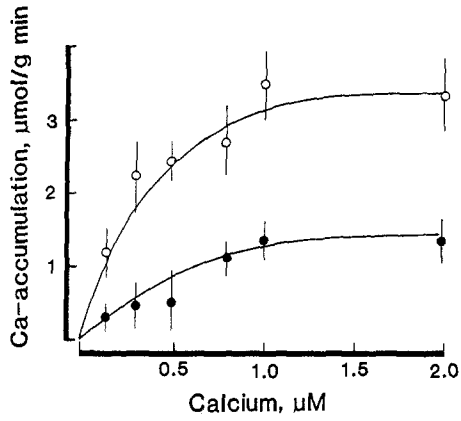

Fig. 4. Ca^{2+} concentration dependence of the apparent initial rate of ATP-dependent Ca accumulation in the presence (●) and absence (○) of 1 mg/ml alloxan. Data are expressed as mean ± SD of four replicates. The apparent initial rate of ATP-dependent Ca-uptake was taken as the Ca^{2+} accumulated in the presence of ATP minus that in the absence of ATP during the first minute (Kwan *et al.*, 1986), and the free Ca^{2+} concentrations were buffered with EGTA and calculated as described previously (Grover *et al.*, 1982).

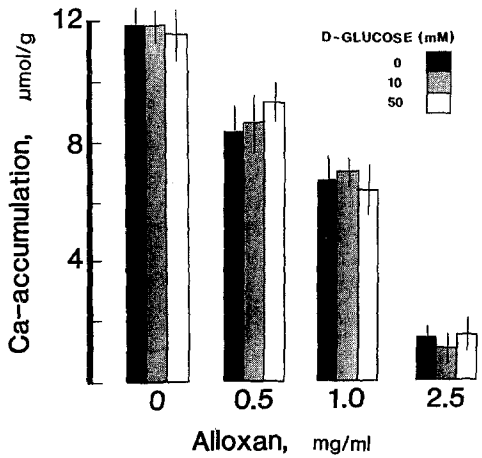

Fig. 5. Effect of D-glucose on the inhibition by alloxan of ATP-dependent Ca accumulation. Glucose solutions were prepared in sucrose/imidazole buffer and mixed in equal volume with alloxan solutions of various concentrations prepared in the same buffer solution. The drug/glucose and drug/buffer solutions (as the control) were then preincubated in an equal volume with the microsomal fraction for 20 min at 37°C. Data are expressed as mean ± SD of three replicates.

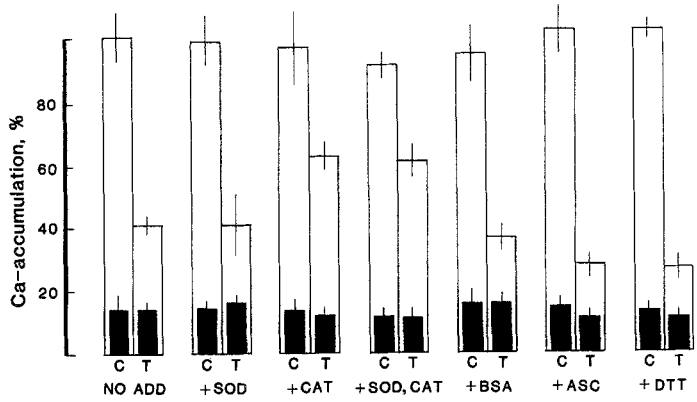

Fig. 6. Effect of free radical scavengers and other reagents on the alloxan inhibition of Ca^{2+} accumulation in the presence (□) and absence (■) of ATP. All reagents were prepared in sucrose/imidazole buffer and diluted with an equal volume of microsomal fraction or the vesicle buffer solution for a 20-min preincubation at pH 7.2, 37°C. T columns represent the values obtained in the presence of 1 mg/ml alloxan in the preincubation medium and C columns represent the control values obtained after the same period of incubation in the absence of alloxan. The final concentrations of the reagents when added to the preincubation medium were: superoxide dismutase (SOD), 200 units; catalase (CAT), 200 units; fatty acid free bovine serum albumin (BSA), 0.2%; sodium ascorbate (ASC) 1 mM; and dithiothreitol (DTT), 1 mM. Data are expressed as mean ± SD of four replicates. Similar results were obtained from two separate experiments.

