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ENHANCED UPTAKE OF SPERMIDINE AND METHYLGLYOXAL-BIS(GUANYLHYDRAZONE) BY RAT LIVER MITOCHONDRIA FOLLOWING OUTER MEMBRANE LYSIS

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Abstract—Isolated rat liver mitochondria rapidly bound the ^{14}C-labeled organic cations spermidine, a physiologically important polyamine, and methylglyoxal-bis(guanylhydrazone) (MGBG), an anticancer drug. This rapid, Mg^{2+}-sensitive, respiration-independent binding is assumed to involve adsorption to anionic surface groups. A slower progressive uptake of the organic cations exhibited respiration dependence, indicating that it involves transport across the inner mitochondrial membrane into the matrix compartment. Addition of digitonin, to lyse the outer mitochondrial membrane, caused an increase in the mitochondrial content of the organic cations and enhanced the rate of progressive, respiration-dependent cation uptake. The data are consistent with the interpretation that the outer mitochondrial membrane limits access of the organic cations, spermidine and MGBG, to the inner mitochondrial membrane. This conclusion is supported also by published data indicating that outer membrane lysis enhances inhibitory effects of the organic cations on mitochondrial respiration. The uptake of spermidine by mitochondria was inhibited by MGBG.

The anticancer drug methylglyoxal-bis(guanylhydrazone) (MGBG), which is structurally similar to the physiologically important polyamine spermidine, blocks cellular synthesis of spermidine and the related polyamine spermine, by inhibiting enzymic decarboxylation of *S*-adenosylmethionine [1]. Various organic cations, including antitumor drugs such as MGBG and adriamycin, the polyamines spermidine and spermine, and alkyl guanidines, inhibit respiratory chain activities of isolated mitochondria [2–10]. Cytotoxic effects of the cationic drugs have been attributed in part to inhibitory effects on oxidative phosphorylation [3, 4, 8, 11]. In addition to directly inhibiting respiration, MGBG inhibits carnitine-dependent oxidation of long chain fatty acids [12].

At submillimolar concentrations, spermine partially restores respiratory control and stimulates state 3 respiration in heat-aged mitochondria [13]. Spermine also alters kinetics of Ca^{2+} transport into and out of mitochondria [14–16], and activates ATPase activity in submitochondrial particles [17]. Slow, respiration-dependent uptake of [^{14}C]spermine by rat liver mitochondria, accompanied by enhanced uptake of P_i, has been demonstrated [18, 19].

Lysis of the outer mitochondrial membrane via treatment with digitonin or osmotic shock has been found not to affect significantly the integrity of the inner membrane, although slight uncoupling has been demonstrated [9, 20–23]. Mitoplasts prepared by digitonin lysis of the outer membrane retain most of their endogenous K^+ [23]. Pretreatment of rat liver mitochondria with digitonin or osmotic shock increases their susceptibility to respiratory inhibition by cationic drugs and polyamines [9].

Voltage-dependent, high conductance channels have been identified in, and purified from, outer membranes of mitochondria from a variety of tissues and species, including rat liver [24–27]. Estimates of the diameter of the open channel range from about 2 to 4 nm [25–28]. The reconstituted channels exhibit greater permeability to anions than to cations [24–27].

The present studies examined the effects of digitonin and the respiratory chain inhibitor antimycin A on the uptake of ^{14}C-labeled spermidine and MGBG by rat liver mitochondria. Some of this work has been presented in abstract form [29], and a brief meeting report has summarized related studies [30].

