Volume 313, number 1, 39–42 FEBS 11755 November 1992

m-Iodobenzylguanidine increases the mitochondrial Ca^{2+} pool in isolated hepatocytes

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Received 24 September 1992

The incubation of isolated hepatocytes with the inhibitor of protein mono ADP-ribosylation, *m*-iodobenzylguanidine (MIBG), resulted in an increase in the size of the mitochondrial Ca^{2+} pool, without alteration of the non-mitochondrial Ca^{2+} store(s). This increase was abolished when the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) was buffered by prior loading of the cells with fluo 3. Elevating $[Ca^{2+}]_i$ by releasing the endoplasmic reticulum Ca^{2+} store with 2,5-di-(*tert*-butyl)-1,4-hydroquinone resulted in a synergistic increase in the magnitude of the mitochondrial Ca^{2+} pool. A role for protein ADP-ribosylation in the intracellular regulation of mitochondrial Ca^{2+} homeostasis is suggested.

m-Iodobenzylguanidine; Mitochondria; Ca^{2+} efflux; Hepatocyte; ADP-ribosylation; 2,5-Di-(*tert*-butyl)-1,4-hydroquinone

1. INTRODUCTION

The electrogenic uptake of Ca^{2+} into mitochondria is counterbalanced by an electroneutral efflux system that functions independently of the membrane potential (for recent reviews, see refs. 1–3). In tissues such as the heart and brain, a Ca^{2+}/Na^+ exchanger is the predominant Ca^{2+} efflux pathway, whereas in the liver, a Na^+-independent pathway involving a Ca^{2+}/H^+ antiporter is the major Ca^{2+} efflux route. Lehninger and associates [4] proposed that Ca^{2+} transport in liver mitochondria is regulated by the redox state of the mitochondrial pyridine nucleotides, such that Ca^{2+} release occurs upon the oxidation of the nucleotides. Similarly, the prooxidant-induced Ca^{2+} release from mitochondria involves the oxidation of mitochondrial pyridine nucleotides [5,6] rather than a reversal of the uniporter or inner membrane damage [3,6–8].

Previous work from Richter's [9] and this laboratory [10] has shown that pyridine nucleotide oxidation is not sufficient to activate the Ca^{2+} efflux pathway but requires the further hydrolysis of the oxidized pyridine nucleotides to nicotinamide and ADP-ribose. The latter observation led Richter and co-workers to propose that the prooxidant-induced Ca^{2+} release from mitochondria is regulated by protein mono ADP-ribosylation [11]. Further support for this contention stems from the recent findings that *m*-iodobenzylguanidine (MIBG), a high affinity competitive inhibitor of protein mono ADP-ribosylation [12], inhibits the prooxidant-induced Ca^{2+} release from rat liver mitochondria without affecting the uniport Ca^{2+} uptake system or pyridine nucleotide oxidation and hydrolysis [13].

In addition to preventing the prooxidant-mediated release of Ca^{2+} from mitochondria, MIBG also inhibits the spontaneous Ca^{2+} efflux [13]. This suggests a physiological role for protein ADP-ribosylation in the regulation of the mitochondrial Ca^{2+} efflux pathway. However, it is not known whether this spontaneous Ca^{2+} efflux pathway found in isolated mitochondria is also operative in an intact cell system and whether there it is also regulated by protein ADP-ribosylation. Hence, this study was aimed at investigating the role of protein ADP-ribosylation in the control of the mitochondrial Ca^{2+} efflux pathway in intact hepatocytes.

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Abbreviations: $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; MIBG, *m*-iodobenzylguanidine; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; tBuHQ, 2,5-di(*tert*-butyl)-1,4-hydroquinone; arsenazo III, 2,2′-[1,8-dihydroxy-3,6-disulfo-2,7-naphthalenebis(azo)]dibenzenearsonic acid; fluo 3, 1-(2-amino-5-(2,7-dichloro-6-hydroxy-3-oxy-9-9-xanthenyl)-phenoxy)-2-(2-amino-5-methylphenoxy)ethane-*N,N,N′,N′*-tetraacetic acid; AM, acetoxymethyl ester; MPP^+, 1-methyl-4-phenylpyridine.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Arsenazo III (98% pure), fura 2-AM and fluo 3-AM were purchased from Sigma Chemical Co. (St. Louis, MO, USA). FCCP was obtained from Aldrich Chemical Co. (Steinheim, FRG) and tBuHQ was purchased from EGA-Chemie (Steinheim, FRG). The Ca^{2+} ionophore, A23187, was purchased from Calbiochem (La Jolla, CA, USA). Collagenase (grade II) and bovine serum albumin were purchased from

Boehringer (Mannheim, FRG). MIBG sulphate (purity >95%) was a generous gift from Dr L.A. Smets, The Netherlands Cancer Institute, Amsterdam. All other chemicals were of the highest purity grade commercially available.

