EFFECT OF THE A23187 IONOPHORE ON MITOCHONDRIAL MEMBRANE Mg^{2+} AND Ca^{2+}

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1. Introduction

In biological membranes, the divalent cations, and chiefly Ca^{2+} and Mg^{2+}, seem to control the stability of the membrane by the formation of a ternary complex between the anionic groups of a protein and a phospholipid molecule. This point which was first suggested for the erythrocyte membrane [1–4] may also be applied to other biological membranes: chloroplasts [5,6], and mitochondria. In mitochondria, it has been shown that the total release of the Mg^{2+} present in the organelle, provoked by an uncoupler, leads to the loss of the impermeability of the mitochondrial membrane [7]. A similar effect is obtained when Ca^{2+}, at a concentration above 100 μM is added to the mitochondria in the absence of a permeant anion e.g. phosphate [8,9]. These experiments, as well as other recent results, [A. Binet and P. Volfin, manuscript in preparation] suggest an important role for Ca^{2+} and Mg^{2+} in the regulation of the integrity of mitochondrial membranes.

In order to clarify this point, we have studied the effect of the ionophore A23187 on mitochondrial Ca^{2+} and Mg^{2+}, both on their movements in the whole mitochondria, and on their interaction with the mitochondrial membrane. Our results suggest that the mitochondrial Mg^{2+} is essential for the maintenance of the functions and the stability of membrane structure. Experiments with a cytosolic factor-CMF* [7–9] indicate that a dual regulation seems to occur at the membrane level both by the external CMF and the intrinsic Mg^{2+}.

* Cytoplasmic Metabolic Factor.

2. Material and methods

Mitochondria were obtained from rat liver by the usual technique [9]. They were suspended in 0.15 M KCl–20 mM Tris pH 7.2. The movements of Ca^{2+} and Mg^{2+} in the membranar phase were visualized by the fluorescence of chlorotetracycline, as described by Caswell [10,11]. Measurements were performed in an Eppendorf spectrofluorimeter. Ca^{2+} and Mg^{2+} were determined in the whole mitochondria by atomic absorption spectrophotometry (with a Perkin-Elmer apparatus, model 300), after rapid centrifugation of mitochondria during the course of the incubation. Preparation of the cytosolic fraction, containing the CMF, was described previously [9,12]. Proteins were determined by the Biuret method. A23187 was a gift from Eli Lilly and Co; chlorotetracycline was purchased from N.B.C.; and all others products were of analytical grade.

3. Results

A23187 is an ionophorous antibiotic which specifically permeabilizes the biological membranes to divalent cations [13,14]. When it is added to rat liver mitochondria at low concentrations (0.04 nmole/mg protein), it induces only a release of Mg^{2+} from the mitochondria (fig. 1). In these conditions, neither Ca^{2+} efflux, nor membranar divalent cations efflux, visualized by a decrease of chlorotetracycline fluorescence, occur. The Ca^{2+} efflux and the decrease of the fluorescence occur simultaneously, when the Mg^{2+} level in mitochondria is very low (less than

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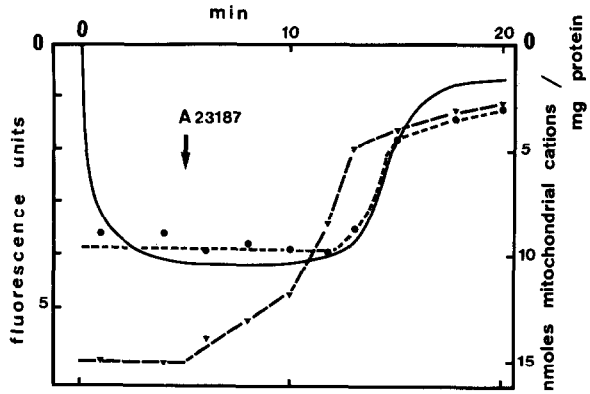

Fig. 1. Effect of A23187 on mitochondrial Mg^{2+} and Ca^{2+}. Rat liver mitochondria (1.9 mg protein/ml) were incubated in 0.15 M KCl, 10 mM Tris pH 7.2 medium, with 10 μM chlorotetracycline and 30 μM ADP, at 30°C. Fluorescence was monitored in an Eppendorf fluorimeter, with excitation wavelength at 366 nm and emission at 530 nm. Reaction was initiated by the addition of mitochondria. Aliquots were taken off at various times, mitochondria were rapidly centrifuged in an Eppendorf mini-centrifuge, and divalent cations were titrated both in mitochondria and supernatant by atomic absorption spectrophotometry. (——) fluorescence recording; (●—●—●) Ca^{2+} in mitochondria; (▲—▲—▲) Mg^{2+} in mitochondria. Addition of A23187 : 5 min : 0.04 nmole/mg protein.

