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PROTECTIVE ACTION OF METHYLGLYOXAL BIS (GUANYLHYDRAZONE) ON THE MITOCHONDRIAL MEMBRANE

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Abstract—At low concentrations (0.5–1.0 mM) methylglyoxal bis (guanylhydrazone) (MGBG) exhibited a clearcut protection of rat liver mitochondria against the deenergizing action of either Ca^{2+}, or oxidizing agents (butylhydroperoxide and oxaloacetate). Such a protection resulted from the prevention of transmembrane potential decay, discharge of accumulated Ca^{2+}, release of mitochondrial Mg^{2+}, adenine nucleotides and pyridine nucleotides and mitochondrial swelling. At high concentrations (5–10 mM) MGBG induced functional alterations of mitochondria (decrease of transmembrane potential, lower capability to accumulate and to retain Ca^{2+}) which can be reversed by resuspension of mitochondria in a MGBG free medium. These reversible mitochondrial alterations by high MGBG concentrations are interpreted as a consequence of an aggregation and coprecipitation of suspended mitochondria.

The interest in methylglyoxal-bis-(guanylhydrazone) (MGBG) as an antiproliferative agent arose when it was recognized to be an inhibitor of *S*-adenosyl methionine decarboxylase (EC 4.1.1.50) therefore capable of restricting the rate of spermidine and spermine biosynthesis [1]. A less specific effect of the drug is to induce functional and structural alterations in mitochondria [2, 3]. Indeed the results obtained by Byczkowski *et al.* [3] indicate that MGBG exhibits a broad damaging action on isolated mitochondria. Mitochondrial energy-linked processes, membrane integrity and the activity of some membrane-linked enzymes, such as CPT and CAT, are all impaired to some extent [4]. A possible explanation is that the drug functions as positively charged MGBG and interacts with the negatively charged surface of the inner mitochondrial membrane [3]. It has been suggested that these effects in mitochondria are the first to occur. Thus according to Pathak *et al.* [5] injury to mitochondria of L1210 leukemia cells precedes the drop in polyamine concentrations and consequent inhibition of cell growth. The observations have always been made using rather high concentrations of the drug (in the 3–20 mM range).

We now report that, at lower concentrations (0.5–1.0 mM), much closer to those presumably attainable in tissues upon MGBG administration [6], MGBG failed to induce the previously reported adverse effects on mitochondria. On the contrary the drug exhibited a clearcut protection against the deenergizing action of either Ca^{2+} or oxidizing agents (butylhydroperoxide [7] or oxaloacetate [8]). The action of MGBG on mitochondrial functions is thus strictly dependent on the concentration of the drug in the suspending medium.

MATERIALS AND METHODS

Rat liver mitochondria were isolated in 0.25 M sucrose and 5 mM Hepes* (pH 7.4) by the conventional centrifugation method. Mitochondrial protein concentration was assayed by a biuret method with bovine serum albumin as standard. Mitochondrial incubations were carried out at 20° with 1 mg mitochondrial protein/ml in the following standard medium: 200 mM sucrose or 100 mM KCl, 10 mM Hepes (pH 6.8), 2 mM Pi, 5 mM succinate, 2.5 μM rotenone, 15 μM $CaCl_2$. Other additions or modification are indicated in the descriptions of specific experiments.

Membrane potential ($\Delta\psi$) was measured in a water-jacketed, thermostatically controlled vessel equipped with a magnetic stirrer, by monitoring the distribution of the lipophilic cation tetraphenylphosphonium (TPP^+) across the mitochondrial membrane with a selective electrode prepared in our laboratory [9, 10] and an Ag/AgCl reference electrode. TPP^+ was included at concentrations of 2 μM in order to achieve high sensitivity in measurements and to avoid toxic effects on proton ATPase activity [11] and calcium movements [12]. The membrane potential measured with the TPP^+ selective-electrode was calibrated according to the equation: $\Delta\psi = (\Delta\psi \text{ electrode} - 66.16 \text{ mV})/0.92$ as proposed by Jensen *et al.* [13].