2.2. *Hepatocyte isolation and incubation*

Hepatocytes were isolated from male Wistar rats (170–210 g, fed ad libitum) as described previously [14]. Between 90–95% of the freshly isolated hepatocytes routinely excluded Trypan blue. Preincubation and incubation conditions were as reported in [15], before exposing the hepatocytes (10^6 cells/ml in Krebs–Henseleit buffer, pH 7.4, supplemented with 12.5 mM HEPES) to the compounds of interest. Controls received the appropriate vehicle additions; the vehicles were deionized water for MIBG and dimethyl sulfoxide for fura 2-AM, fluo 3-AM and tBuHQ.

2.3. *Experimental design*

The measurement of the mitochondrial Ca^{2+} pool in intact hepatocytes was estimated as the FCCP-releasable Ca^{2+} pool after separation of the hepatocytes from the incubation mixture by centrifugation and resuspension of the cells in Ca^{2+}- and Mg^{2+}-free Hanks' solution. [15,16]. Under these conditions the treatment of hepatocytes with mitochondrial uncouplers did not mobilize the endoplasmic reticular Ca^{2+} pool [17]. The cytosolic Ca^{2+} buffering capacity of hepatocytes was increased by loading the cells with the Ca^{2+} chelator fluo 3 [18]. The cells were incubated with 15 μM fluo 3-AM for 15 min using conditions previously described [19]. $[Ca^{2+}]_i$ and Ca^{2+} entry across the plasma membrane were measured fluorimetrically with fura 2, as reported in [19,20]. Cytotoxicity was monitored by a change in $[Ca^{2+}]_i$, the appearance of cell surface blebs and the loss of the ability of the cells to exclude Trypan blue.

2.4. *Statistical analysis*

All data are presented as mean ± S.E. of three to seven separate experiments. Duncan's multiple range test was performed to determine the significance of differences among individual group means. The significance level chosen for all statistical analyses was $P<0.05$. The statistical analyses were carried out with the SPSS/PC$^+$ statistical package.

3. RESULTS AND DISCUSSION

The exposure of isolated hepatocytes to MIBG for 60 min resulted in a concentration-dependent increase in the size of the mitochondrial Ca^{2+} pool (Fig. 1). The threshold concentration of MIBG to produce a detectable change in mitochondrial Ca^{2+} content was 10 μM MIBG, and half-maximal and maximal accumulation of Ca^{2+} occurred with approximately 100 μM and 250 μM MIBG, respectively. With 250 μM MIBG, the content of the mitochondrial Ca^{2+} pool was increased to 1.20 nmol $Ca^{2+}/10^6$ cells as compared with 0.29 nmol $Ca^{2+}/10^6$ control cells. No significant change in Ca^{2+} content was detected in the other (Ca^{2+}-ionophore-releasable) cellular compartments (0.55 ± 0.06 and 0.52 ± 0.08 nmol $Ca^{2+}/10^6$ cells with 100 μM and 250 μM MIBG, respectively, *vs.* 0.61 ± 0.06 nmol $Ca^{2+}/10^6$ cells in control cells). The changes in the magnitude of the mitochondrial Ca^{2+} pool occurred in the absence of any signs of toxicity to the hepatocytes. However, the exposure of hepatocytes to 1 mM MIBG resulted in extensive plasma membrane blebbing and after 2 h produced a 86% decrease in cell viability. This cytotoxicity was probably due to ATP depletion resulting from the inhibition of site 1 of the respiratory chain by MIBG [21].