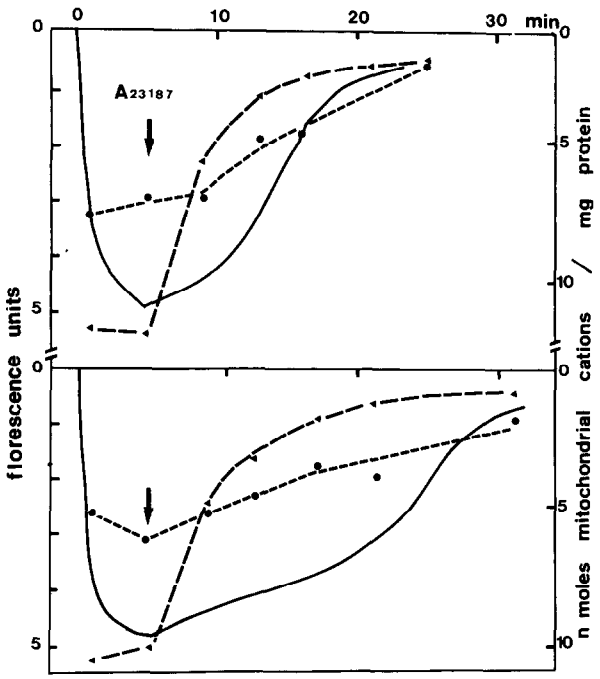

Fig. 2. Effect of A23187 on mitochondrial Mg^{2+} and Ca^{2+}, in the presence of CMF. Rat liver mitochondria (1.9 mg protein/ml) were incubated under the same conditions as in fig. 1. Fig. 2 top: absence of CMF; Fig. 2 bottom: presence of CMF. A23187 (0.07 nmole/mg protein) is added at 5 min.

5 nmoles/mg protein). The fluorescence decrease and the Ca^{2+} efflux are concommitant with a loss of the impermeability of the mitochondrial membrane.

The effect of CMF, an unknown factor present in the cytosol, which prevents the release of Mg^{2+} from mitochondria in various conditions: uncoupler action [7], aging [15], or action of Ca^{2+} [8,9] has been previously described. The results of the fig. 2 show that CMF has no influence on the ionophore-induced Mg^{2+} efflux, but slows down significantly both the Ca^{2+} efflux and the decrease of chlorotetracycline fluorescence provoked by A23187.

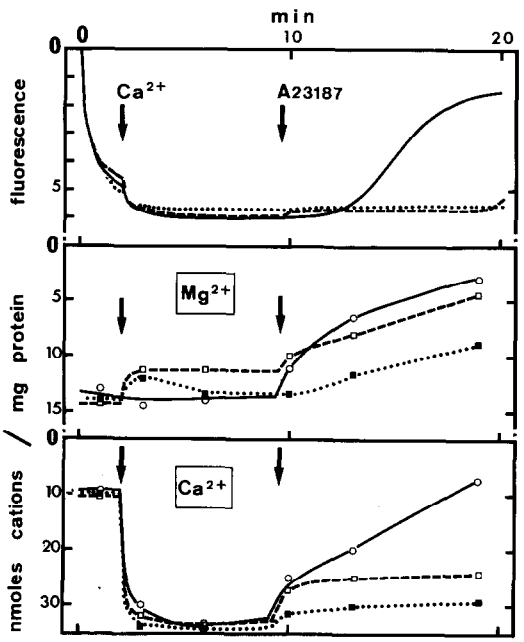

Fig. 3. Effect of A23187 on mitochondrial Mg^{2+} and Ca^{2+}, in presence of external Mg^{2+} and Ca^{2+}. Rat liver mitochondria (2.4 mg protein/ml) were incubated under the same conditions as in fig. 1. (○—○—○) control; (□—□—□) + 0.5 mM Mg^{2+}; (■ .. ■ .. ■) + 0.5 mM Mg^{2+} + CMF; 50 μM Ca^{2+} was added at 2 min, and 0.10 nmole A23187/mg protein was added at 9 min.

The preceding experiments have been carried out in the absence of divalent cations in the incubation medium. Fig. 3 shows the results obtained in the presence of low concentrations of Ca^{2+} (50 μM, corresponding to 20 nmoles/mg protein) and of Mg^{2+}. This amount of added Ca^{2+} is completely bound to the mitochondria, and is insufficient to induce the total Mg^{2+} release which occurs at higher Ca^{2+} concentrations; this Ca^{2+} uptake is persistent [9]. The addition of 0.10 nmoles A23187/mg protein to Ca^{2+}-loaded mitochondria induces a fast release of Mg^{2+}, followed by the efflux of Ca^{2+} and a decrease of chlorotetracycline fluorescence. In the presence of 0.5 mM Mg^{2+}, the release of Mg^{2+} is unaffected but the efflux of Ca^{2+} is limited to 8–10 nmoles/mg protein, and no decrease in the fluorescence can be observed 10 min after the addition of the ionophore. When the medium is supplemented with CMF, the rate of Mg^{2+} efflux is slightly decreased, and the amount of Ca^{2+} ejected is reduced to 2–4 nmoles/mg protein when the ionophore is added.

