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Note

The interaction of methylmercury with lysosomes from rat liver

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Abstract

The interactions of methylmercury with rat liver lysosomes were investigated. The results indicate that methylmercury enhances the proton permeability in membranes. Since this uncoupling mechanism necessarily implies the transport of a charged ion (in this case CH_3Hg^+) through the membrane, this fact can help to explain why a preferential target of methylmercury are nerve cells, whose plasma membrane potential has the highest value, when compared with other cells. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

The presence of methylmercury in natural waters is a problem of great environmental concern since this compound possesses a high toxicity very well documented in animals and humans [1–6].

Methylmercury is prevalently neurotoxic and studies have been performed in cells and in subcellular structures [7–10] in order to establish the molecular mechanism (or mechanisms) which is responsible for the toxicity in whole animals.

Methylmercury is reported to increase cytosolic Ca^{++} concentration in rat cerebrum synaptosomes [7], in PC12 cells [8], and in rat T lymphocytes [9]. In other cellular systems, the inhibition of ATP synthesis and apoptosis have also been reported [10,11].

Among the subcellular structures, mitochondria have been extensively investigated since a damage on mitochondria, which produce ATP for the cell, gives rise to a corresponding cell damage [12–17]. In mitochondria, methylmercury interacts with all the mechanisms, which are involved in ATP synthesis. In fact, it has been reported that methylmercury inhibits the respiratory chain [14,15], and, in the same way as uncoupler, it

collapses $\Delta\psi$ and causes a Ca^{++} release [14–16]. Furthermore, it induces a cytochrome *c* release [17] and the opening of a transition pore [18]. In mitochondria, therefore, it is difficult to establish the effective toxic effect (the lowest dose which causes a failure in ATP synthesis) since many interaction mechanisms are present. Furthermore, the effective toxic effect in cells and, in particular, the reason why nerve cells are the preferential target for methylmercury has not yet been established.

In this paper our attention is focused on lysosomes. Up to now, the interactions of methylmercury with these organelles have never been examined and our aim is to verify whether a particular transport system of this compound is present in the lysosomal membrane.

Moreover, a study of a membrane transport mechanism in lysosomes offers some advantages since, in this biological system, many functions such as the respiratory chain, selective transition pore and antiporters are absent or present in a limited number. This facilitates the approach and could permit to propose a general peculiar behaviour also for other membranes. The results, in reality, indicate that methylmercury behaves as an uncoupler. This behaviour, being a membrane property, could explain not only one aspect of the toxicity of methylmercury since uncouplers are toxic compounds, but in particular the prevalent neurotoxicity of the compound.

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2. Materials and methods

Rat liver lysosomes were prepared according to Sawant et al. [19] and resuspended before use at 0 °C in a medium (mother solution) containing 0.25 M sucrose, 10 mM Mops-Hepes pH 7.4 and 5 mM MgSO₄. Acridine Orange (AO) (Merck) was purified according to Pal and Schubert [20]. Aliquots of lysosomes were withdrawn from the mother solution and added to the operating medium. The uptake of AO by lysosomes was monitored spectrophotometrically at room temperature using a Jenway 6400 spectrophotometer, by measuring the change in dye absorbance at 492 nm. After the addition of the lysosomes and AO to the operating medium (final concentration of lysosomes 0.2 mg ml⁻¹), the spectrophotometer was adjusted to 0 absorbance. The protein concentration was determined by the Lowry method [21].

ATPase inhibition measurements were performed following the procedure suggested by Nishimura and Cahnce [22] by means of a pH meter (PHM 84 Radiometer Copenhagen). The medium contained Oligomycin in order to prevent spurious mitochondrial contaminations and 100% inhibition of the rate of ATP hydrolysis was measured in the presence of bafilomycin.

Determination of the concentration of CH₃HgCl in the supernatant of energized lysosomes. The suspension containing energized lysosomes (medium and conditions as in Fig. 1) was centrifuged at 14,000 rpm by means of a Sorvall centrifuge RC2B, rotor SS34. The mercury content in the supernatant was obtained by flow injection (Perkin–Elmer 3.100) cold vapor atomic absorption Spectroscopy (Perkin–Elmer FIAS 100) using SnCl₂ as reductant.

All the reagents were of an analytical grade. The bafilomycin, duramycin, valinomycin and 2,4-dinitrophenol (DNP) were purchased by Sigma (Milan).