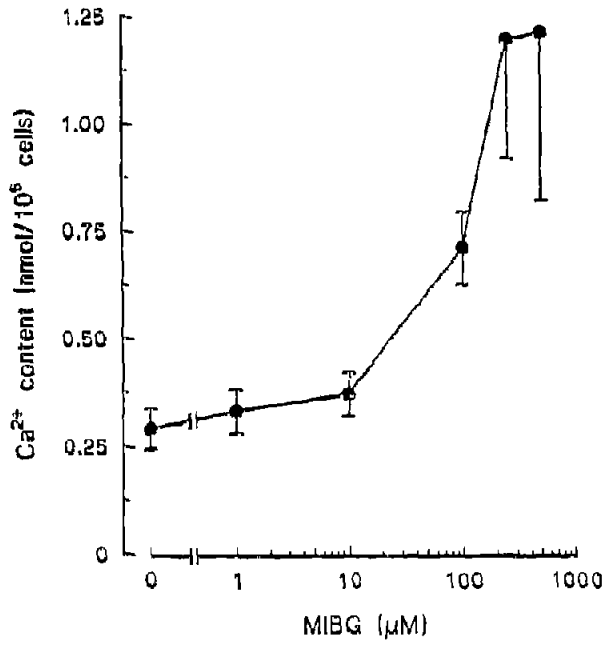

Fig. 1. MIBG-stimulates a concentration-dependent increase in the content of the mitochondrial Ca^{2+} pool. Isolated hepatocytes were treated with the indicated concentrations of MIBG for 60 min, before measuring the size of the mitochondrial Ca^{2+} pool as described in section 2. Each point represents the mean ± S.E. of three to six independent experiments.

The time course of Ca^{2+} accumulation in the mitochondrial pool by 100 μM MIBG is shown in Fig. 2. The mitochondrial pool in MIBG-treated cells rose rapidly to a maximal concentration of 0.92 nmol $Ca^{2+}/10^6$ cells by 100 min vs. 0.26 nmol $Ca^{2+}/10^6$ cells for controls. Again, no change was found in the ionophore-releasable Ca^{2+} stores (data not shown). The size of the mitochondrial Ca^{2+} pool in the control cells remained unchanged for the first two hours. Subsequently, a small elevation occurred, probably as a result of a slowly developing net accumulation of extracellular Ca^{2+} by the cells.

These results suggest that the inhibition of the spontaneous Ca^{2+} release from isolated mitochondria by MIBG [13] also takes place in intact cells. Thus, our findings are compatible with Richter's hypothesis [11] that protein mono ADP-ribosylation is a physiological regulatory mechanism of mitochondrial Ca^{2+} homeostasis. Additionally, it appears that even under resting conditions, there is substantial Ca^{2+} cycling occurring between the cytosol and the mitochondria. Although the affinity of the uniporter for Ca^{2+} is generally regarded as being low [1], our time-course experiments indicate that Ca^{2+} enters the mitochondria at a considerable rate. It is possible that physiological regulators, such as polyamines, inorganic phosphate ions and ADP, enhance the uniporter's affinity for Ca^{2+} sufficiently for significant Ca^{2+} uptake to occur under basal

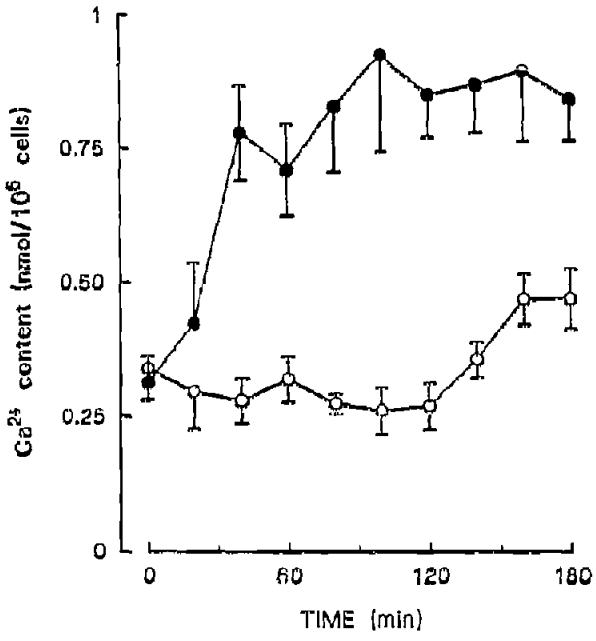

Fig. 2. Time-course of the MIBG-stimulated increase in the content of the mitochondrial Ca^{2+} pool. Isolated hepatocytes were incubated in the absence (○) or presence (●) of MIBG (100 μM), and the size of the mitochondrial Ca^{2+} pool was measured at the indicated time points as described in section 2. Each point represents the mean ± S.E. of four to seven independent experiments.

$[Ca^{2+}]_i$ conditions [22,23]. In the absence of MIBG to prevent ADP-ribosylation reactions, the Ca^{2+}/H^+ antiporter is likely to be constitutively active in order to keep the in situ mitochondrial Ca^{2+} content low [24].

