AN IN VITRO STUDY OF THE INTERACTION OF HEART MITOCHONDRIA WITH TROPONIN-BOUND Ca^{2+}

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Summary: Rat heart mitochondria are able to extract a large fraction of the Ca^{2+} tightly bound to rabbit skeletal muscle troponin, or to the 18.300 daltons, Ca^{2+} receptor fragment of the troponin molecule (TN-C). The amount of Ca^{2+} removed may reach 100 % in the case of TN-C- but substantially less with intact troponin. The reaction is fairly rapid, often reaching completion in seconds, and is inhibited by uncouplers and by the classical inhibitor of Ca^{2+} transport in mitochondria, ruthenium red.

In the last few years, it has been repeatedly suggested that mitochondria can play a role in the Ca^{2+} cycles in heart cells (Patriarca and Carafoli, 1968; Fehmers, 1968; Haugaard et al., 1969; Gillis, 1972; Carafoli, 1972, 1974). Severa parameters of the Ca^{2+} binding and transport process have been examined: one of the most important is certainly the affinity for Ca^{2+}. Clearly, if mitochondria were to be involved in the beat to beat regulation of Ca^{2+} in heart, they would have to be able to transport it with optimal efficiency from concentrations not too far removed from 1 μM, which is supposed to be the concentration of free Ca^{2+} in heart sarcoplasma above which contraction of the myofibrils ensues. Several Authors have indeed been able to measure K_m's of heart mitochondria for Ca^{2+} in the vicinity of 1 μM. Carafoli and Azzi (1972) have used the redox shift of cytochrome b as an indicator for the energy-linked interaction of mitochondria with Ca^{2+}, Reynafarje (1974) has used the stimulation of oxygen consumption by low concentration of Ca^{2+}, Bygrave et al. (1971) have measured the ATP hydrolysis induced by the addition of Ca^{2+} to mitochondria. It is important to note that in all these cases care was taken to relate the K_m to the actual free Ca^{2+} concentration in the system, an obvious precaution in media containing several substances able to complex Ca^{2+}. Recently, however, the problem of the affinity of heart mitochondria for Ca^{2+} has been reexamined by Scarpa and Graziotti (1973), who have measured the removal of Ca^{2+} from the medium with the "direct" murexide spectrophotometric method. They have concluded that the K_m for Ca^{2+}, at least in

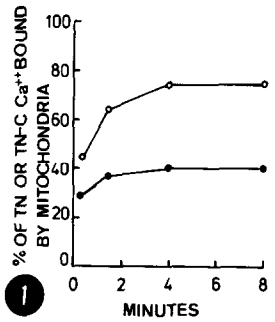
rodent-heart mitochondria, is between one and two orders of magnitude higher than that measured by the other Authors, and have therefore concluded that heart mitochondria play no major role in the beat to beat regulation of Ca^{2+}. The reasons for the difference between the results of Scarpa and Graziotti (1974) and those of the other Authors mentioned above are not clear, although one could question the use of Mg^{++}, which is known to depress the ability of heart mitochondria to transport Ca^{++}, or the fact that no correction was applied by Scarpa and Graziotti for the Ca^{2+} complexed by components of the reaction medium.

Since all the methods used to determine the affinity of mitochondria for Ca^{2+} have limitations, and since mitochondria must eventually be able to extract Ca^{2+} from troponin, similarly to sarcoplasmic reticulum of skeletal muscle (FSR), if they are to be involved in the beat to beat regulation of Ca^{2+} in heart, it was thought desirable to study directly the ability of heart mitochondria to extract Ca^{2+} from troponin (TN) or from its Ca^{2+} binding component (TN-C). The results obtained have shown that the affinity of heart mitochondria for Ca^{2+} is indeed high enough for removing most of the tightly bound Ca^{2+} from TN-C, either free, or present in the troponin complex.

Materials and Methods: Mitochondria were prepared from the hearts of albino rats (Wistar strain) essentially according to the method of Pande and Blanchaer (1971), slightly modified to obtain a lower proteinase (Nagarse) to tissue ratio. Troponin, and the TN-C, Ca^{2+}-binding protein, were prepared from rabbit skeletal muscles according to Drabikowski et al. (1971), and were labeled with $^{45}Ca^{2+}$ (New England Nuclear, Frankfurt, carrier-free). Free and loosely bound Ca^{2+} were removed by treating the protein solutions with Dowex-50 (Drabikowski and Barylko, 1971).

The incubation of mitochondria with TN or TN-C was carried out in small flasks, at 25°, with continuous stirring, in a final volume of 2 ml. The medium contained 0.21 M mannitol, 0.07 M sucrose, 0.01 M Tris-Cl, pH 7.4, 0.005 M Na-pyruvate, 0.005 M Na-malate, 5 mg mitochondrial protein, 1 mg TN or 0.2 mg TN-C. When present, ATP was 0.0025 M, $MgCl_2$ 0.005 M, inorganic phosphate 0.005 M, ruthenium red 7 μM, carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP) 5 μM, antimycin 1.5 μg, oligomycin 1.5 μg, rotenone 1.5 μM. At the required time, 50 μl aliquots of the incubation medium were rapidly withdrawn and filtered through Millipore filters (0.45 μ pore size) which retained mitochondria and let TN and TN-C through. The filters were rapidly washed with approximately 1 ml cold incubation medium, and counted in a Beckman LS-100 scintillation counter.

