The plasma-membrane component is the primary site of action of alloxan on ATP-driven Ca^{2+} transport in vascular-muscle microsomal fractions

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The direct effects of alloxan *in vitro* on the handling of Ca^{2+} by microsomal fractions from dog aortic smooth muscle were investigated. Preincubation of the vascular-muscle microsomal membranes with alloxan showed a suppression of both binding of Ca^{2+} (in the absence of ATP) and ATP-driven Ca^{2+} transport. Alloxan substantially inhibited the microsomal ATP-driven Ca^{2+} transport stimulated by P_i , but not that stimulated by oxalate. Studies using subfractions isolated from the microsomal membranes on a sucrose density gradient indicated that plasma membrane is the primary site of action of alloxan on the ATP-driven Ca^{2+} transport.

INTRODUCTION

In addition to its well-known diabetogenic action (Malaisse, 1982), administration of alloxan *in vivo* has long been shown to cause transient vasoconstriction at the site of injection and slight elevation of blood pressure (Webb, 1966; Macqueen, 1952). At high doses, alloxan has also been observed to cause pulmonary vasoconstriction in dogs (Aviado & Schmidt, 1957). The long-term vascular effect of alloxan *in vivo* also includes altered vascular reactivity (Cavaliere *et al.*, 1980) and defects in Ca^{2+} handling by vascular smooth muscles (Turlapaty *et al.*, 1982). To test the hypothesis that alloxan may exert a direct deleterious effect on the vascular-muscle membranes leading to derangement of the regulation of cytoplasmic Ca^{2+} , we have previously demonstrated in microsomes (microsomal fractions) isolated from rat small mesenteric arteries that alloxan, but not streptozotocin, elicited an inhibitory effect on ATP-driven Ca^{2+} transport (Kwan & Beazley, 1987). We have also shown that oxygen-derived free radicals generated from the cyclic oxidoreduction of alloxan and its product dialuric acid (Munday, 1988) were responsible for such an inhibitory effect (Kwan & Beazley, 1988). In the present study, we have further investigated these inhibitory effects of alloxan *in vitro* on the Ca^{2+} handling by microsomes and submicrosomal-membrane fractions isolated from dog aorta, with a specific aim to determine the primary subcellular site of action of alloxan.

MATERIALS AND METHODS

Animals and tissue trimming

Aortae from one or two mongrel dogs weighing 10–20 kg were used for each experiment. Dogs were killed by pentobarbital overdose, and the thoracic aortae were removed and placed in ice-cold 250 mM-sucrose solution buffered with 10 mM-imidazole, pH 7.2 (SUC/IM). The vasculature was trimmed to separate the medial smooth-muscle layer from the endothelium and adventitia, as previously described in detail (Kwan *et al.*, 1986).

Preparation of microsomal membranes

Isolated muscle strips were homogenized in SUC/IM

buffer (10 ml/g wet wt.) with a Polytron, and the homogenate was separated into various fractions by differential centrifugation as previously described (Kwan *et al.*, 1986). 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 10000 *g* for 10 min to remove the mitochondrial membranes and then at 100000 *g* for 40 min to sediment the crude microsomal membranes. The pellet was resuspended and centrifuged again at 10000 *g* for 10 min to remove further 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 the distribution of marker enzymes, to be highly enriched in plasma membranes, with slight contamination by endoplasmic reticulum (Kwan *et al.*, 1984, 1986).

Subfractionation of microsomes

Refined microsomal membranes (MC) in 8% (w/v) sucrose containing 10 mM-imidazole buffered at pH 7.2 were layered on top of a discontinuous sucrose density gradient (15%-, 30%-, and 60%-sucrose bands). After centrifugation at 105000 *g* for 120 min, membrane fractions F1, F2 and F3, formed as distinct bands respectively at the 8/15%-, 15/30%-, and 30/60%-sucrose interfaces, were collected from the top of the gradient by aspiration. The plasma-membrane contents in the microsomal subfractions were reflected by the enrichment of two separate plasma-membrane marker enzymes, 5'-nucleotidase and Mg^{2+} -ATPase, in these microsomal subfractions. These two enzymes have been established as plasma-membrane-associated enzymes in several smooth muscles (Kwan *et al.*, 1981, 1984, 1986). Protein content and enzyme activities were determined as previously described (Kwan *et al.*, 1984).