Mitochondrial matrix volume was calculated from the distribution of [^{14}C]sucrose and ^3H_2O according to Palmieri and Klingenberg [14].

* Abbreviations used: CPT, carnitine palmitoyl transferase; CAT, carnitine acetyl transferase; EGTA, ethylene-bis-(oxyethilenenitrilo) tetracetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid; ΔE, electrode potential; Pi, inorganic phosphate.

During $\Delta\psi$ measurements, samples of 1 ml of mitochondrial suspension were withdrawn at the indicated time intervals, placed in 1.5 ml Eppendorf 5414 centrifuge tubes and centrifuged for 1 min at 12,000 rpm. The supernatant was used for Mg^{2+} and Ca^{2+} determinations by atomic absorption spectroscopy [15]. The pellet was washed twice with a cold solution of 0.25 M sucrose and 5 mM Hepes. Nine-hundred and fifty microlitres of water was added, the suspension was shaken in Vortex until complete solubilization of the pellet and then 50 μl of 70% PCA was added. The samples were cooled for 2 min and 130 μl of a solution of 3 M K_2CO_3 in 2 M Tris pH 7.4 was added. After shaking and pH control (about 6.3) the samples were centrifuged on Eppendorf 5414 for 2 min at 12,000 rpm, the supernatant was withdrawn and placed in freezer for 24 hr. Individual endogenous adenine and pyridine nucleotides in PCA extracts were measured using high performance liquid chromatography (HPLC) by a Beckman 110A liquid chromatograph.

A 10 μm Ultrasil AX ion-exchange column (25 cm × 4.6 mm i.d.) was used. The elution was performed by a gradient of 10 mM KH_2PO_4 pH 4 and 300 mM K_2HPO_4 pH 6.5. The concentrations of the individual nucleotides were calculated by comparison with the elution pattern of known mixtures of adenine and pyridine nucleotides.

Mitochondrial swelling was monitored at 540 nm in a Perkin Elmer Lambda 5 spectrophotometer.

Ruthenium red was purified according to Luft [16]. The solutions of the dye were prepared daily and the concentrations of ruthenium red were determined spectrophotometrically on the basis of an ε of 68 $mM^{-1}\,cm^{-1}$ at 533 nm [17].

RESULTS

As reported in Fig. 1A, 1 mM MGBG added to rat liver mitochondria incubated in sucrose–Hepes medium, in the presence of 50 μM Ca^{2+} and 2 mM Pi (A) fully prevented the collapse of the transmembrane potential ($\Delta\psi$) otherwise induced by these ions [18]. An analogous protective action has been observed when rat liver mitochondria were incubated in the presence of either 50 μM *tert*-butylhydroperoxide or 0.1 mM oxaloacetate (Fig. 1, B and C). Both EGTA, a Ca^{2+} chelator and ruthenium red, an inhibitor of Ca^{2+} uptake, not only prevented, as expected, the decay of $\Delta\psi$ induced by Ca^{2+} and Pi, but also that produced by *tert*-butylhydroperoxide or oxaloacetate. This indicates that in the absence of Ca^{2+}, or when the transport of Ca^{2+} ions into mitochondria was inhibited, both *tert*-butylhydroperoxide and oxaloacetate are without effect. Very similar results have been obtained in a KCl–Hepes medium (see dashed lines in Fig. 1).

As shown in Fig. 2 the action of MGBG on $\Delta\psi$ of liver mitochondria suspended in a medium containing 50 μM Ca^{2+} and 2 mM Pi was dose dependent. The same dependence has also been observed in *tert*-butylhydroperoxide or oxaloacetate-treated mitochondria (results not reported).

It may be noted that the maximum protection of MGBG on $\Delta\psi$ was attained at 1 mM concentration.