MATERIALS AND METHODS

Mitochondria were isolated from rat liver by standard procedures, in medium containing 225 mM mannitol, 75 mM sucrose, and 2 mM sodium EDTA, pH 7, supplemented during the initial stages of preparation with 0.1% bovine serum albumin (fatty acid free, Sigma Chemical Co.). Mitochondrial protein was measured by the biuret procedure [31]. Mitochondria (5.6 to 7.0 mg protein/ml) were incubated at 20° in medium containing, unless specified otherwise, 240 mM mannitol, 9 mM KCl, 4.4 or 24.4 mM

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$MgCl_2$, and 9 mM P_i plus 22 mM succinic acid (adjusted to pH 7.0 with NaOH). Radioisotope tracers added were ^3H_2O (about 1.5 μCi/ml, NEN Products), and [^{14}C]spermidine (about 0.3 μCi/ml, NEN Products or Amersham Corp.) or [^{14}C]MGBG (about 0.4 μCi/ml, Amersham Corp.). For some samples in some experiments, [^{14}C]sucrose or [^{14}C-*carboxy*]dextran (NEN Products) was substituted for the labeled cation. Media included 5 mM spermidine and/or MGBG (Sigma Chemical Co.). These concentrations may be considered close to physiologically relevant levels, based on reports of the spermidine contents of animal tissues and the known ability of cells to concentrate polyamines and MGBG [e.g. see Refs. 6 and 32–38]. When added, digitonin (Fisher Scientific Co.) was at 0.1 mg/mg protein, and antimycin A (Sigma Chemical Co.) at 0.2 μg/ml (31–35 ng/mg protein).

Mitochondria were separated from the incubation medium by centrifugation through silicone into 15% $HClO_4$ [39], using Eppendorf or Beckman microcentrifuges. Where incubation time is specified, it refers to the time when the microcentrifuge was started. The actual time of separation of mitochondria from the incubation medium would be 15–30 sec later. Radioisotopes were assayed by liquid scintillation counting. The distribution space of each labeled compound was calculated from the counts in the acid layer and the supernatant specific activity, as in previous studies [40]. Distribution spaces presented correspond to 0.2-ml samples of the incubation mixture, or 1.1 to 1.4 mg protein. About 20% of [^{14}C]MGBG counts sedimenting with the mitochondria were found to adhere to the denatured membranes pelleted below the $HClO_4$. Therefore each pellet, as well as the acid extract, was counted to determine the total uptake of MGBG. The other labeled compounds were found not to bind to the denatured membranes in the presence of $HClO_4$. In some experiments, the K^+ content of mitochondrial samples was determined by atomic absorption analysis of the $HClO_4$ extracts. For the values reported, K^+ in the sucrose penetrable space was subtracted from the total K^+ sedimenting with the mitochondria.

RESULTS AND DISCUSSION

The data of Table 1 show rapid initial binding of each of the labeled cations, spermidine and MGBG, followed by additional uptake during the subsequent 4 min of incubation. Spermidine and MGBG distribution spaces equalled or exceeded the distribution space of ^3H_2O under all conditions tested. [^{14}C]Sucrose, which is sometimes used as a marker for the fluid space external to the mitochondrial matrix [39], was found to penetrate approximately 70% of the water space under the conditions of these experiments. [^{14}C-*carboxy*]Dextran, in the size range 50,000–70,000 daltons, used as a marker for entrained medium external to the outer mitochondrial membrane, was found to penetrate approximately 40–45% of water sedimenting with the mitochondria under equivalent conditions. Distribution ratios greater than 1.0 for the organic cations could result from accumulation against a gradient and/or adsorption to membrane surfaces. The rapidity and lack of respiration dependence (see below) of the initial uptake of each organic cation are consistent with the interpretation that this uptake, in excess of that in the entrained medium, is at least partly due to adsorption to anionic membrane groups.

These studies have, in part, been aimed at testing whether enhanced uptake of the organic cations, corresponding to penetration of the outer mitochondrial membrane, results following lysis of the outer membrane with digitonin, as predicted by earlier findings [9]. The space between the inner and outer membranes corresponds to about 30% of the total water space under the conditions studied. The ability to detect uptake into this space is limited by the background of surface binding of the labeled cations. The electrophoretic mobility of mitochondria is altered by polyamines and MGBG, suggesting binding of these organic cations to negative surface charges [6, 7]. Experiments such as those shown in Table 1 have examined whether the sensitivity of the uptake assays could be increased by decreasing surface adsorption via addition of inorganic cations to compete with the organic cations for binding to anionic membrane groups. As shown in Table 1, increasing the Mg^{2+} content of the medium decreased both the initial and progressive uptakes of spermidine and MGBG. In most of the remaining experiments, media were used containing the higher concentration of Mg^{2+}.

