KINETICS OF CALCIUM ACCUMULATION BY MITOCHONDRIA, STUDIED IN SITU, IN SQUID GIANT AXONS

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Received 26 April 1978

1. Introduction

The relevance of in vitro studies of mitochondrial function to physiological situations remains conjectural until critical aspects of this work can be verified in situ.

This report permits comparison of in vitro and in situ rates of calcium uptake by cyanide sensitive entities presumed to be mitochondria, as a function of free calcium concentration. Calcium transport was measured kinetically in situ, in squid axons, by following the differential absorbance changes of calcium sensitive metallochromic indicators, in the presence and absence of inhibitors of mitochondrial energy-linked functions. At very high concentrations of free calcium (~ 50 μM), the rate of in situ uptake is reasonably close to published rates for several in vitro mitochondrial preparations. However, at physiological concentrations (less than 100 nM), the rate is so low as to suggest that mitochondria play a relatively limited role in physiological calcium homeostasis in squid axons.

In these experiments we have no evidence, other than the sensitivity of the uptake to cyanide, that the calcium was actually sequestered within mitochondria. However, in closely similar experiments in squid axons [1], it has been reported that calcium does appear within mitochondria as well as within other organelles.

2. Methods

The basic techniques used in these experiments, i.e., multiwavelength differential absorption microspectrophotometry in combination with metallochromic dyes, microinjected into single isolated squid axons, as well as the calibration of the absorbance changes, have been described [2–4]. In addition to arsenazo III, used to measure low concentrations of free calcium, another dye, antipyrylazo III, (3,6-bis(4-antipyrylazo)-4,5-dihydroxy-2,7-napthalene-disulphonic acid), was used to measure high concentrations. The properties and purification of this dye are described [5]. Free calcium was measured at wavelength pairs 685–675 nm with arsenazo III and 685–660 nm with antipyrylazo III.

Experiments were performed during May and June, 1977, using living specimens of *Loligo pealei* obtained from the Marine Biological Laboratory, Woods Hole, MA. Isolated axons were stored at 10°C, in 3 mM calcium artificial sea water, in place of the usual 10 mM calcium, to prevent gain of intracellular calcium [2].

Arsenazo III was purchased from Sigma Chemical Co., St Louis, MO and purified as in [2]. The commercial purified product had approx. 4% (mol fraction) calcium contamination, which was reduced to approx. 0.5% by repeated passage over a Chelex 100 column. Antipyrylazo III was obtained from ICN Pharma-

ceuticals, Plainview, NY and twice recrystallized from alcohol. The dye was essentially free of calcium (less than 0.1% mol fraction), but contained about 15% of a nontoxic dye contaminant which was not sensitive to calcium. FCCP (carbonyl cyanide 4-trifluoro-methoxyphenylhydrazone) was obtained from Pierce Chemical Co., Rockford, IL and was dissolved in 2 mg/ml DMSO (dimethyl sulphoxide). Aliquots, 5 μl, were applied directly to the surface of the axon. Apyrase (adenosine 5′-triphosphatase and adenosine 5′-diphosphatase), was obtained from Sigma Chemical Co., and purified of calcium by passage through a Chelex 100 column.

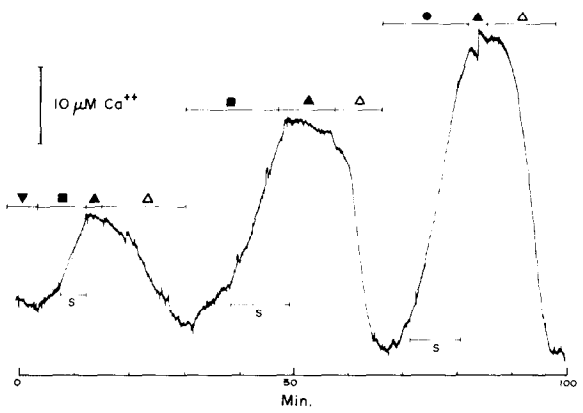

Fig.1. In situ calcium uptake by mitochondria, in intact squid axon, at 16°C. Axon preinjected with antipyrylazo III to final conc. 494 μM, and apyrase to final conc. 0.3 units/ml axoplasm. Axon diam. 445 μM. Horizontal bars labelled 'S' indicate periods of electrical stimulation of axon at 100 pulses/s. Symbols indicate composition of medium bathing axon as follows: (▼) 3 mM Ca^{2+} artificial sea water plus 2 mM CN^-; (■) 56 mM Ca^{2+} artificial sea water plus 2 mM CN^-; (▲) Ca^{2+}-free artificial sea water plus 2 mM CN^-; (△) Ca^{2+}-free artificial sea water; (●) 112 mM Ca^{2+} artificial sea water plus 2 mM CN^-.

3. Results and discussion

The rate of calcium uptake by mitochondria was evaluated at physiological concentrations of free calcium, i.e., 50–100 nM [2], and also at high concentrations, i.e., 5–50 μM. The procedures varied in the 2 series of experiments.

