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THE PROOXIDANT-INDUCED AND SPONTANEOUS MITOCHONDRIAL CALCIUM RELEASE: INHIBITION BY *META*-IODO-BENZYLGUANIDINE (MIBG), A SUBSTRATE FOR MONO (ADP-RIBOSYLATION)

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The norepinephrine analogue *meta*-iodo-benzylguanidine (MIBG), a substrate for mono(ADP-ribosylation) and inhibitor of eukaryotic ADP-ribosyltransferases, inhibits the prooxidant-induced and spontaneous calcium release from intact rat liver mitochondria without affecting pyridine nucleotide oxidation and hydrolysis. This finding strongly suggests regulation of calcium release by ADP-ribosylation in mitochondria, and may be relevant for the cellular and pharmacological effects of MIBG.

KEY WORDS: ADP-ribosylation, calcium, mitochondria, prooxidants.

INTRODUCTION

Cytosolic calcium plays a central role in cellular regulation.[1] Its cellular homeostasis is maintained by the concerted action of ATP-driven calcium pumps in the plasma and the endoplasmic reticular membrane, and by respiring mitochondria. Due to their relatively low calcium affinity and very high calcium storage capacity mitochondria may act as a safety device against a toxic increase of cytosolic calcium.[1,2]

Prooxidants like hydroperoxides,[3] menadione,[4,5] alloxan,[6,7] divicine,[8] N-methyl-4-phenylpyridine (MPP^{+1}), [9] and hydroperoxyeicosatetraenoic acids[10] induce calcium efflux from mitochondria. The prooxidant-induced efflux is accompanied by pyridine nucleotide oxidation and hydrolysis, and occurs from intact mitochondria (reviewed in ref.[2])

There is strong, albeit indirect evidence for the regulation of mitochondrial calcium release by protein mono(ADP-ribosylation). Thus, in mitochondria a NAD^+ glycohydrolase[11] and three classes of ADP-ribose acceptor proteins[12,13] have been identified whose ADP-ribose moiety turns over rapidly; ATP inhibits both, mitochondrial calcium release and ADP-ribosylation;[14] both, mitochondrial pyridine nucleotide hydrolysis and calcium release have the same sigmoidal dependence on the calcium load.[15]

Abbreviations: arsenazo III 2,2-(1,8-dihydroxy-3,6-disulfonaphthalene-2,7-BIS-azo)bis(benzenearsonic acid); EGTA [ethylenebis(oxyethelenenitrilo)]tetraacetic acid; MIGB *meta*-iodo-benzylguanidine; MPP^+ N-methyl-4-phenylpyridine.

The cationic norepinephrine analogue *meta*-iodo-benzylguanidine (MIBG), used in its radioiodinated form for the detection and therapy of neuronal tumors,[16–19] is a high-affinity substrate for mono(ADP-ribosyl)ating enzymes and a strong inhibitor of intracellular ADP-ribosyltransferases.[20] Since organic cations such as rhodamine 123,[21] MPP^+,[9] and dequalinium[22] are taken up by energized mitochondria in response to the membrane potential, the cation MIBG is a promising tool to further investigate the regulation of calcium release by ADP-ribosylation in mitochondria. Here we report the inhibition of the prooxidant-induced and spontaneous mitochondrial calcium release by MIBG, discuss its relationship to mitochondrial and cellular functions, and briefly consider the pharmacological and therapeutic potential of MIBG.

MATERIALS AND METHODS

MIBG, synthesized according to Smets *et al.*,[23] was a generous gift of Drs. C. Loesberg and L. Smets, The Netherlands Cancer Institute, Amsterdam. Other reagents were of the highest purity commercially available.

Mitochondria were isolated from rat liver as described.[7] The movement of calcium across the inner mitochondrial membrane was followed under standard conditions (defined in ref.[15]) with radioactive calcium, or spectrophotometrically with the calcium indicator arsenazo III. MIBG was added, when appropriate, before rotenone. The redox state of mitochondrial pyridine nucleotides was measured spectrophotometrically at 340–370 nm.[5] Oxygen uptake was measured with a Clark-type electrode, and the mitochondrial membrane potential as described by Lötscher *et al.*[24]

RESULTS

Mitochondria possess independent calcium uptake and release pathways. For this reason calcium release from intact mitochondria is initially followed by re-uptake (calcium "cycling")[25] until the release leg predominates and net calcium release occurs. This is illustrated in the filtration experiment shown in Figure 1 where the prooxidants menadione and *t*-butylhydroperoxide are used to stimulate the release leg. In the absence of EGTA, *i.e.*, under conditions which allow calcium to be cycled, the menadione-induced net release of calcium occurs only after some lag period. This is in agreement with the result obtained spectrophotometrically using arsenazo III (c.f. Figure 2). To minimize the contribution of calcium cycling during calcium release, and to obtain information about the true prooxidant-induced calcium release, it was measured in the presence of EGTA. As a reflection of the continuous steady-state cycling,[25] addition of EGTA alone leads to a slow release of calcium.