As shown in Fig. 1, addition of digitonin, at a concentration sufficient to make the outer mitochondrial membrane permeable to cytochrome *c* [9], caused an additional uptake of labeled spermidine.

Table 1. Effect of increased Mg^{2+} on uptake of the organic cations spermidine and MGBG

Expt.	Labeled cation	Mg^{2+} (mM)	min	Cation uptake (nmol/mg protein)	Cation space / Water space
A	Spermidine	4.4	0.5	14.5 ± 0.7	1.31 ± 0.02
		4.4	5.0	20.4 ± 0.6	1.71 ± 0.03
		24.4	0.5	9.5 ± 0.5	1.03 ± 0.01
		24.4	5.0	11.0 ± 0.1	1.13 ± 0.04
B	MGBG	4.4	0.5	24.2 ± 0.6	2.31 ± 0.07
		4.4	5.0	41.8 ± 3.7	3.64 ± 0.08
		24.4	0.5	17.4 ± 0.6	1.82 ± 0.03
		24.4	5.0	24.9 ± 1.1	2.48 ± 0.13

Values shown are means of three determinations ± SD.

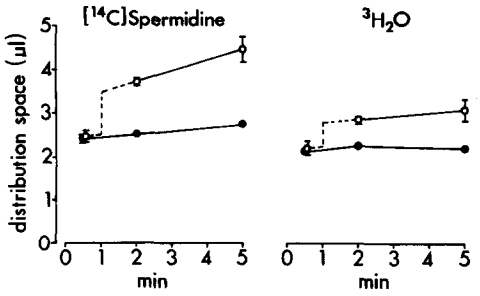

Fig. 1. Effect of digitonin addition on spermidine uptake. The medium contained 24.4 mM Mg^{2+}. The distribution spaces of [^{14}C]spermidine and ^3H_2O are plotted as a function of incubation time. The values shown are means of three determinations ± SD. Symbols: (●) control samples; and (○) digitonin was added at 1 min.

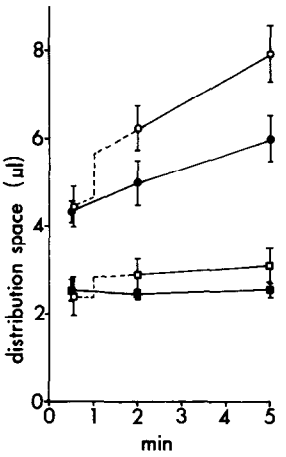

Fig. 2. Effect of digitonin addition on MGBG uptake. The medium contained 24.4 mM Mg^{2+}. Distribution spaces of [^{14}C]MGBG (○, ●) and ^3H_2O (□, ■) are plotted as a function of incubation time. The values shown are averages of distribution spaces determined in three separate experiments ± SD. The solid symbols (●, ■) represent control samples, whereas the open symbols (○, □) depict samples to which digitonin was added at 1 min.

The slower increase in spermidine content, which followed the initial rapid binding, was also enhanced after digitonin addition. The observed increase in the total water space may result from an increase in entrained medium, as the cristae become everted following digitonin treatment [20]. The matrix volume, estimated from the difference between ^3H_2O and [^{14}C]sucrose distribution spaces, showed no significant change with the digitonin treatment. In the experiment of Fig. 1, the matrix volume corresponding to a 1.12 mg protein sample, after 5 min of incubation, was 0.68 to 0.74 µl for control mitochondria and 0.72 to 0.75 µl for digitonin-treated mitochondria. Measurements of K^+ sedimenting with the mitochondria indicate retention of endogenous K^+ following the digitonin addition, consistent with earlier studies [23]. In the experiment of Fig. 1, after 5 min of incubation the mitochondrial K^+ content was 61 ± 5 nmol/mg protein for control samples and 70 ± 7 for digitonin-treated samples. Thus, the inner mitochondrial membrane permeability to both sucrose and K^+ does not appear to be grossly altered by the digitonin treatment under the conditions of these experiments.