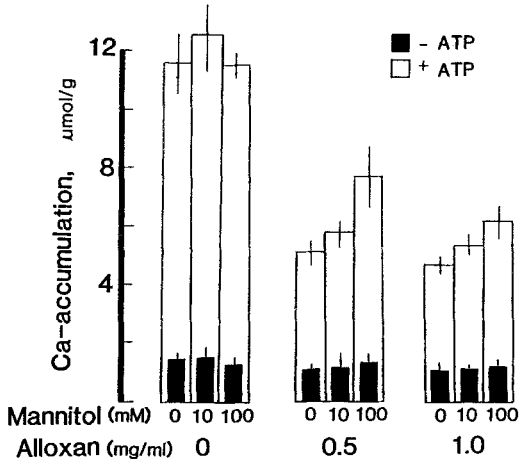

Fig. 7. Effect of preincubation of microsomes with mannitol on the inhibition by alloxan of the Ca^{2+} accumulation in the presence and absence of ATP. Preincubation and Ca^{2+} accumulation conditions are as described in the legand of Fig. 5.

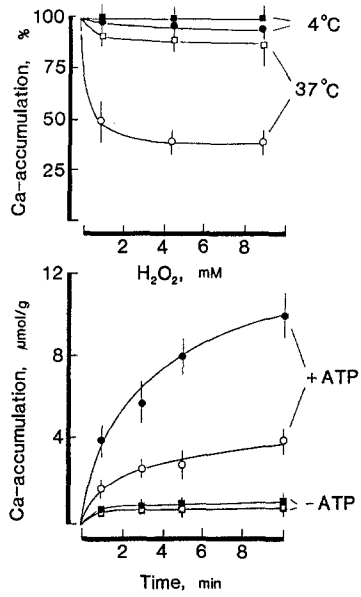

Fig. 8. Concentration, temperature, and time dependence of inhibition by hydrogen peroxide of the Ca^{2+} accumulation in the presence and absence of ATP. Preincubation conditions are as described in Fig. 3 and Table 3 except for the use of freshly prepared H_2O_2 instead of alloxan. Ca^{2+} accumulation was performed in the presence (circles) and absence (squares) of ATP as well as with (open symbols) and without (close symbols) preincubation of H_2O_2 (preincubation condition of 4.5 mM H_2O_2, at 37°C and pH 7.2, for 20 min, if not specified). Data are expressed as mean ± SD of four replicates in two separate experiments.

100 mM, but not 10 mM, mannitol in the preincubation mixture partially and significantly protected against the inhibition of Ca^{2+} by alloxan. Such a reversal by mannitol of alloxan inhibition seemed to be more pronounced at lower alloxan concentrations.

The protection offered by CAT as well as by mannitol from the inhibitory effect of alloxan on Ca^{2+} transport suggests the involvement of H_2O_2 intermediate in the inhibitory events caused by preincubation of membranes with alloxan. If it is indeed so, preincubation of microsomal membranes with H_2O_2 would also be expected to cause potent inhibition of the Ca^{2+} transport in the presence of ATP and have much less effect on Ca^{2+} binding in the presence of ATP. The results shown in Fig. 8 strongly support such a hypothesis. Furthermore, it also demonstrated that the inhibitory effect of H_2O_2 was also temperature dependent, as was the case for the inhibition of alloxan (see Table I). This was further supported by the finding (not shown) that addition of 4.5 mM H_2O_2, which caused about 60% inhibition of the ATP-driven Ca^{2+} transport (see Fig. 8), to microsomes

preloaded with Ca^{2+} in the presence of ATP did not accelerate the release of Ca^{2+} (not shown, but similar to the effect of alloxan on the Ca^{2+} efflux shown in Fig. 3).