Results and Discussion: Figure 1 shows that heart mitochondria, incubated under conditions that permit energy-linked uptake of Ca^{2+}, are able to remove about 40 %



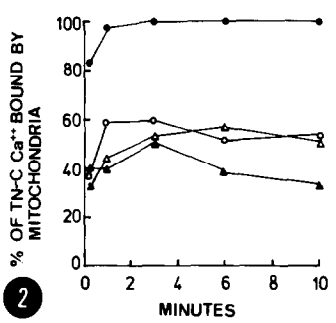


Fig. 1. Removal of Ca^{2+} from TN and TN-C by heart mitochondria. Technical details are found in the Materials and Methods Section. The incubation medium contained ATP and $MgCl_2$. -●- TN. -○- TN-C.

Fig. 2. Removal of Ca^{2+} from TN-C by heart mitochondria. Effect of ATP, $MgCl_2$, antimycin, oligomycin. Technical details are found in the Materials and Methods Section. -●- standard incubation medium. -○- plus ATP, $MgCl_2$, inorganic phosphate. -▲- plus ATP, $MgCl_2$, inorganic phosphate, antimycin, rotenone. -△- plus ATP, $MgCl_2$, inorganic phosphate, oligomycin.

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of the Ca^{2+} bound to TN and almost 80 % of the Ca^{2+} bound to TN-C, in 2 to 4 minutes. Under the experimental conditions of Figure 1, the removal of Ca^{2+} from TN varied in different experiments between 40 and 60 %. It has also been found that the removal from TN-C could reach 100 % in less then one minute (see Figure 2), if the medium did not contain ATP and $MgCl_2$. This is probably due to the inhibitory effect of $MgCl_2$ against the transport of Ca^{2+} by heart mitochondria (Jacobus and Carafoli, in preparation). The observation is of interest from a physiological standpoint, since heart mitochondria in situ must presumably operate in the presence of fairly high concentration of Mg^{2+}. It is also of interest that mitochondria seem to be more active against TN-C-bound Ca^{2+} than against TN-bound Ca^{2+}, similarly to rabbit skeletal FSR (Drabikowski et al., 1973). This observation may indicate a "protective" role against the removal of Ca^{2+} by the other fragments of the troponin molecule, and is currently being extended. It is also in agreement with the finding by Potter et al. (1973) that the affinity of Ca for TN is 10 times higher than for TN-C alone. It is also worth noting that in rodent hearts the concentration of troponin is about 1.5 mg/g (Tsukui and Ebashi, 1973) whereas that of mitochondria is about 80 mg of protein per g of tissue (Scarpa and Graziotti, 1973). In living hearts, then, the ratio of mitochondria to troponin, on a protein basis, is about 50 to one, that is,

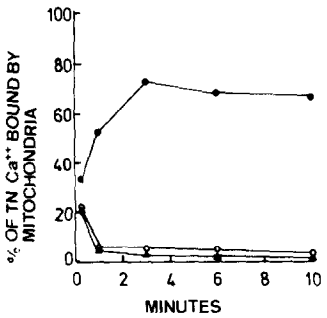

Fig. 3. Removal of TN-C-bound Ca^{2+} by heart mitochondria. Effect of ruthenium red and FCCP. Technical details are found in the Materials and Methods Section. The medium contained ATP, $MgCl_2$, inorganic phosphate (-●-), ATP, $MgCl_2$, inorganic phosphate, ruthenium red (-○-), ATP, $MgCl_2$, inorganic phosphate, FCCP (-▲-).

10 times higher than in the experiments described in the present paper. It is easy to see, thus, that the conditions employed in the present paper would tend to underestimate the total capacity of heart mitochondria to remove Ca^{2+} from troponin. Figure 2 also shows that oligomycin and the respiratory inhibitors antimycin and rotenone have at best a marginal effect on the ability of mitochondria to remove Ca^{2+} from TN-C, when the medium contains both respiratory substrates and ATP. This indicates that in the system used both ATP and the respiratory substrates contribute energy for the uptake of Ca^{2+}. Both the ATP-supported, and the respiration supported processes have thus the ability to extract Ca^{2+} from TN-C.

Uncouplers of oxidative phosphorylation, which completely prevent the energy-linked interaction of Ca^{2+} with mitochondria, and ruthenium red, which abolishes both the energy-linked transport (Moore, 1970), and the energy-independent binding of Ca^{2+} (Vasington et al., 1972), eliminate almost completely the ability of mitochondria to remove Ca^{2+} from TN-C (Figure 3). Since uncouplers do not affect the non-specific binding of Ca^{2+} to the so-called low-affinity sites of the mitochondrial membrane (Carafoli and Lehninger, 1972) the experiment shows that only the energy-linked system has the ability to remove Ca^{2+} from TN-C. It is not necessary to postulate, however, a trans-membrane transport of Ca^{2+} to the inner mitochondrial compartment. TN binds a maximum of 4 moles of Ca^{2+} per mole at two classes of binding sites in the absence of Mg^{2+}, and at one class in its presence. Considering the total amount of TN in heart (see above),this would correspond to less than 100 nmoles of Ca^{2+} per g of tissue, as compared to 80 mg of mitochondria protein. In the presence of energy, these minute amounts could easily be complexed

by superficial, or intramembrane, high-affinity binding sites of the mitochondrial membrane. It appears, then, that the affinity of mitochondria for Ca^{2+} exceeds that of troponin, which binds it with an affinity constant of the order 10^6-$10^8 M^{-1}$ depending on the concentration of Mg^{2+} (Potter et al. 1973). Obviously, the experiments presented here do not establish the involvement of mitochondria in the beat to beat regulation of Ca^{2+} in heart. It is clear, however, that they cannot be ruled out on the basis of their affinity for Ca^{2+}.

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