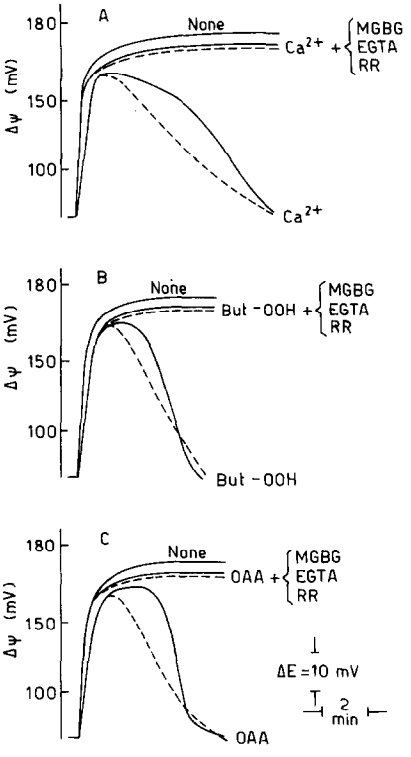

Fig. 1. Protective action of MGBG on the collapse of $\Delta\psi$ in damaging conditions induced by Ca^{2+}, *tert*-butylhydroperoxide and oxaloacetate in the presence of phosphate. Rat liver mitochondria were incubated in sucrose Hepes (full lines) or in KCl–Hepes medium (dashed lines) as described in Materials and Methods. When present 1 mM MGBG, 1 mM EGTA and 0.16 μM ruthenium red (RR). 50 μM $CaCl_2$ (A), 50 μM tert-butylhydroperoxide (But-OOH) (B), 100 μM oxaloacetate (OAA) (C). The results reported in Figs 1, 3 and 5 were obtained from the same incubations.

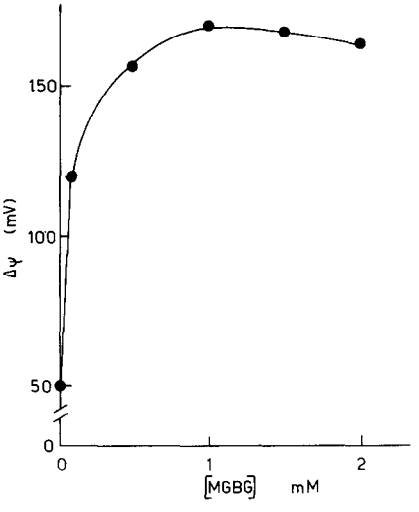

Fig. 2. Dose–response curve of MGBG protective action on the collapse of $\Delta\psi$ induced by Ca^{2+} and phosphate. $\Delta\psi$ values have been measured upon 9 min of incubation of liver mitochondria in the presence of 50 μM Ca^{2+} and 2 mM Pi (see conditions in Fig. 1A), in the absence (full deenergization) and in the presence of increasing amounts of MGBG.

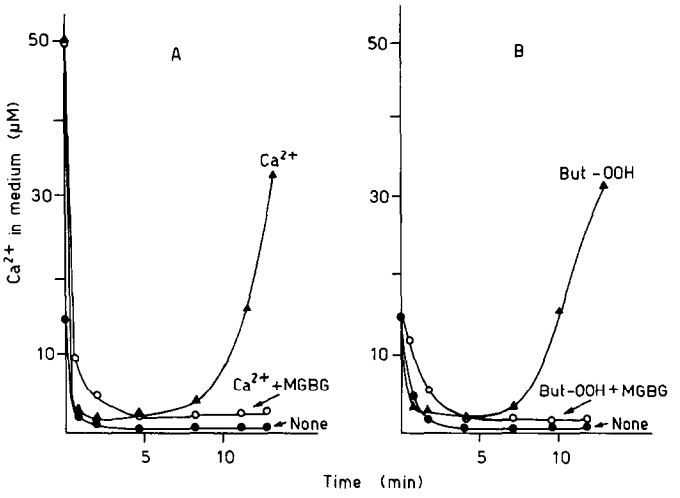

Fig. 3. Action of MGBG on calcium efflux induced by damaging conditions. Rat liver mitochondria were incubated in the same conditions as in Fig. 1 A and B.