When the change in the average spermidine distribution space was compared to the change in the average water space, it was apparent that there was a greater increase in the spermidine space. This difference may be interpreted as reflecting penetration of spermidine into the space between the inner and outer membranes, and perhaps binding to now accessible membrane sites facing this compartment. A similar effect of digitonin on the uptake of MGBG is shown in Fig. 2.

Tables 2 and 3 examine the effect of the respiratory chain inhibitor antimycin A on uptake of spermidine and MGBG, in the presence and absence of digitonin. Spermidine and MGBG themselves cause only partial inhibition of respiration (less than 50%) under the conditions tested [9]. Antimycin A blocked the progressive uptake of each of the organic cations in the absence of digitonin and prevented the enhanced rate of uptake following digitonin addition. A similar effect of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone in inhibiting the progressive uptake of spermidine has been observed [30]. Activity of the respiratory chain, which is localized in the inner mitochondrial membrane, is associated with outward transport of protons [41]. The resulting electrical gradient is considered to be the driving force for accumulation of permeant cations in the

Table 2. Effect of antimycin A on spermidine uptake in the presence and absence of digitonin

Additions	min	Spermidine space (µl)	Water space (µl)	ΔSp. space ± digitonin (µl)	ΔW space ± digitonin (µl)
None	1	2.13 ± 0.12	1.89 ± 0.05		
	5	2.31 ± 0.10	2.00 ± 0.07		
Digitonin	1	3.45 ± 0.15	2.68 ± 0.14	1.32	0.79
	5	4.15 ± 0.05	2.93 ± 0.11	1.84	0.93
Antimycin A	1	2.04 ± 0.06	1.88 ± 0.03		
	5	2.05 ± 0.05	1.89 ± 0.08		
Anti. A + Dig.	1	3.27 ± 0.16	2.75 ± 0.12	1.23	0.87
	5	3.25 ± 0.16	2.77 ± 0.13	1.20	0.88

All reagents were present in the medium from zero time. The Mg^{2+} concentration was 24.4 mM. Values shown are means of three determinations ± SD.

Table 3. Effect of antimycin A on MGBG uptake in the presence and absence of digitonin

Additions	min	MGBG space (μl)	Water space (μl)	ΔMGBG space ± digitonin (μl)	ΔW space ± digitonin (μl)
None	1	4.55 ± 0.15	2.40 ± 0.08		
	5	6.13 ± 0.06	2.52 ± 0.06		
Digitonin	1	5.61 ± 0.34	3.09 ± 0.18	1.06	0.69
	5	7.58 ± 0.38	3.21 ± 0.10	1.45	0.69
Antimycin A	1	4.28 ± 0.17	2.47 ± 0.18		
	5	4.40 ± 0.22	2.49 ± 0.35		
Anti. A + Dig.	1	5.16 ± 0.18	3.01 ± 0.34	0.88	0.54
	5	5.47 ± 0.10	3.15 ± 0.15	1.07	0.66

All reagents were present in the medium from zero time. The Mg^{2+} concentration was 24.4 mM. Values shown are means of four determinations ± SD.

matrix compartment, in accordance with the chemiosmotic model [41]. Thus, the respiration dependence of the progressive uptake of organic cations indicates that this uptake involves transport across the inner mitochondrial membrane into the matrix compartment.

The increased rates of uptake following lysis of the outer membrane with digitonin may be explained as resulting from increased access of the organic cations to transport sites on the outer surface of the inner mitochondrial membrane. The slow progressive uptake of the organic cations in the absence of digitonin may be attributable to a subfraction of the isolated mitochondria having damaged outer membranes (found to vary from 10 to 40% in a previous study [9]) or to slow penetration of the outer membrane channels. Published studies have shown that spermine uptake by mitochondria exhibits respiration dependence and sensitivity to inhibition by Mg^{2+} [18], as does the uptake of spermidine and MGBG described here. However, in the published studies of spermine uptake [18], effects of digitonin were not tested, and experiments were not designed to evaluate whether the outer membrane permeability barrier might be limiting rates of spermine uptake.