Measurements of rate of calcium uptake at high free-calcium concentrations required considerable prior loading of the fibers with calcium. In these experiments, the axons were first injected with apyrase to final conc. 0.1–0.3 units/ml axoplasm. This enzyme reduces cellular ATP from the normal value of 4 mM to ~ 50 μM [6]. The axons were then treated with cyanide to block the respiratory chain and loaded with calcium by electrical stimulation at 100 pulses/s in high calcium sea water. Figure 1 shows such an experiment in which the first load was imposed upon an axon by electrical stimulation in artificial sea water containing 56 mM Ca^{2+} and 2 mM CN^-. This procedure raised the cytoplasmic ionized calcium to about 12 μM. The initial concentration of ionized calcium after injection of antipyrylazo III and apyrase is uncertain, but should have been about 50–100 nM [2] which is less than 1% of the first increment produced by loading. The loading period was terminated by bathing the fiber in Ca^{2+}-free artificial sea water containing 2 mM CN^-. In this solution, a decrease in free-calcium concentration presumably reflects loss via the axolemma since the mitochondria are still inactivated by CN^-. After a baseline had been obtained the CN^- was removed, presumably activating the mitochondria, and resulting in a much more rapid decrease in free calcium. This decrease can not be the result of ATP activation of the calcium efflux [7,8] because the presence of apyrase keeps the axoplasmic ATP at a low level. Therefore, the inference is that the difference in rate of loss of free calcium in Ca^{2+}-free artificial sea water with and without CN^-, reflects the activity of a cyanide-sensitive entity, presumably mitochondria. Ordinarily, the rate of decrease in free calcium in the Ca^{2+}-free artificial sea water was much less than seen in this experiment, and was not considered in the calculation of CN^--sensitive uptake. The procedure was repeated by re-exposing the fiber to cyanide and loading it with more calcium to obtain a higher free concentration. Figure 1 shows that raising the level of ionized calcium increased the maximum rate of uptake. It is possible to convert the rate of disappearance of free calcium from the axoplasm to mitochondrial calcium uptake by knowing the volume fraction of mitochondria in axoplasm (1%) [9], the fraction of calcium load which appears as ionized calcium under these conditions (~ 5%) [3,4], and the fraction of mitochondria which is protein (3.43 kg

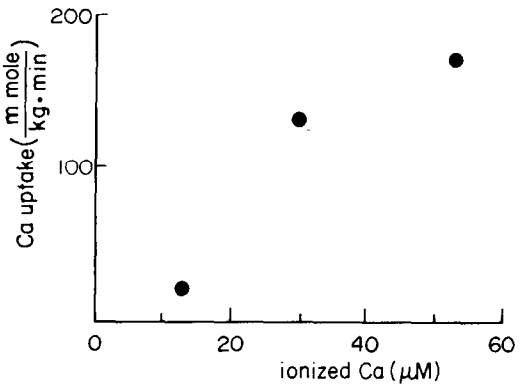

Fig.2. Plot of data of fig.1 to show calcium uptake, converted to mmol/kg mitochondrial protein.min, as a function of ambient calcium, at high calcium concentration. Rate was calculated from maximum uptake slopes, using parameters described in text.

wet wt/kg protein) [3]. The data of fig.1 are replotted in fig.2 to show the calculated rate of calcium uptake by mitochondria as a function of ionized calcium concentration. Although the rate of uptake does not saturate at the highest ionized calcium concentration obtainable with this method of loading (~ 50 μM), it is clear that the uptake is less than proportional to the calcium concentration. The rate of uptake at a free-calcium concentration of 50 μM, is about 27 μmol/kg axoplasm.min, which is equivalent to a calculated rate of uptake by mitochondria of about 170 mmol/kg mitochondrial protein.min.

It is somewhat difficult to compare the absolute rates of uptake in these in situ experiments with data for experiments in vitro, because squid axoplasm has higher ionic strength (~ 0.4 M), higher free-magnesium concentration (2–3 mM) [10,11] and lower physiological temperature (16°C), than more familiar preparations. However, the rate of uptake in the present experiments is about the same as or somewhat higher than in vitro rates. Table 1 in [12] gives a range of 3–14 mmol/kg mitochondrial protein . s, at 26°C, which is fairly close to the value reported here, 3 mmol/kg mitochondrial protein . s, especially if one assumes a reasonable value of Q_{10} 2.5 for the temperature dependence of the uptake, which raises our value to about 8 mmol/kg mitochondrial protein . s.