Preincubation of alloxan and other chemicals with membranes

Solutions of alloxan at the desired concentrations were prepared in 250 mM-sucrose buffered with 50 mM-imidazole at pH 7.2 (since alloxan solution is acidic, a higher concentration of imidazole is required to maintain the pH of the medium). Equal volumes of the drug solutions

Fig. 1. Time course of the ATP-dependent Ca^{2+} accumulation (Ca^{2+} uptake) in the presence of 5 mM-oxalate (□, ■) or 20 mM- P_i (▲, △) and in their absence (○, ●)

Symbols: □, △, ○, control microsomes (not treated with alloxan); ■, ▲, ●, microsomes preincubated with alloxan (1 mg/ml) at 37 °C for 20 min. Data are expressed as means $\pm$ S.D. for triplicates: * significantly different from control values ($P < 0.05$).

and microsomal membranes suspended in SUC/IM were mixed and incubated in a water bath with constant shaking and with the temperature controlled at 37 °C for 20 min (Kwan & Beazley, 1987). The control membrane fraction was treated similarly 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

This was done by a Millipore filtration technique (Kwan *et al.*, 1981, 1984, 1986). 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 $\text{Mg}^{2+} = 0.5$ mM), 100 μM total Ca^{2+} with trace amount of $^{45}\text{Ca}^{2+}$ (free $\text{Ca}^{2+} = 20$ μM), 0.5 mM- NaN_3 in 250 mM-sucrose solution buffered with 50 mM-imidazole at pH 6.8, 37 °C. In order to study the binding of Ca^{2+} under similar conditions, the medium for Ca^{2+} binding contained 0.5 mM- Mg^{2+} , 20 μM total Ca^{2+} and 0.5 mM- NaN_3 in the same buffer solution. NaN_3 was always included in the Ca^{2+} -accumulation media to eliminate the contribution to Ca^{2+} transport by any contaminating mitochondrial membranes. A 10 min incubation time was routinely used unless otherwise specified, because the optimal Ca^{2+} transport and binding occurred about 5–10 min after addition of microsomal membranes to the assay medium (Kwan *et al.*, 1984). Membrane fractions (50 μl, containing 15–25 μg of protein) were added to 200 μl of assay medium to start the Ca^{2+} accumulation, and 200 μl of the mixture was removed and filtered.

Fig. 2. Distribution of Ca^{2+} binding and transport (a) and of plasma-membrane marker-enzyme activities (b)

Data are expressed as means $\pm$ S.D. for two or three separate experiments with three replicates each. Microsomes (MC) and submicrosomal fractions (F1, F2 and F3) obtained from the sucrose density gradient were assayed for Ca^{2+} binding and transport with (■, ●, ▲) and without (□, ○, △) alloxan preincubation (1 mg/ml, for 20 min at 37 °C), and for marker enzyme activities.

RESULTS AND DISCUSSION

In pilot experiments, we demonstrated that alloxan inhibited the Ca^{2+} binding as well as the Ca^{2+} transport by dog aortic muscle microsomes in a time-, temperature- and dose-dependent manner. These results were very similar to those reported in our previous studies using microsomes isolated from rat (Kwan & Beazley, 1987) and dog (Kwan & Beazley, 1988) mesenteric arteries, and thus are not shown here. Since the microsomal fractions isolated from these vascular tissues including dog aorta were usually heterogeneous, containing variable amounts of subcellular membranes (Kwan *et al.*, 1981, 1983, 1984), we wished to determine the subcellular site of action of alloxan, i.e. plasma membranes or endoplasmic

Fig. 3. Distribution of Ca^{2+} binding and transport (a) and of plasma-membrane marker-enzyme activities (b) by sub-fractions isolated from control microsomes not treated with alloxan (□, ○, △) and microsomes preincubated with alloxan (1 mg/ml) at 37 °C for 20 min (■, ●).

Other conditions were the same as described in the legend of Fig. 2.

reticulum? This was done by three different approaches. First, the effect of alloxan on the stimulation of Ca^{2+} transport by oxalate or P_i anions was studied. P_i anions are known to stimulate preferentially the transport of Ca^{2+} into the plasma-membrane vesicles isolated from vascular (Kwan *et al.*, 1981; Kwan, 1985; Sharma & Bhalla, 1986) and non-vascular smooth muscles (Kwan, 1985; Raeymaekers *et al.*, 1983). However, the sub-cellular site of action of oxalate anions is still very obscure. Both endoplasmic reticulum (Raeymaekers *et al.*, 1983; Grover, 1985; Wibo *et al.*, 1981) and plasma-membrane vesicles in vascular (Kwan, 1985; Sharma & Bhalla, 1986) and non-vascular (Grover *et al.*, 1980; Batra, 1986) smooth muscles have been suggested. Fig. 1 shows that 5 mM-oxalate only slightly and significantly stimulated the Ca^{2+} transport at 10 min, but not at 40 min. On the other hand, P_i greatly stimulated Ca^{2+}

transport in a time-dependent manner (20 mM- P_i is required, owing to the lower solubility product of the Ca^{2+} -phosphate complex compared with the Ca^{2+} -oxalate complex). Alloxan substantially inhibited these anion-stimulated Ca^{2+} transports. These results confirmed the preferential effect of alloxan on the active-transport process, and further suggested that alloxan acts primarily on the plasma-membrane vesicles in the microsomal fraction.