3. Results and discussion

In lysosomes the pH in the matrix is acid, about 5.2 as a consequence of a Donnan effect [23]. The lysosomes possess an ATP-driven proton pump, which, in the presence of ATP, pumps protons into the matrix. The acidification in lysosomes can easily be monitored using the AO dye [24]. This dye is a weak permeant base and, following the classical mechanism of weak bases, it crosses the membrane as an electroneutral compound and accumulates in the matrix, since the pH is acid inside. The accumulation gives rise to a chemical shift [25]. Therefore, the accumulation and the internal acidification can easily be monitored by following the absorbance quenching at 492 nm, as shown in Fig. 1.

In Fig. 1, the absorbance quenching signal before the addition of ATP is due to the non-energized entry of

AO, as a consequence of the internal pH of 5.2 as mentioned above. The addition of ATP causes a further acidification, since the ATP-driven proton pump pumps the protons into the matrix, but the acidification is appreciable and monitorable only if Cl⁻ ions are present in the medium. This is due to the fact that the pumping of protons induces a positive-inside potential, which is a force that opposes further significant acidification. In the presence of Cl⁻ ions, a selective channel allows for the Cl⁻ uptake by the positive-inside potential. The Cl⁻ entry neutralizes, although not completely (see below) the internal potential, and, as a consequence, the protons are ‘easily’ pumped from the pump into the matrix without the opposing force of the positive-inside potential [26]. Under these conditions the internal pH is about 4.5 [26].

Analogously, since the mechanism which regulates internal acidification implies the presence of a positive-inside potential which is an opposing force, a marked and monitorable ATP dependent acidification is observed in a medium not containing Cl⁻, but containing valinomycin, the potassium carrier [27]. Under these experimental conditions, valinomycin allows for a potassium efflux from the matrix (the matrix contains 0.1 M potassium) to the suspending medium, which is equivalent (with regard to the electrical balance) to a Cl⁻ uptake (Fig. 2).

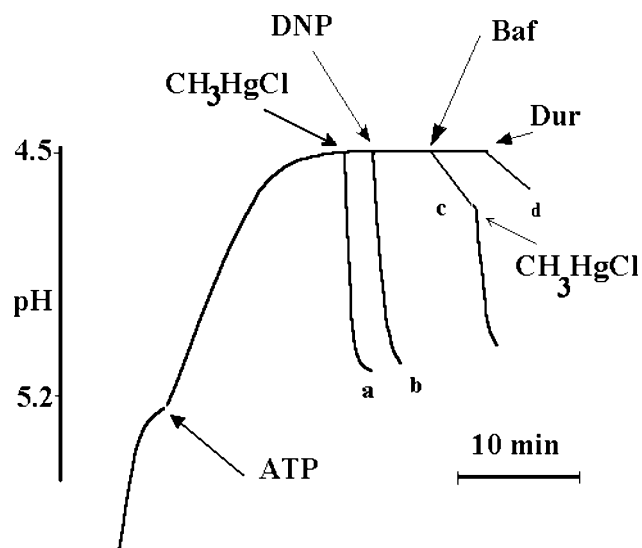


Fig. 1. Alkalinization induced by methylmercury or by DNP, bafilomycin (Baf) and duramycin (Dur) in energized lysosomes resuspended in a chloride medium. Medium composition: 125 mM KCl, 20 mM Hepes pH 7.4, 5 mM MgCl₂. Immediately after addition to the medium (2.5 ml) of lysosomes and AO (final concentration 3 mg ml⁻¹ and 5 μM, respectively) the instrument was adjusted to zero absorbance. Under these conditions, the trace, before the addition of ATP, measures the internal pH of non-energized lysosomes (pH 5.2). After the addition of ATP (2 mM), a further acidification occurs. In a steady state, the traces show the addition of: 20 μM CH₃HgCl (trace a), 40 μM DNP (trace b), 50 nmol bafilomycin (trace c), 50 ng duramycin (trace d).