Discussion

Major Novel Aspects

In this study, we have presented two major pieces of novel information. First, alloxan exerts an *in vitro* dose- and time-dependent inhibition of Ca^{2+} handling by isolated vascular smooth muscle microsomes. This inhibitory effect of alloxan is more pronounced at physiological temperature and more potent on ATP-driven Ca^{2+} transport than on Ca^{2+} binding in the absence of ATP. Second, we have demonstrated that such an inhibition by alloxan of the ATP-driven Ca^{2+} transport in vascular smooth muscle microsomes is not simply due to the direct effect of alloxan on the binding of Ca^{2+} or on the permeability of membrane vesicles to Ca^{2+}. Apparently, the inhibition of the energy-dependent Ca transport by alloxan is primarily due to the indirect action of alloxan via the formation of free radical intermediate, H_2O_2, and perhaps hydroxyl free radicals. This conclusion is based upon the observations that catalase and mannitol, a "scavenger" for H_2O_2 and hydroxyl free radicals, substantially protected the microsomes from alloxan inhibition of the Ca^{2+} transport and that H_2O_2 added directly *in vitro*, on the contrary, inhibited the Ca^{2+} in the absence of alloxan. Not only do these findings provide novel information about the toxic offects of alloxan acting directly on the Ca-transport function of vascular smooth muscle microsomes, but they also provide a biochemical basis at both the membrane and molecular levels for the mechanism action of alloxan.

Physiological Significance

Suppressed active transport of Ca^{2+} across cellular and intracellular membranes in vascular muscle under the influence of alloxan can conceivably lead to elevation of cytoplasmic Ca^{2+}, thus causing vasoconstriction (Macqueen, 1952; Aviado and Schmidt, 1957). Such an *in vivo* vasoconstriction caused by administration of alloxan, however, was not long lasting (Macqueen, 1952), presumably due to the rapid breakdown of alloxan in the blood and other adaptive responses (Webb, 1966), and this may explain the observation of a transient elevation of blood pressure after administration of alloxan (Macqueen, 1952). Whether the sustained mild hypertension, observed in experimental animals made chronically diabetic (>4 weeks) by a single bolus injection of a high concentration of alloxan

(Igarashi *et al.*, 1979), is causally related to the observations made in this study is an intriguing question. Nethertheless, altered vascular reactivity (Owen and Carrier, 1980; Pfaffman *et al.*, 1982; MacLeod and McNeill, 1985) and altered Ca^{2+} handling by intact vascular muscle strips (Turlapaty *et al.*, 1982) have indeed been reported in alloxan-induced diabetes. Furthermore, we and others have repeatedly demonstrated a diminished level of ATP-driven Ca transport across the vascular muscle membrane vesicles isolated from hypertensive animals as compared to the corresponding control groups (reviewed in Kwan, 1985). We are currently investigating the *in vivo* acute as well as chronic effect on alloxan on the Ca^{2+} transport and binding by vascular muscle microsomes isolated from rats treated with alloxan. The direct *in vitro* effects observed in the present study would serve as an important reference point for those studies of the *in vivo* effects of alloxan.

Subcellular Site of Action

Due to the low yield of smooth muscle membranes from vascular tissues, we used the heterogeneous microsomal fraction for general characterization on the effects of alloxan. It is therefore possible that alloxan could inhibit both plasma membrane and endoplasmic reticulum Ca pumps. However, it is likely that alloxan may act primarily on the cell membrane Ca pump in vascular smooth muscle for a number of reasons. First, we have previously shown that most of the Ca^{2+} transport and binding activities in the microsomal fractions isolated from mesenteric arteries of rat (Kwan *et al.*, 1979) or dog (Kwan *et al.*, 1983) was primarily due to the plasma membranes. Second, upon further subfractionation of vascular muscle microsomes on a sucrose density gradient to purify the plasma membrane fraction (Kwan *et al.*, 1983), the relative extent of inhibition by alloxan of ATP-driven Ca^{2+} transport in microsomes or enriched plasma membranes remained unaltered (data not shown). Third, Preincubation of crude vascular muscle microsomes (containing a high level of endoplasmic reticulum fragments) with dithiothreitol (DTT) enhanced the ATP-driven Ca transport (Muchlin *et al.*, 1978). DTT also protected against the inhibition of ATP-driven microsomal Ca transport during oxidative stress produced by peroxides (Jones *et al.*, 1983). In this study, DTT at its effective concentration, had no effect on the ATP-driven Ca transport and showed no protective effect against the alloxan-induced inhibition of the Ca transport. Finally, Grover and Samson (1987) have shown that superoxide anion free radicals generated by xanthine and xanthine oxidase preferentially inhibited the ATP-driven Ca^{2+} transport in endoplasmic reticulum-enriched fraction prepared from pig coronary artery. However, the lack of effect of SOD on the alloxan inhibition of the plasma