Addition of menadione together with EGTA stimulates calcium release, the duration and extent being dependent on the menadione dose. 100 μM menadione is about as effective as 100 μM *t*-butylhydroperoxide. With 50 menadione, calcium release can be evoked again by a second addition of menadione after 7 min (not shown). The menadione-induced release is inhibited by ATP, albeit less than that induced by hydroperoxides.[14]

MIBG is taken up by succinate-energized mitochondria as indicated by a transient stimulation of respiration and a transient change in the mitochondrial membrane

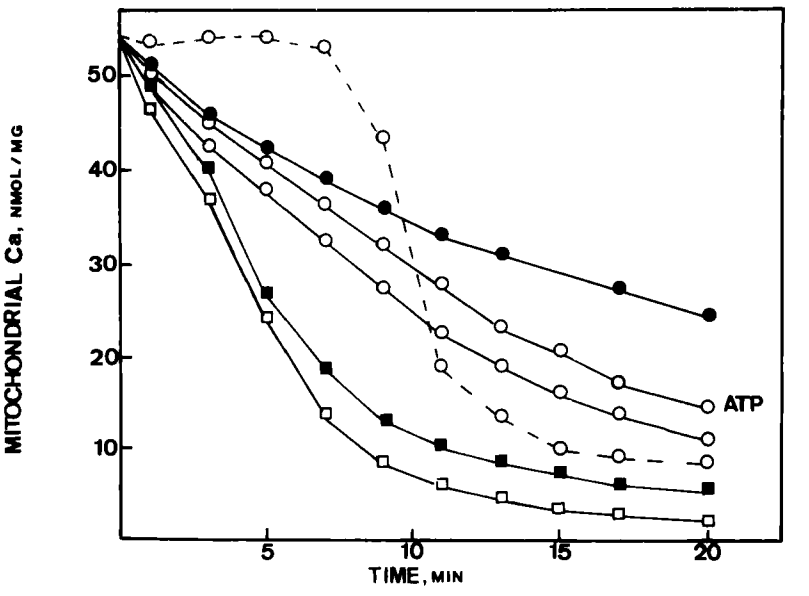

FIGURE 1 Prooxidant-induced Ca^{2+} release from rat liver mitochondria. Mitochondria were loaded with 54 nmol of $^{45}Ca^{2+}$ (specific activity 2100 dpm/nmol)/mg of protein. At zero time, efflux was initiated with 0.5 mM EGTA (●), 0.5 mM EGTA together with 50 μM menadione (○) with or without 0.2 mM ATP, 0.5 mM EGTA together with 100 μM menadione (□), or 0.5 mM EGTA together with 100 μM *t*-butylhydroperoxide (■). The dashed curve was obtained with 50 μM menadione in the absence of EGTA. The decrease of intramitochondrial radioactivity was followed by Millipore filtration.

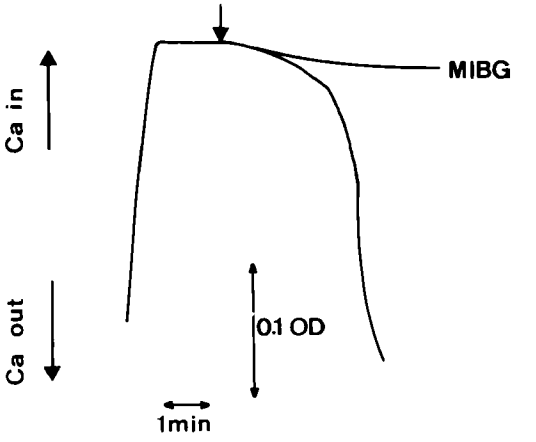

FIGURE 2 *t*-Butylhydroperoxide-induced Ca^{2+} release from rat liver mitochondria. In the presence of 50 arsenazo III, mitochondria were loaded under standard conditions with 60 nmol Ca^{2+} of protein. At the *arrow*, 100 μM *t*-butylhydroperoxide was added. *MIBG*: In the presence of 100 μM MIBG.