4. Discussion

During the course of this work, we have followed the movement of membranar divalent cations by the fluorescence of their complex with the chlorotetracycline. Before discussing these results, it is important to emphasize the contribution and the limits of this technique. The chlorotetracycline is only fluorescent when it is complexed by divalent cations in an apolar medium [10,11]. In consequence, the fluorescence we measure corresponds only to the Ca^{2+} and the Mg^{2+} present in the membrane, and eventual binding sites for divalent cations on the surface of the membrane are not accounted for the fluorescence. So, it is difficult to say whether the 'non-fluorescent' Mg^{2+} ejected comes from the inside of the mitochondria, or from a site on the surface of the mitochondrial membrane.

From the results presented in this paper, it seems apparent that, when an ionophore, specific for divalent cations, is added to mitochondria, Mg^{2+} is always the first cation to be ejected from the mitochondria. This Mg^{2+} comes from a non-membranar site, because no fluorescence decrease occurs during this efflux. Moreover, the shape of the kinetic curve of the fluorescence decrease, as shown in the fig. 2 up for example, indicates that the decay of chlorotetracycline fluorescence is rather slow during the earlier minutes after A23187 is added, then it is accelerated until all the fluorescence ceases definitely. This rapid decrease is parallel to the efflux of the mitochondrial Ca^{2+}. In this case, an irreversible situation is reached for the mitochondrial membrane when more than 80% of the mitochondrial Mg^{2+} is ejected.

Taking these results and the first remarks into account, we can make some inferences concerning the localisation of some divalent cations in the mitochondria. The simultaneous efflux of the mitochondrial Ca^{2+} and the decrease of the fluorescence indicate that nearly all mitochondrial Ca^{2+} (6–8 nmoles/mg protein) is tightly bound to the membrane. On the other hand, only a small amount of Mg^{2+} (less than 2 nmoles/mg protein) is located in the membrane. Moreover, the results obtained at the time of the addition of 50 μM Ca^{2+} to the mitochondria, indicate that a part of the uptake of the Ca^{2+} consists of bound Ca^{2+} (there is about 20% increase of the chlorotetracycline fluorescence), and a part of 'non-fluorescent' Ca^{2+}. The ratio between these two pools of Ca^{2+}, and the localisation (surface sites on the membrane, or in the matrix) of the Ca^{2+} incorporated in the mitochondria cannot be discussed in this paper. This question will be studied in a forthcoming paper.

The results of the experiments carried out in the presence of external Mg^{2+} show that this Mg^{2+} prevents more efficiently the release of the mitochondrial Ca^{2+}, than the release of Mg^{2+}, and it helps to maintain a sufficient concentration of both these divalent cations in the membrane since the chlorotetracycline fluorescence remains at its maximum value. The same phenomenon has been obtained with higher concentrations of Mg^{2+}, and seems to be independent of the Mg^{2+} concentrations in the range 0.5–5 mM. The addition of CMF, which normally prevents completely the damaging effect of Ca^{2+} on the mitochondria [8,9] has only a small antagonistic action against the mobilisation of divalent cations by A23187. CMF counteracts essentially the A23187 induced-Ca^{2+} release in addition to its well-known effect on the Ca^{2+}-induced Mg^{2+} release [9]. The absence of inhibition of the A23187-induced Mg^{2+}

efflux may suggest that the CMF regulation applies to a more specific membranar Ca^{2+}–Mg^{2+} interaction (A. Binet and P. Volfin, manuscript in preparation).

In conclusion, these results may be interpreted according to the following hypothesis. Ca^{2+} is the major divalent cation constituent of the mitochondrial membrane, but the stability of the membrane is regulated by the presence of a low amount of a Mg^{2+} bound in, or on the surface of the membrane. If this Mg^{2+} is released, by the means of the ionophore, the membranar Ca^{2+} becomes labile and this process leads to the loss of the inpermeability of the mitochondrial membrane. This hypothesis is consistent with two recent reports. Leblanc and Clauser [16] have shown that Mg^{2+}, together with ADP, is required for energy coupling between calcium phosphate accumulation and oxygen uptake in heart mitochondria. Also, Romero [4] has indicated that a membrane bound Mg^{2+} is required to maintain a low permeability to K^+ in erythrocyte membrane. The nature and the characteristics of the interactions of this Mg^{2+} with the mitochondrial membrane, as well as the mechanism of the regulation of this binding by CMF are under investigation.

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