Above 5 mM concentrations, $\Delta\psi$ was not apparently fully preserved (see below).

The stabilizing action on the mitochondrial inner membrane was confirmed by the observation that MGBG strongly delayed the discharge of accumulated Ca^{2+} from liver mitochondria exposed to the same damaging agents (the results relative to oxaloacetate are not reported) (Fig. 3). (This action on Ca^{2+} retention shows that MGBG does not interfere with the recording of $\Delta\psi$ by the TPP^+ selective electrode.)

Additional evidence for the protective action of MGBG on the mitochondrial inner membrane was provided by results (reported in Fig. 4) showing that under the described conditions MGBG completely prevented mitochondrial swelling induced by Ca^{2+}, *tert*-butylhydroperoxide and oxaloacetate.

As previously demonstrated [18–20] alterations of the mitochondrial membrane leading to $\Delta\psi$ decay and loss of capability to retain accumulated Ca^{2+} also induce a release of mitochondrial components such as endogenous adenine and pyridine nucleotides and Mg^{2+}. As shown in Fig. 5, 1 mM MGBG fully prevented the efflux of these compounds when mitochondria were exposed to *tert*-butylhydroperoxide. Similar results were obtained in the presence of Ca^{2+} and Pi ions or in the presence of oxaloacetate (results not reported).

As shown in Fig. 6 MGBG not only prevented but also reversed the effects of Ca^{2+}, *tert*-butylhydroperoxide or oxaloacetate on $\Delta\psi$, provided that ATP was also added. The full restoration has been achieved with 1 mM MGBG; at lower concentrations dose-related effects were observed (Fig. 6A). In spite of the ATP requirement the restorative effect of MGBG was not abolished by atractyloside, the known inhibitor of adenylate translocase.

In the presence of high MGBG concentrations, in the range of those employed by the previous workers [3], mitochondria were unable to attain normal $\Delta\psi$ values, even in the absence of any damaging agent (Ca^{2+} or oxidants). Thus in the presence of 10 mM MGBG the maximum $\Delta\psi$ value reached upon energization with 5 mM succinate was much below that attained in the absence of the drug. In spite of this lower value, $\Delta\psi$ was maintained constant for a considerable length of time. Since, even in the presence of 10 mM MGBG, $\Delta\psi$ did not collapse, a decrease in the active mitochondrial surface (e.g. because of mitochondrial aggregation [3]) is indi-

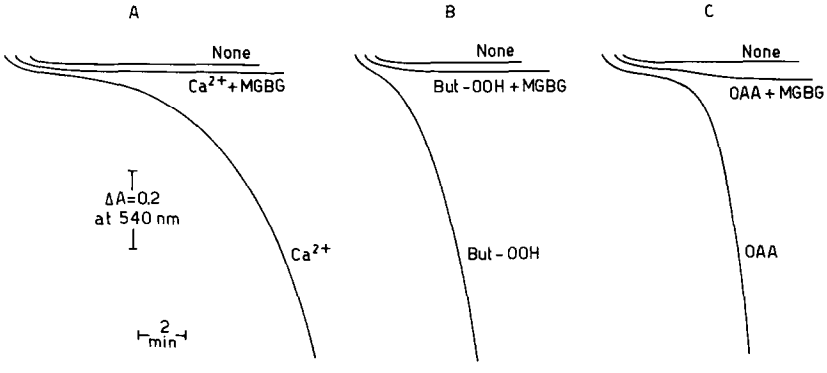

Fig. 4. Action of MGBG on mitochondrial swelling induced by damaging conditions. Rat liver mitochondria were incubated in the same conditions as in Fig. 1.