The cycling of Ca^{2+} by mitochondria was further investigated by (i) pharmacologically altering the cytosolic Ca^{2+} buffering capacity and (ii) raising $[Ca^{2+}]_i$. The cytosolic Ca^{2+} buffering capacity was increased by loading the hepatocytes with the Ca^{2+} chelator fluo 3 [18]. This treatment abolished the ability of MIBG to stimulate an increase in the size of the mitochondrial Ca^{2+} pool (data not shown). In contrast, treatment of the hepatocytes with tBuHQ (which mobilizes the endoplasmic reticular Ca^{2+} store [19] and stimulates capacitative Ca^{2+} entry across the plasma membrane [20] thereby raising $[Ca^{2+}]_i$ to a level similar to that produced by a Ca^{2+}-mobilizing hormone) stimulated a very large increase in the size of the mitochondrial Ca^{2+} store in the presence of MIBG (Table I). Although elevating $[Ca^{2+}]_i$ with tBuHQ was by itself sufficient to raise the mitochondrial Ca^{2+} content, the combination of MIBG and tBuHQ produced a synergistic response. The treatment with tBuHQ also elevated the non-mitochondrial ionophore-releasable pool; this was most likely due to the net accumulation of Ca^{2+} in the cell following the sustained activation of capacitative Ca^{2+} influx across the plasma membrane [20]. The treatment of hepatocytes with MIBG alone did not produce any change in $[Ca^{2+}]_i$ and did not increase Ca^{2+} influx across the plasma membrane (data not shown). Taken together, these results show that mitochondria in situ continuously take up and release Ca^{2+} and that the degree of Ca^{2+} cycling by the mitochondria is dictated by the availability of Ca^{2+} from the cytosol.

Table I

Effect of tBuHQ on the increase in the mitochondrial Ca^{2+} pool in isolated hepatocytes induced by MIBG

Treatment	Mitochondrial Ca^{2+} pool (nmol $Ca^{2+}/10^6$ cells)	Non-mitochondrial Ca^{2+} pool (nmol $Ca^{2+}/10^6$ cells)
Control*	0.25 ± 0.05	0.62 ± 0.08
100 μM MIBG	0.55 ± 0.14	0.66 ± 0.16
10 μM tBuHQ	0.83 ± 0.31	1.09 ± 0.17
100 μM MIBG + 10 μM tBuHQ	1.97 ± 0.47**	0.53 ± 0.10

Isolated hepatocytes were treated with MIBG (100 μM) or tBuHQ (10 μM) or a combination of both compounds for 20 min before measuring the Ca^{2+} content of the mitochondrial and non-mitochondrial pools as described in section 2. Each value is the mean ± S.E. of five separate experiments except for the 10-μM tBuHQ treatment which is the mean ± SE for four experiments.

*Controls contained 0.1% dimethyl sulfoxide.

**Statistically different from other treatment groups ($P < 0.05$).

We have recently reported [15] that the immunosuppressant cyclosporin A stimulates a similar sequestration of Ca^{2+} mitochondria in hepatocytes. Like MIBG, cyclosporin A also prevents the prooxidant-induced Ca^{2+} release from mitochondria [25]. However, the immunosuppressant has been shown to inhibit the prooxidant-induced pyridine nucleotide hydrolysis [9,10] and is therefore believed to act prior to the step regulated by MIBG [13].

MIBG is actively accumulated by cells and is predominantly concentrated in the mitochondria [26]. MIBG can also function as a complex I inhibitor of the mitochondrial respiratory chain and this is probably the mechanism which accounts for the cytotoxicity observed with high concentrations of the inhibitor [21]. Hence, MIBG is very similar in a number of aspects to 1-methyl-4-phenylpyridine (MPP^+), the active metabolite of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Both compounds are actively accumulated in mitochondria [27,28] and have been shown to inhibit mitochondrial respiration at the level of site 1 [28,29]. However, in clear contrast with MIBG, MPP^+ produced a rapid discharge of mitochondrial Ca^{2+} in hepatocytes [30], demonstrating that the increase in the mitochondrial Ca^{2+} pool by MIBG was not linked to the action of MIBG as an inhibitor of NADH dehydrogenase or its ability to be accumulated by mitochondria.

In conclusion, this study has shown that the inhibitor of protein mono ADP-ribosylation, MIBG, stimulates the selective accumulation of Ca^{2+} by mitochondria in intact hepatocytes, probably by affecting the Ca^{2+} efflux pathway. Our results therefore suggest that the activity

of the inner mitochondrial membrane Ca^{2+}/H^{+} antiporter and thereby mitochondrial Ca^{2+} homeostasis is controlled by protein ADP-ribosylation.

Acknowledgements: This work was supported by the Swedish Medical Research Council (Project no. 03X-2471) and by grants from Karolinska Institutet and the Konrad and Svea Ulins Foundation (to GENK). We thank Dr. L.A. Smets for the generous gift of MIBG.

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