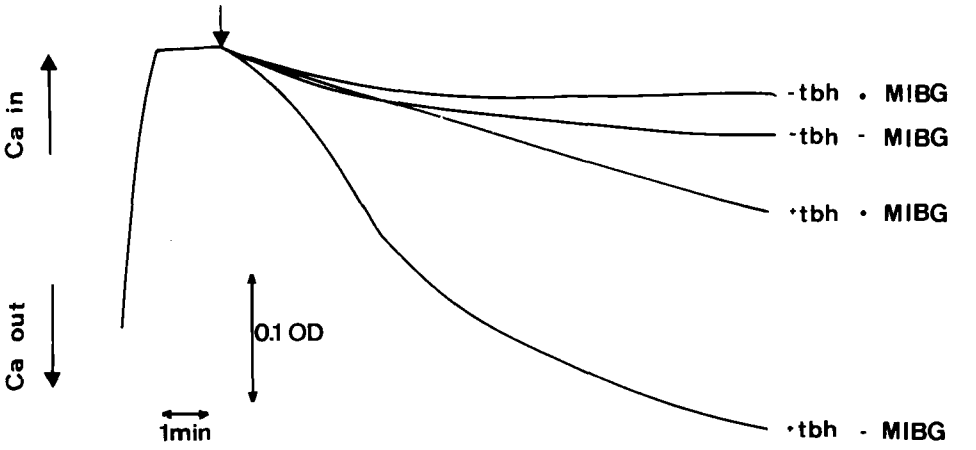

FIGURE 3 *t*-Butylhydroperoxide-induced Ca^{2+} release from rat liver mitochondria. In the presence of 50 μM arsenazo III, mitochondria were loaded under standard conditions with 60 nmol Ca^{2+}/mg of protein. At the *arrow*, ruthenium red (2 nmol/mg of protein) was added, immediately followed by 100 μM *t*-butylhydroperoxide as indicated for traces + *tbh*. + *MIBG*: In the presence of 100 μM MIBG.

potential upon MIBG addition (not shown). At 100 μm, MIBG totally prevents the *t*-butylhydroperoxide-induced net calcium release (Figure 2). In fact, no release was observed with MIBG during 45 min, the longest observation time. Between 80 and 300 μM, MIBG effectively inhibited the hydroperoxide-induced calcium release. At higher concentrations it interfered with mitochondrial calcium uptake. MIBG did not affect calcium release induced by uncoupler.

Figure 3 shows an experiment identical to that in Figure 2 except that ruthenium red, a specific inhibitor of the mitochondrial calcium uptake pathway, is added just before *t*-butylhydroperoxide to prevent calcium cycling. Calcium release is again very effectively inhibited by 100 μM MIBG. Remarkably, it not only inhibits the prooxidant-induced calcium release (see lower two traces in Figure 3), but also release in the absence of the prooxidant (upper two traces in Figure 3).

A spectrophotometric determination of prooxidant-induced pyridine nucleotide oxidation and hydrolysis[3] revealed no inhibition by MIBG.

DISCUSSION

Besides ATP,[14] MIBG is the only substance known so far to inhibit distally to pyridine nucleotide oxidation the prooxidant-induced calcium release from intact mitochondria. MIBG is a high-affinity substrate for mono(ADP-ribosylation),[20] and acts distal to pyridine nucleotide oxidation and hydrolysis. These findings strongly support the previously proposed regulation of calcium release by ADP-ribosylation in mitochondria.[2] It will be interesting to see to what an extent the modification of the different mitochondrial ADP-ribose acceptor proteins is affected by MIBG. Inhibition by MIBG of the spontaneous calcium release, the release leg of calcium cycling, indicates that this is not due to leakiness of the inner membrane, but is also controlled by ADP-ribosylation. This notion is consistent with and gives emphasis to the high

steady-state protein mono(ADP-ribosylation) (more than 200 pmol ADP-ribose bound per mg of protein) in normal rat liver mitochondria.[13]

Apart from mitochondria, the importance of the endogenous eukaryotic mono(ADP-ribosylation) reactions for cellular functioning is presently unclear,[26] partly because appropriate inhibitors of mono(ADP-ribosylation) were not available. MIBG is cytostatic and, upon prolonged exposure, even cytotoxic to various cell lines.[23] On the basis of MIBG inhibition studies it was recently suggested that the cytolytic action of glucocorticoid hormones in leukemic cells is negatively controlled by mono(ADP-ribosylation) of receptor proteins.[20] Thus, MIBG has great promise as a tool to study eukaryotic ADP-ribosylation. In this context it appears important to determine whether the killing of neuronal tumors by ^{131}J-MIBG[2-5] is caused by radiation or interference with cellular ADP-ribosylation. One intracellular target of MIBG could be mitochondria since they actively accumulate it. Because MIBG inhibits mitochondrial calcium release, calcium-regulated mitochondrial dehydrogenases may become inhibited in the presence of MIBG, resulting in the long run in cellular ATP depletion. The primary target of ^{131}J-MIBG radiation may be mitochondrial DNA. Since it is oxidatively very vulnerable,[27] not covered by proteins, and its oxidative damage is repaired inefficiently,[28] oxygen radicals derived from water radiolysis may compromise mitochondrial DNA integrity.

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