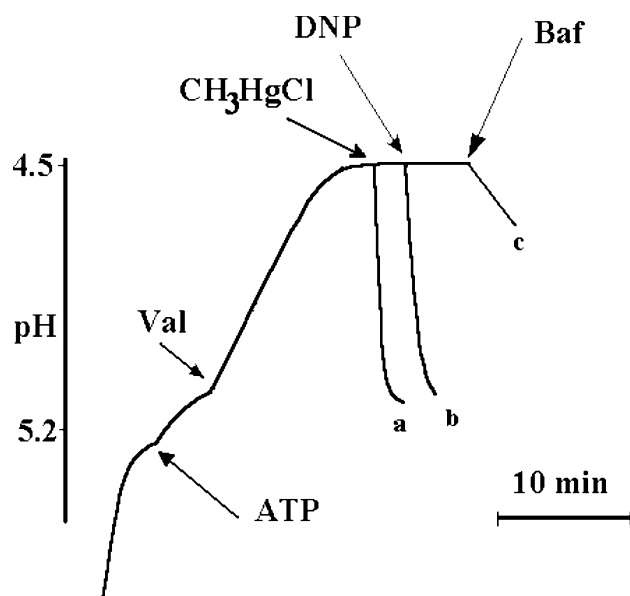


Fig. 2. Alkalinization induced by methylmercury or by DNP and bafilomycin (Baf) in energized lysosomes resuspended in a chloride-free medium and in the presence of valinomycin. Medium composition: 25 mM sucrose, 20 mM Hepes pH 7.4, 5 mM MgSO₄. The instrument was adjusted, before the addition of ATP to the medium (2.5 ml) to zero absorbance, with the same concentration of lysosomes and AO as in Fig. 1. After the addition (2 mM) of ATP, 50 nmol of valinomycin (Val) were added. In a steady state, the traces indicate the addition of: 20 μM CH₃HgCl (trace a) or 40 μM DNP (trace b), 50 nmol bafilomycin (Baf) (trace c).

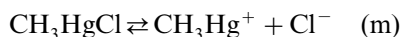
In all cases (in the presence of chloride or valinomycin in the medium) about 10 min after addition of ATP, the system is in a steady state condition, in which the internal pH is about 4.5 [25] and the internal potential $\Delta\psi$ is low, about +40 mV [28]. If, under these conditions, methylmercury is added, a rapid alkalinization occurs (Figs. 1 and 2, traces a).

In general, a rapid alkalinization can be due to many factors:

a) *An uncoupling effect* [29]: The lysosomal membrane is not permeable to protons. If a protonophor (or uncoupler) is added, internal acidification occurs. Figs. 1 and 2 (traces b) show examples of (rapid) internal alkalinization, induced by DNP, which is a classical uncoupler. DNP permits the permeation of protons since it enters the matrix as a phenate negative ion, the driving force being the positive-inside potential. Once inside, it is extruded as an electroneutral undissociated compound, since DNP is a weak acid and the pH is acid inside. The balance of this cyclic mechanism is the transport of a proton through the membrane during any cycle. This mechanism, being cyclic, well explains the low doses of uncoupler (40 μM in the case of DNP) required to obtain a rapid and complete internal pH alkalinization.

- b) *Weak base effect* [30]: Weak bases such as ammonia enter the membrane and accumulate inside, since the pH is acid inside. The alkalinization occurs only in the presence of high doses of a weak (permeant) base, above millimolar [30].
- c) *Inhibition of the ATPase* [31]: Many chemical compounds inhibit the ATPase. Bafilomycin is a potent inhibitor [31]. Its addition in a steady state causes a slow alkalinization (Figs. 1 and 2, traces c).
- d) *Inhibition of the Cl⁻ channel* [32]: Duramycin is a potent inhibitor of the Cl⁻ channel [32]. Its addition in a steady state causes a slow alkalinization (Fig. 1, trace d).
- e) Presence of an antiporter system, such as an OH⁻/Cl⁻ exchanger [33,34]. This situation has been proposed as an explanation for the toxicity of many organometal compounds, such as alkyl₃SnCl and alkyl₃PbCl [33,34]. This mechanism is, in theory, proposable for methylmercury, since it forms the CH₃HgCl complex [35,36].

In solution, methylmercury chloride gives rise to the following equilibria: [35,36]



The dose of methylmercury necessary to obtain a fast alkalinization such as that indicated in Figs. 1 and 2 is in the order of 1 μM. Therefore, a weak-base mechanism (mechanism b), as methylmercury is a weak base (equilibrium n) should be excluded, even if theoretically possible, since weak bases require high doses, of above 10 mM.

Analogously, even though we cannot exclude the fact that methylmercury inhibits ATPase (see below), such behaviour should be excluded, since the ATPase inhibition induced by bafilomycin [31], which is a potent and instantaneous ATPase inhibitor, gives rise to a slow alkalinization, even slower than that induced by methylmercury.

In the same way, even though methylmercury inhibits the Cl⁻ channel, this is not the reason why alkalinization is induced, since Fig. 1 shows that the addition of duramycin, the chloride channel inhibitor [32] gives rise to a slow alkalinization.

In any case, methylmercury also induces alkalinization in a chloride free medium, as shown in Fig. 2. Therefore, even though we cannot exclude an inhibitory effect on the chloride channel, this is not the cause of the rapid alkalinization shown in Figs. 1 and 2.

An antiporter mechanism (mechanism e) has been proposed as an explanation of the toxicity mechanism of alkyl₃SnCl and alkyl₃PbCl in mitochondria [32,33]. Since methylmercury forms stable chloride complexes (eq m), this mechanism can explain the behaviour in Fig.