membrane-enriched microsomes from dog mesenteric artery does not support the involvement of superoxide anion free radicals. The difference between these two studies is unlikely due to the different arteries or animal species used, because similar findings of this alloxan effect were also observed in microsomes prepared from rat mesenteric artery (Beazley and Kwan, 1987) and dog aorta (unpublished observation). Microsomes prepared from both vascular tissues have been well characterized and shown to contain primarily plasma membranes (Kwan *et al.*, 1979, 1984, 1986; Kwan, 1986).

Role of Oxygen Free Radicals and Other Oxidative Products

Since both SOD and mannitol partially reversed the inhibition by alloxan of ATP-driven Ca transport, it is possible that hydroxyl free radicals, which can be generated from H_2O_2 nonenzymatically, may contribute to the inhibitory mechanism of alloxan. Participation of alloxan anion radicals was considered unlikely, because the formation of alloxan anion radicals requires the complexation of a relatively high concentration of divalent metal ions, such as Zn^{2+}, Mg^{2+}, and Cd^{2+} (Daul *et al.*, 1983), which were not present in the preincubation medium containing microsomal membranes and alloxan. The involvement of free radical-induced lipid peroxidation is also considered unlikely, because freshly isolated microsomal membranes from vascular muscle are resistant to lipid peroxidation (Kostka and Kwan, 1987). Besides, lipid peroxidation may be expected to cause a more generalized membrane damage rendering the membrane vesicles more permeable to Ca^{2+}. Our results do not support such a hypothesis. Alloxan apparently acts primarily on the Ca-pump molecule. Unfortunately, the current technical difficulties in unequivocally identifying the nature of Ca-ATPase responsible for the Ca-pump activity in vascular smooth muscle microsomes (Grover, 1985; Kwan *et al.*, 1986) preclude us from studying the effect of alloxan on the Ca-pump ATPase activity at this stage.

Comparison with Other Studies

To the best of our knowledge, the direct *in vitro* effect of alloxan on the Ca^{2+} transport by isolated microsomal membranes has not been reported previously in either vascular smooth muscle or other cell types. However, there is limited information concerning the effect of alloxan on the Ca^{2+} movement across liver mitochondria. Unlike the present findings using vascular muscle microsomes, alloxan did not inhibit the ATP-dependent uptake of Ca^{2+} by liver mitochondria *in vitro* (Frei *et al.*, 1985). Since redox cycling of alloxan is known to produce H_2O_2 (Deamer *et al.*, 1971; Ishibasshi and Howard, 1981; Grankvist, 1981; Cohen, 1984), as also suggested in the present study, the lack of effect of exogenously added H_2O_2 on the

mitochondrial Ca uptake (Levine *et al.*, 1983) is consistent with the lack of effect of alloxan on the mitochondrial Ca uptake (Frei *et al.*, 1985). The effect of alloxan on vascular muscle microsomes also differed from that on the liver mitochondria in other perspectives. For example, alloxan inhibited Ca^{2+} release from liver mitochondria (Nelson and Boquist, 1982). It was also demonstrated that redox cycling of alloxan was not involved in the inhibitory mechanism of mitochondrial Ca release, and oxidation of critical sulfhydryl groups seemed to be the key event (Frei *et al.*, 1985). This is further supported by the finding by Boquist (1984) that the mitochondrial Ca release induced by alloxan was inhibited by dithioerythritol.

Conclusions

The bulk evidence discussed above collectively suggests that the Ca-handling properties of different subcellular membranes may be affected differently by alloxan or associated oxygen free radicals. Our findings are consistent with the contention that cell membrane is the primary site of action of alloxan (Cooperstein and Watkins, 1981) or associated free radicals (Freeman and Crapo, 1982), and that any intracellular effects are probably secondary and not critical to its cytotoxicity. Our studies also support the contention that the cytotoxic effect of alloxan is not selective for the β-cells of Islet of Langerhans and provide further evidence for the free radical theory of the diabetogenic action of alloxan (Malaisse, 1982; Cohen, 1984).

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