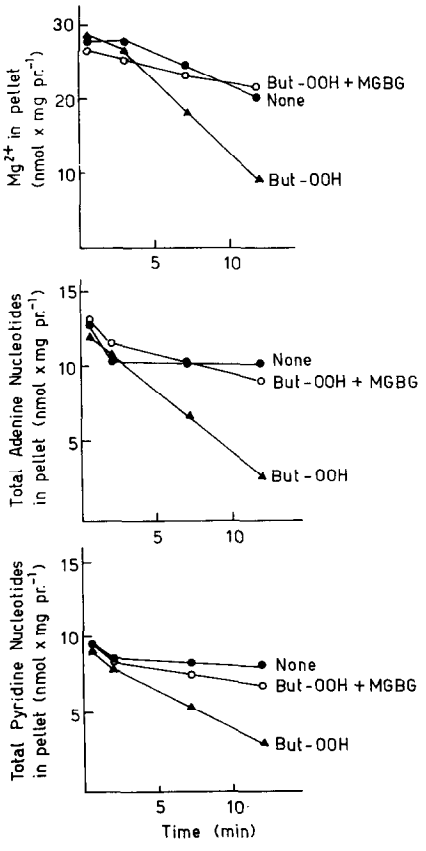

Fig. 5. Protective action of MGBG on the efflux of Mg^{2+}, adenine and pyridine nucleotides induced by *tert*-butylhydroperoxide. Rat liver mitochondria were incubated in the same conditions as in Fig. 1B. To simplify the graphs, individually measured nucleotides have been reported in total amounts.

cated rather than a progressive alteration of the permeability properties of the inner membrane. Such an assumption was further supported by the behaviour of the Ca^{2+} transport systems. As it can be seen in the insert to Fig. 7, in the presence of 10 mM MGBG mitochondria were still able to take up Ca^{2+} from the medium although to a much smaller extent than normal mitochondria. Moreover accumulated Ca^{2+} was also retained, again showing the conservation of membrane potential.

Finally, resuspension in a standard medium of mitochondria pretreated with 10 mM MGBG brought about a significant increase in $\Delta\psi$, a result compatible with a reversible aggregation rather than an irreversible alteration of the membrane. Such an assumption is further supported by the results in the insert to Fig. 7, showing that also the extent of Ca^{2+} uptake by MGBG treated mitochondria was substantially enhanced upon resuspension of mitochondria in an MGBG free medium.

DISCUSSION

The results reported in the present paper show that MGBG at 0.5–1.0 mM concentrations has a protective action on the energy linked processes of mitochondria exposed to noxious conditions such as high Ca^{2+} concentrations or oxidizing agents like *tert*-butylhydroperoxide or oxaloacetate.

These oxidants have been recognized noxious to mitochondrial membrane as a consequence of the enhanced Ca^{2+} cycling [21]. In particular, as confirmed in the present paper (Fig. 1), Bellomo *et al.* [21] have found that membrane damage by *tert*-butylhydroperoxide is prevented by ruthenium red.

Considering that MGBG considerably decreases the rate of both Ca^{2+} influx and efflux in liver mitochondria [22], thus considerably dampening Ca^{2+} cycling, it may be inferred that the protective action of MGBG, even against the noxious effects of oxidants, is primarily related to the action of Ca^{2+}.

In the presence of ATP, MGBG can reverse, as well as prevent, the effect of Ca^{2+} on transmembrane potential whereas ruthenium red cannot do this. MGBG must therefore have some additional, albeit undefined, effect which is also dependent on the presence of ATP. Since the synergistic effect of added ATP was not abolished by atractyloside, ATP acts, probably as MGBG does, on the external domain of the inner membrane.

