Hypothesis

Does calmitine, a protein specific for the mitochondrial matrix of skeletal muscle, play a key role in mitochondrial function?

B. Lucas-Heron*, B. Le Ray, N. Schmitt

Laboratoire de Physiologie, Faculté de Médecine, 1 rue Gaston Veil, 44035 Nantes Cédex, France

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Abstract The effect of the myotoxic drug chlorpromazine was studied in vitro on proteins of sarcoplasmic reticulum and mitochondrial matrix of skeletal muscle in the normal mouse. Our results indicate that the drug is specific for calcium-binding proteins (calcium ATPase, calsequestrin and calmitine). Its proteolytic effect on these proteins, apparently due to the stimulation of specific proteases, could account for its myotoxic action. Moreover, calsequestrin (sarcoplasmic reticulum) and calmitine (mitochondrial matrix) were not sensitive to the same proteases. Proteases acting on calmitine were inhibited by α2-macroglobulin but not those acting on calsequestrin. Despite some similarities between these two proteins, their characteristics of localization and sensitivity of their proteases indicate that calmitine has a specificity within the mitochondrial matrix and very probably plays a major role in the mitochondrial regulation of free calcium, which controls the activity of various enzymes of the mitochondrial matrix involved in ATP synthesis.

Key words: Calmitine; Mitochondrial matrix; Skeletal muscle

1. Introduction

Calmitine, a mitochondrial calcium-binding protein specific for fast skeletal muscle [1], shows some similarities with calsequestrin [2]. The fact that it has recently been localized more specifically in the mitochondrial matrix [2] suggests that it plays a primordial role in the regulation of mitochondrial bound and free calcium [3] and thus in the cellular functioning and particularly the synthesis of ATP. There is increasing evidence that mitochondrial calcium, which influences the activity of various matrical enzymes involved in oxidative phosphorylation and the production of cellular energy, plays a second messenger role triggering mitochondrial response to cytosolic signals [4]. For example, at the time of muscle contraction, when the need for ATP is very great, the concentration of cytosolic calcium (released by sarcoplasmic reticulum) increases, producing an augmentation of mitochondrial calcium serving to stimulate the activity of certain matrical enzymes (dehydrogenases [5–7], ATP synthase [8]) responsible for ATP production. It is noteworthy that calcium is the only known second messenger capable of entering mitochondria. The concentration of free calcium in the mitochondrial matrix influences certain enzymes of the electron transport chain. Calmitine, by binding calcium, could regulate the mitochondrial balance between free and bound calcium in response to cellular demand. This could account in part for muscle degeneration in patients with Duchenne's or Becker's muscular dystrophy and in dy/dy mice for which a large calmitine deficit has been observed [9]. Moreover, an experimental calmitine deficit, associated with increased mitochondrial free calcium and muscle degeneration, has been obtained in the normal mouse after a single injection of the myotoxic drug chlorpromazine [10]. According to data in the literature, this drug has an affinity for calcium-binding proteins [11,12] and could act more specifically at the mitochondrial level [13]. We studied the effect of chlorpromazine in vitro on subcellular fractions (sarcoplasmic reticulum and mitochondrial matrix) to determine whether the proteolytic action observed in vivo on calmitine was the same for other calcium-bound proteins (calcium ATPase and calsequestrin) or whether calmitine in fact showed particularities differentiating it from calsequestrin and according it a role and a very specific function within the mitochondrial matrix.

2. Experimental

2.1. Subcellular fractioning

Fractions of sarcoplasmic reticulum and mitochondria from gastrocnemius muscle of Swiss mice were obtained as previously described [14]. The preparation of mitochondrial matrix from isolated mitochondria was performed as reported in [15]. Pure calmitine was obtained by electroelution of matrical calmitine. The protein concentration of the fractions was determined by the method of Lowry [16].

2.2. Incubation of subcellular fractions and protein separation

Fractions of sarcoplasmic reticulum, mitochondrial matrix and eluate collected in MOPS-KCl (10% triton X 100) buffer were incubated for 0.5 to 10 min at 37°C or 4°C in the presence or absence of chlorpromazine (5 mg/mg of protein) and α2 macroglobuline (2 mg/mg of protein). The proteins of the different fractions were then separated in duplicate by SDS polyacrylamide gel electrophoresis [17]. One gel was stained with Coomassie brilliant blue, and the other was used for blotting on nitrocellulose. Ca^{45} binding was performed on blots, as described in [18].

3. Results and discussion

Chlorpromazine, a phenothiazine derivative, is a myotoxic drug in vivo which seems more specifically to have a degenerative action on mitochondria [13] and a preferential effect on calcium-binding proteins [11,12]. During a previous study [10], we found that a single intramuscular injection of chlorpromazine in the normal mouse caused a decrease in mitochondrial calmitine associated with an increase in mitochondrial free calcium and a degeneration of muscle fibers. The in vitro results reported here confirm this specific effect of chlorpromazine on

*Corresponding author. Fax: (33) (40) 403686.

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calcium-binding proteins and provide a possible explanation for its mechanism of action.

Fig. 1 (A and B) shows that the drug caused nearly total degradation of calcium ATPase and calsequestrin in sarcoplasmic reticulum (Fig. 1A), and of calmitine in the mitochondrial matrix fraction (Fig. 1B). It can be seen that the proteolysis increased as a function of time. Conversely, calmitine obtained by electroelution of the matrical protein showed no degradation as a result of chlorpromazine action (Fig. 2A, lanes 7 and 8).