The second approach involved the subfractionation of microsomes in a sucrose density gradient and to relate the pattern of inhibition by alloxan of these sub-microsomal fractions to the plasma-membrane content in these fractions. Fig. 2 shows very clearly that plasma membranes were further enriched about 4-fold over MC in fraction F2 (collected at the 15/30%-sucrose interface), as judged by the two plasma-membrane marker enzyme activities, thus confirming our previous finding in a more detailed study on the same tissue (Kwan *et al.*, 1984). In that study, we also showed that 5'-nucleotidase and Mg^{2+} -ATPase had a similar pattern of subcellular distribution, with the highest enrichment in F2 fraction. They were quite different from the distribution of several other internal-membrane marker enzymes, which showed the highest enrichment in F3 fraction (Kwan *et al.*, 1984). In the present study, a similar degree of inhibition of the Ca^{2+} binding (25–30 %) and Ca^{2+} transport (60–70 %) in the submicrosomal fractions and the close resemblance of the distribution of plasma-membrane marker enzymes and those of Ca^{2+} binding and transport strongly suggest that alloxan acts primarily on the plasma membranes.

The third approach involves the pretreatment of microsomes with alloxan and the examination of the Ca^{2+} binding and transport in the subfractions of the treated membranes isolated from the sucrose density gradient. Fig. 3 clearly indicated a finding similar to the observation shown in Fig. 2, i.e. both Ca^{2+} binding and transport are primarily associated with the vascular-muscle plasma membranes, and the major site of action of alloxan on Ca^{2+} transport seems to be the plasma-membrane vesicles. These results also suggest that the inhibitory effect of alloxan is irreversible, since the subfractions of pretreated microsomes collected from the gradient would have been highly diluted and contained little or no alloxan. The 5'-nucleotidase and Mg^{2+} -ATPase activities were not affected by alloxan treatment. Whether or not this reflects a selective effect of alloxan on the ion-transport function of the vascular-muscle plasma membranes requires investigation.

The results taken collectively from the above three approaches are consistent with the contention that the primary site of action of alloxan is the plasma membrane of the vascular muscle. Although our data do not completely rule out the possibility of its inhibitory action on the endoplasmic-reticulum Ca^{2+} pump, the finding of plasma membrane as the major membrane component in the microsomal fraction of dog aortic muscle in the present and previous studies (Kwan *et al.*, 1984, 1986; Kwan, 1985) make such a possibility less attractive. In the present study, we were not concerned with mitochondrial contamination in the microsomes, because NaN_3 was included in the incubation medium for the measurement of Ca^{2+} accumulation. Besides, both biochemical and histological studies have indicated that mitochondrial Ca^{2+} transport in vascular muscle was not

physiologically important in the regulation of cytoplasmic Ca^{2+} concentration (Vallieres *et al.*, 1975; Somlyo *et al.*, 1979). Nevertheless, there is indeed some information about the effect of alloxan on Ca^{2+} movement across the liver mitochondria. Unlike the present findings for vascular-muscle microsomes, alloxan did not inhibit the ATP-dependent uptake of Ca^{2+} (Frei *et al.*, 1985), but it inhibited the release of Ca^{2+} in liver mitochondria *in vitro* (Frei *et al.*, 1985; Nelson & Boquist, 1982; Boquist, 1984).

In conclusion, the present study has confirmed our previous finding of an inhibitory action of alloxan *in vitro* on the Ca^{2+} binding and transport by microsomes isolated from small arteries (Kwan & Beazley, 1987, 1988). Our finding has also provided supportive evidence for the subcellular site of action of alloxan. Since alloxan has commonly been used to induce diabetes mellitus via bolus intravenous injection at high concentrations (65–100 mg/kg), our finding that alloxan at 0.5 mg/ml could cause > 50% inhibition of the ATP-driven Ca^{2+} transport would be of pharmacological and toxicological relevance. Such a direct inhibitory action of alloxan on the Ca^{2+} binding and transport of vascular smooth muscle may contribute to the local vasoconstriction induced by the intravenous administration of alloxan (Macqueen, 1952; Aviado & Schmidt, 1957).

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