Since the replacement of sucrose with KCl does not substantially effect both the protective (Fig. 1) and the restorative (results not reported) effects of

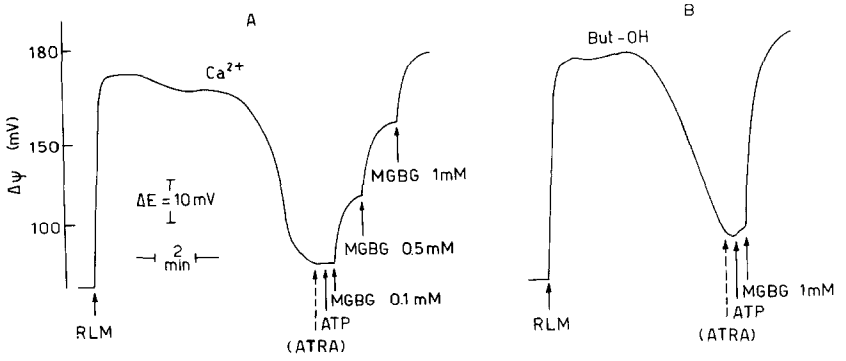

Fig. 6. Restoration of $\Delta\psi$ induced by MGBG in fully deenergized mitochondria. Rat liver mitochondria were incubated in the standard medium as described in Materials and Methods. When present $CaCl_2$ 50 μM (A) and 50 μM *tert*-butylhydroperoxide (B). At the arrows additions of 0.4 mM ATP and MGBG at various concentrations as indicated. When added: 10 μM atractyloside (ATRA).

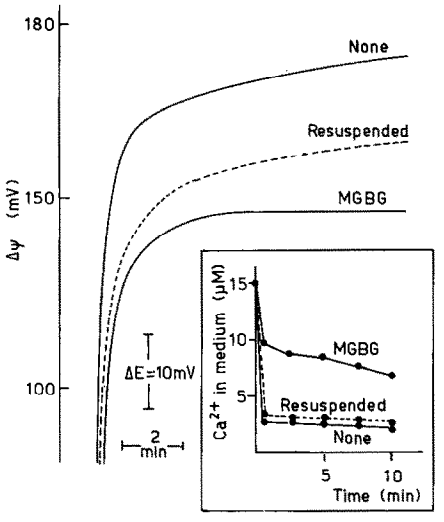

Fig. 7. Reversible action of MGBG at high concentration on $\Delta\psi$ and Ca^{2+} accumulation. Rat liver mitochondria were incubated in the standard medium as described in Materials and Methods at pH 7.4. When present: 10 mM MGBG. Dashed line represents $\Delta\psi$ and Ca^{2+} accumulation (see insert) of mitochondria previously incubated for 2 min in the presence of 10 mM MGBG, centrifuged at 12,000 *g* for 10 min and resuspended in the same medium without MGBG. The results reported in the insert were obtained from the same incubations of $\Delta\psi$ measurements as reported in Materials and Methods.

MGBG, the described action of this agent seems to be independent on the composition of the suspending medium.

When exposed to high MGBG concentrations (above 5 mM) mitochondria attain a lower but stable $\Delta\psi$. This circumstance and the capability of mitochondria treated with MGBG concentration to accumulate and to retain Ca^{2+}, although at lower extent than normally, would indicate a decrease of the overall surface of suspended mitochondria as might occur upon aggregation as described by Byczkowski *et al.* [3]. Indeed an irreversible alteration of membrane permeability properties would imply a progressive decay of both $\Delta\psi$ and the capability to accumulate and retain Ca^{2+}.

The concept that mitochondrial aggregation may limit but not suppress the energy-linked properties of mitochondria is also supported by the observation that both $\Delta\psi$ and the capability to accumulate Ca^{2+} increase towards its normal value when mitochondria pretreated with high MGBG concentrations are subsequently resuspended in a drug free standard medium (Fig. 7).

These two effects of MGBG (protecting at low concentrations and aggregating at high concentrations) could both be a common consequence of an electrostatic interaction between the drug cation and the negatively charged external surface of the inner membrane. This view is also supported by the finding that on removing most of the drug (by resuspension on fresh medium) the mitochondria largely regain their original properties. The extent of such an interaction might either increase the stability of the inner mitochondrial membrane antagonizing the effects of high Ca^{2+} concentrations, or induce an aggregation and coprecipitation of suspended mitochondria.

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