Table 4A shows the effect of MGBG on uptake of spermidine, present in the medium at the same concentration, while Table 4B summarizes similar observations of the effect of spermidine on uptake of equimolar MGBG. The relatively high distribution spaces obtained in these experiments relate, in part, to the fact that the medium contained a lower concentration of Mg^{2+} (5 mM) than was used in most of the other experiments presented in this paper. As shown in Table 4A, MGBG decreased the rapid binding of spermidine, both in the presence and absence of digitonin. The progressive uptake of spermidine, which was enhanced in the mitochondria with lysed outer membranes, was diminished in the presence of MGBG. Thus, MGBG appears to inhibit both adsorption of spermidine to the mitochondrial membranes and transport of spermidine into the matrix compartment. The small decrease in the average 5-min uptake of MGBG caused by equimolar spermidine, which is apparent in Table 4B, was within the range of variability of the data. Whether the difference in degree of cross inhibition by the two organic cations may reflect different affinities of these compounds for binding and transport sites requires further study.

The mechanism(s) by which spermidine and

Table 4. Effect of MGBG on spermidine uptake and effect of spermidine on MGBG uptake

	Labeled cation	Additions	Distribution space (μl) 1 min	Distribution space (μl) 5 min	ΔSpace (μl) 1–5 min
A	Spermidine	None	8.4 ± 1.2	10.1 ± 0.9	1.8 ± 0.3
		Digitonin	12.2 ± 1.1	17.2 ± 2.5	5.0 ± 1.4
		MGBG	4.7 ± 0.4	6.1 ± 0.6	1.4 ± 0.2
		Dig. + MGBG	7.4 ± 1.0	10.2 ± 1.1	2.9 ± 0.4
B	MGBG	None	9.1 ± 0.9	12.0 ± 0.6	2.9 ± 0.7
		Digitonin	12.2 ± 0.8	17.3 ± 1.4	5.1 ± 0.9
		Spermidine	8.1 ± 0.6	10.9 ± 0.9	2.8 ± 0.6
		Dig. + Sperm.	11.2 ± 1.0	14.8 ± 1.5	3.6 ± 1.4

The incubation medium included 300 mM mannitol, 10 mM KCl, 10 mM KP_i, 5 mM $MgCl_2$, and 12 mM succinic acid, and was adjusted to pH 7.0 with NaOH. All reagents were present from zero time. In A the medium included 5 mM spermidine with [^{14}C]spermidine, and 5 mM MGBG was present only as indicated. Distribution spaces shown are means of average values determined in three experiments ± SD. In B the medium included 5 mM MGBG with [^{14}C]MGBG, and 5 mM spermidine was present only as indicated. Distribution spaces shown are means of five to six determinations pooled from two separate experiments ± SD.

MGBG penetrate the inner mitochondrial membrane remains to be determined. Transporters mediating uptakes of the inorganic cations Ca^{2+} [42], K^+ [43], and Mg^{2+} [44, 45], and the cationic amino acids ornithine and lysine [40, 46–50] by rat liver mitochondria have been characterized to varying degrees. Plasma membranes of various cell types contain a specific transport system which mediates cellular accumulation of the polyamines and MGBG [34–38, 51]. It remains to be determined whether the inner mitochondrial membrane contains a transporter specific for the polyamines and related compounds.

Published evidence has been interpreted as indicating that the outer mitochondrial membrane limits access of the anionic solutes, adenine nucleotides and glyceraldehyde-3-phosphate, to enzymes within or facing the intermembrane space [52]. Results of the present studies are consistent with the interpretation that the outer mitochondrial membrane may limit access of the organic cations spermidine and MGBG to the inner membrane. This conclusion is also supported by metabolic studies indicating that outer membrane lysis enhances inhibitory effects of organic cations on mitochondrial respiration [9]. The ability of the outer mitochondrial membrane to act as a barrier to penetration of spermidine and MGBG would appear to be physiologically significant, considering the concentrations of these organic cations which may exist within mammalian cells under normal or clinically relevant conditions [6, 32–38], and the known inhibitory effects of these compounds on mitochondrial function [2–12].

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