It is very likely that the lack of any effect of chlorpromazine on eluted calmitine means that the drug acts through enzymes isolated respectively in sarcoplasmic reticulum and mitochondrial matrix and totally absent in the eluate (owing to the way in which it was obtained). Chlorpromazine would appear to stimulate the specific proteases of these proteins. Our results (Fig. 2) indicate that the proteolytic activity of chlorpromazine on calmitine was abolished in the mitochondrial matrix fraction in the presence of the protease inhibitor α2-macroglobulin. Conversely, α2-macroglobulin did not prevent the proteolytic activity of chlorpromazine on the calcium ATPase and calsequestrin of sarcoplasmic reticulum (Fig. 2). These observations, clearly showing that calsequestrin and calmitine, despite certain similarities [2], are not dependent on the same protease, suggest that each has a distinct specificity in addition to a different localization. It may be noted that in all cases Ca^{45} binding to specific proteins (Fig. 2B) reflected the quantitative protein variations observed after Coomassie brilliant blue staining (Fig. 2A).

In vitro and in vivo experiments were performed by replacing chlorpromazine with phenothiazine which had no proteolytic effect on the proteins studied. Thus, the specific effect found in our study is attributable only to the derivative chlorpromazine. (This drug may unblock calmitine synthesis in genetically dystrophic mouse [19]). We also tested another myotoxic drug, metoclopramide, which had no proteolytic effect on the proteins studied.

Thus, our study shows that the myotoxic effect of chlorpromazine could be due to a stimulation of the specific proteases of calcium-binding proteins (calcium ATPase, calsequestrin and calmitine). These proteins play a primordial role in muscular function, and their degradation could lead to muscle degeneration. Despite their similarities, calsequestrin and calmitine have different localizations (respectively sarcoplasmic reticulum and mitochondrial matrix) and do not respond to the same proteolytic enzymes. The proteases of calsequestrin are insensitive to α2-macroglobulin, whereas those of calmitine are not. These observations seem to indicate a real specificity of calmitine within the mitochondrial matrix of skeletal muscle. The role of calmitine could thus prove of considerable importance in mitochondrial response to cellular energy demands. Calmitine, as a function of cellular ATP needs, would continually adapt the balance between free and bound calcium within the mitochondrial matrix, either activating or inhibiting (as circumstances required) the matrical enzymes involved in ATP synthesis.

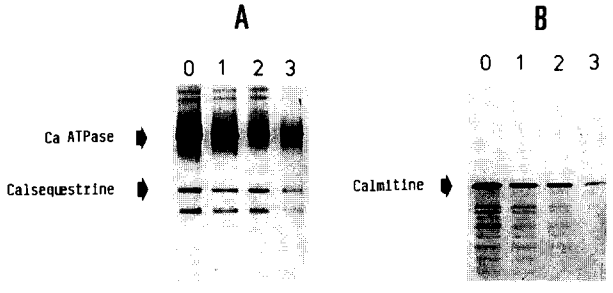

Fig. 1. Separation of subcellular fraction proteins by SDS polyacrylamide gel electrophoresis and Coomassie brillant blue staining. (A) Sarcoplasmic reticulum fractions (40 μg) were incubated for 30 sec (lane 1), 1 min (lane 2), 5 min (lane 3), in the presence of chlorpromazine; lane 0: control without chlorpromazine. (B) Mitochondrial matrix fractions (30 μg) were incubated for 30 s (lane 1), 1 min (lane 2) and 5 min (lane 3) in the presence of chlorpromazine; lane 0: control without chlorpromazine.

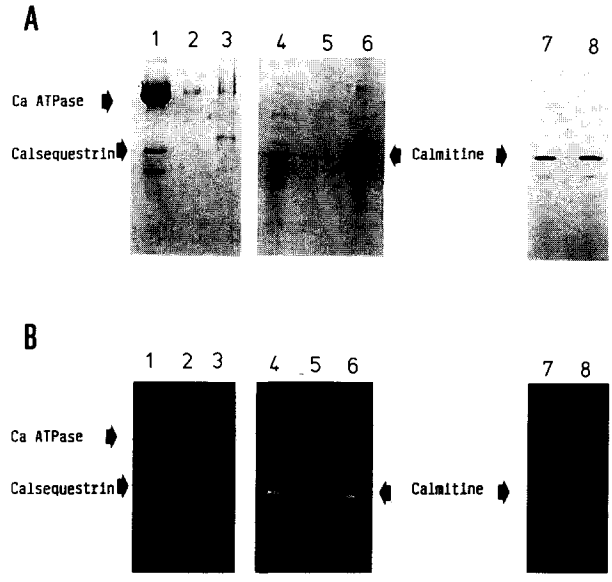

Fig. 2. Separation of subcellular fraction proteins by SDS polyacrylamide gel electrophoresis. All fractions were incubated for 10 min in the presence or absence of chlorpromazine and α2 macroglobulin. lanes 1–3: sarcoplasmic reticulum (60 μg): lane 1, control; lane 2: with chlorpromazine; lane 3: with chlorpromazine and α2 macroglobuline. Lanes 4–6: mitochondrial matrix (30 μg): lane 4: control; lane 5: with chlorpromazine; lane 6: with chlorpromazine and α2 macroglobuline. Lanes 7 and 8: eluated calmitine (10 μg): lane 7 control; lane 8 with chlorpromazine. (A) Coomassie brilliant blue staining. (B) ^{45}Ca binding proteins after autoradiography.

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