1, but not in Fig. 2 since in Fig. 2, the chloride is absent from the medium.

On the basis of these observations, we have concluded that methylmercury behaves as an uncoupler. In the case of methylmercury, the mechanism corresponds to the entry of methylmercury as an undissociated, electro-neutral CH_3HgOH compound, which tends to accumulate inside, the pH being acid in the matrix. Once inside, the CH_3HgCl^+ cation is extruded from the positive-inside potential, thus giving rise to a cyclic mechanism, resulting in the transport of a proton during any cycle, as in the case of uncouplers. The cyclic mechanism clearly explains, as it also does in the case of uncouplers, the low doses of methylmercury necessary to induce a fast internal pH alkalization.

This mechanism, as already been mentioned, does not exclude the use of the antiporter mechanism (mechanism e) in the presence of chloride ions.

However, the possibility that CH_3HgCl induces not only an uncoupling transport mechanism, but also a vacuolar ATPase inhibition, cannot be excluded. This is supported by the experiment shown in Fig. 1 (trace c), where the addition of CH_3HgCl after that of bafilomycin induces the same alkalization rate as that which occurs in the presence of bafilomycin alone (Fig. 1, trace a). A direct measurement of ATPase inhibition by CH_3HgCl has been performed (Fig. 3) in order to clarify this point. The graph shows that CH_3HgCl actually does inhibit the ATP hydrolysis, but the doses necessary to inhibit the ATPase are higher than those necessary to induce the alkalization rate shown in Fig. 1 (trace a). This is demonstrated by the given data in Fig. 3, in which 20 μM CH_3HgCl induces only a

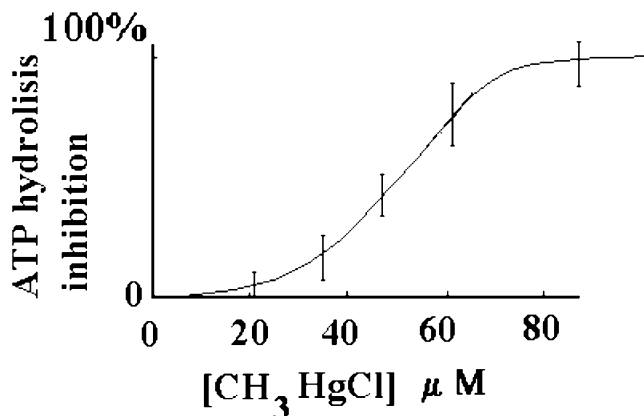


Fig. 3. The vacuolar ATPase inhibition induced by CH_3HgCl . The graphic reports the inhibition of the ATP hydrolysis induced by increasing amounts of CH_3HgCl . *Experimental conditions:* Medium composition: 0.125 M KCl, 0.5 mM Hepes-Mops pH 7.4, 10 mM MgCl_2 , oligomycin 2 γ , FCCP, 60 nM. Each point in the graph (the mean value obtained after five replicate measurements) is the initial acidification rate obtained after addition of 400 μM ATP to the medium (4 ml) containing the lysosomes (0.3 mg ml^{-1}) in the presence of various amounts of methylmercury. As reference point, the acidification rate was measured in the presence of 2 nM bafilomycin.

negligible effect on the ATPase. This also demonstrates that the toxic effect of CH_3HgCl (with the lowest dose) is the uncoupling effect.

The importance of the possibility that CH_3HgCl can behave as an uncoupler arises from the fact that an uncoupling mechanism necessarily implies the passage through the membrane of a charged compound, in this case CH_3Hg^+ . This is made possible since under the operating conditions, the concentration of methylmercury in the supernatant of energized lysosomes (measured by means of atomic absorption after the centrifugation of the lysosomes) results as 8 μM . This implies that not all methylmercury added is bound to the membrane proteins and that the free methylmercury is available to be used in the uncoupling mechanism, as mentioned above.

The transport of the CH_3Hg^+ cation can explain not only the toxicity (in mitochondria, where the Ca^{++} release and other correlated phenomena can be seen to be due to an uncoupling effect) but, in particular, the neurotoxicity, since the passage of the ion through a membrane occurs by means of a potential-driven mechanism. The above mentioned passage depends exponentially on the applied potential [37] and nerve cells possess the highest membrane potential in the cytoplasmic membrane. For this reason, we retain that only the uncoupling mechanism, even though it is probably not the only cause of toxicity, can explain why nerve cells are the preferential target for methylmercury.

4. Supplementary material

The material is available from the authors on request.

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