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A DIRECT EVIDENCE OF THE LOCALIZATION OF MITOCHONDRIAL CALMODULIN

Osamu Hatase, Akitaka Doi, Toshifumi Itano, Hideki Matsui, and Yoshiaki Ohmura

Dept. of Physiology, Kagawa Medical School, Miki-Cho, Kagawa, Japan 761-07

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The presence and localization of mitochondrial calmodulin was directly proved immuno-electron microscopically by the protein A-gold technique. In the ultra-pure mitochondria the complexes of anti-calmodulin antibody and protein A-gold clearly showed the localization of mitochondrial calmodulin on the inner membrane and in the matrix space.

We communicated the presence and localization of mitochondrial calmodulin (Mt-CaM) in rat liver and bovine heart mitochondria: Biochem. Biophys. Res. Commun. 104, 673, 1982 (1) and idem 113, 633, 1983 (2). A critical paper was communicated to be open question on the presence of real Mt-CaM (3). To clarify the localization of real Mt-CaM we present the direct evidences of the CaM presence in the ultra-pure rat liver mitochondria by immuno-electron microscopy.

Materials and Methods

SOURCES: Phenylmethylsulfonyl fluoride (PMSF) from Sigma Chemical Co. (St Louis); Percoll from Pharmacia (Uppsala); rabbit gamma-globulin from Cooper Biomedical (Malvern); protein A-gold from Janssen (Beerse). The anti-CaM antibody was kindly supplied by Dr. J.H. Wang, Calgary, Canada. The chemical reagents used were all reagent grade.

MITOCHONDRIA: Rat liver mitochondria were prepared from over night fasted Wister rats by the method previously described (1) in the isotonic sucrose media with EGTA and Percoll gradient centrifugation: Rat livers were homogenized in the preparation medium that contained 0.25 M sucrose, 20 mM Tris-HCl, pH 7.4, 1 mM β-mercaptoethanol, 1 mM magnesium acetate, 1 mM imidazole, 0.1 mM PMSF, and 1 mM ethyleneglycol bis(β-aminoethylether)-N,N,N',N',-tetraacetic acid (EGTA). The mitochondrial fraction (Mt) was washed three times in the same media at 7,000 X g, for 10 min. The thirty percent and 50 % (V/V) pH-adjusted-Percoll, pH 7.4, was prepared in 0.25 M sucrose solution. The three times washed Mt (5 ml) were mixed with 30 % Percoll solution (30 ml), and were centrifuged at 32,000 X g, for 30 min. The lower Mt fraction (5 ml) was again mixed with 50 % Percoll solution (30 ml), and was centrifuged in the same conditions. The lower Mt fraction was again washed with the preparation media.

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IMMUNO-ELETRON MICROSCOPY: The pure Mt were fixed with 4 % paraformaldehyde and 0.25 % glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 1 h at room temperature, and embedded in Epon 812 by the method of Luft (4). Immunostaining was performed in two steps: The first antibody reaction was done in the ultra-thin sections on mesh; the samples were treated with 1 % bovine serum albumin (BSA) solution in phosphate buffered saline (PBS) for 30 min at room temperature, and then the samples were treated with anti-CaM antibody (50 µg/ml --- 2.4 mg/ml) in the same BSA solution for 24 h at 4° C. As a control the samples were treated with rabbit gamma-globulin (25 µg/ml --- 1.2 mg/ml) in the BSA solution instead of anti-CaM antibody. The second antibody reaction was performed with the protein A-gold complexes (10 times diluted) for 1 h at room temperature. The samples were well washed with PBS, and were counterstained with uranyl acetate and lead acetate.

Results and Discussions

Fig. 1 (A) showed the control pattern of the mitochondria immuno-stained by the protein A-gold technique (5) with rabbit gamma

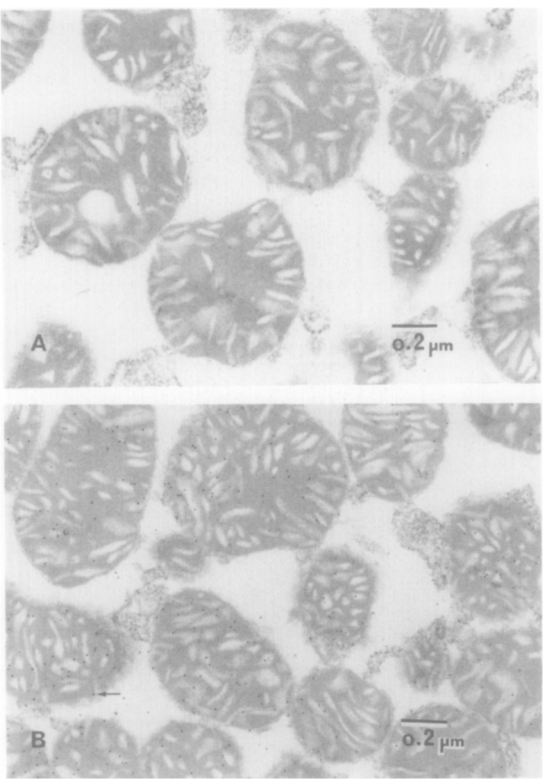

Fig. 1. (A) A control mitochondrial pattern immuno-stained without anti-CaM antibody treatment. (B) A typical experimental Mt pattern immuno-stained with anti-CaM antibody treatment. The preparation procedures of the samples were described in Materials and Methods; the concentration of the first antibody reaction was 1.2 mg/ml for rabbit gamma globulin as control (A) and 2.4 mg/ml for anti-CaM antibody (B). The final magnification was 50,000 times. An arrow indicates a protein A-gold complex.

globulin as control. The particle ratio of the protein A-gold complexes in the Mt space was $0.36/cm^2$, and it was $0.16/cm^2$ in the non-Mt background space, respectively. There was only a few non-specific background particles in the control system.

The experimental Mt system treated with anti-CaM antibody clearly showed the presence of the specific immuno-complexes on the inner membrane and in the matrix space; the particle ratio was $3.4/cm^2$ in the Mt space and $0.48/cm^2$ in the background space, respectively (Fig. 1, B). The localization of the Mt-CaM was about half on the inner membrane, and the other half in the matrix space. These results consist with the data presented previously (1). It is noteworthy that there is no outer membrane-bound CaM in these EGTA-washed Mt. The Mt samples used was pure enough (Fig. 2) and functionally active in oxidative phosphorylation (data not shown).

The physiological significance of Mt-CaM is still under discussion. But the presence of CaM-binding protein (2,6), nucleotide phosphodiesterase (7) and nucleotide cyclase (8), protein kinases (9,10), and cyclic AMP receptor protein (11) in Mt suggests certain physiological roles of CaM in Mt.

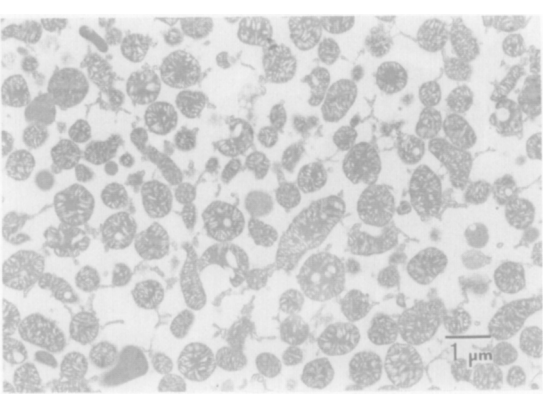

Fig. 2. A pattern of mitochondrial population used. The final magnification was 10,000 times.

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