Experiments at low ionized calcium were done somewhat differently as shown in fig.3. In these

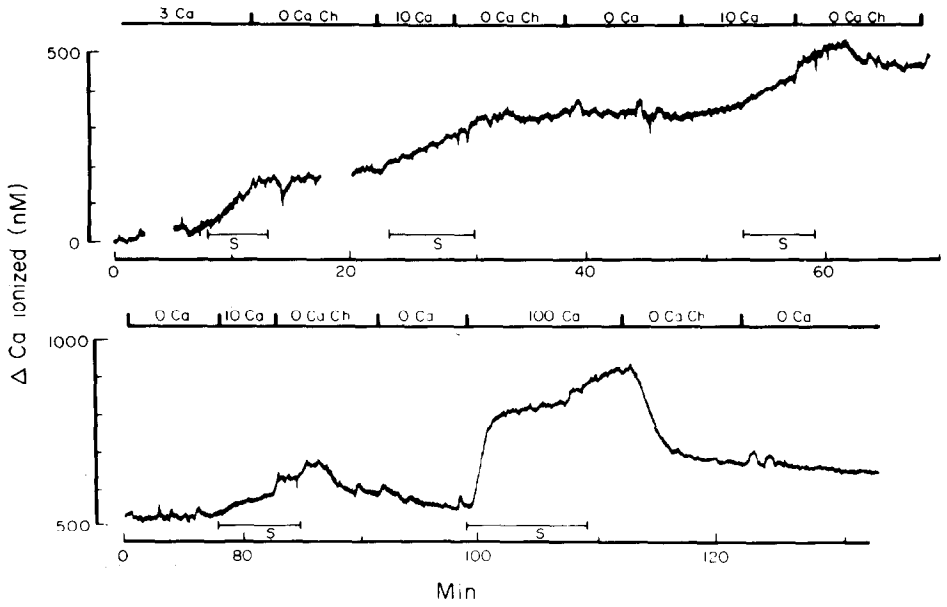

Fig.3. In situ calcium uptake by mitochondria of an intact squid axon, at 16°C. Axon preinjected with arsenazo III to final conc. 450 μM. Horizontal bars labelled 'S' indicate electrical stimulation at 100 pulses/s. Upper bars indicate composition of medium bathing the axon. (Ch: choline replacing sodium in artificial seawater).

experiments, the axon was not poisoned with cyanide but instead was only given a very light calcium load, by electrical stimulation in 3 mM or 10 mM calcium sea water. After the load was imposed, the fiber was bathed in Ca^{2+}-free choline solution to prevent either gain of calcium by leak, or loss of calcium by the sodium–calcium exchange mechanism [13]. Assuming that the total calcium content remains constant in the recovery solution, any decrease in free calcium must reflect uptake by an internal sink. Since there was little change in ionized calcium during the first two periods in Ca^{2+}-free choline solution, it is concluded that mitochondria were not active in sequestering calcium from axoplasm at these ambient free-calcium concentrations, i.e., 200–300 nM.

However, following the third loading period which raised the free-calcium concentration in the axoplasm to about 500 nM, a small but clear reduction in ionized calcium occurred when the fiber was soaked in the Ca^{2+}-free bathing solution. During the fourth and fifth loading periods, when the ionized calcium was allowed to rise to 650 nM or 950 nM before recovery, there was a clear and progressively more rapid disappearance of calcium, reflecting increased rates of calcium sequestration.

The data of fig.3, showing uptake at low concentrations of free-calcium, are replotted in fig.4 to permit comparison with the experiment in fig.2, obtained at very high concentrations. Although a sigmoidal relation between concentration and uptake is not obvious from these data, such is implied. The calculated rate of uptake increases by a factor of 120 between free-calcium concentrations of 0.5 μM and 10 μM, although the concentration increases by only a factor of 20. This indicates a relation between uptake and free calcium which increased more than linearly. Below 0.5 μM, one cannot be sure that the rate of uptake is independent of concentration, since it is too low for accurate measurement.

Considered together, the data of fig.2 and fig.4 show qualitatively that calcium uptake by entities presumed to be mitochondria, at low free-calcium concentration is undetectable until the free calcium is in the high physiological range of several hundred nM, and above 10 μM rises less than proportionately. Quantitatively, the data show that although in situ mitochondria of squid axons are able to take up calcium from a high free-calcium concentration, at

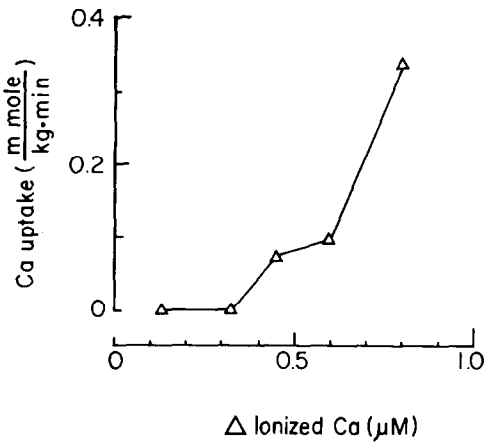

Fig.4. Plot of data in fig.3 to show calcium uptake calculated in mmol/kg protein.min, as a function of ambient calcium, at a near physiological concentration of free calcium.

about the same rate as isolated mitochondria studed in vitro, they are not a rapid buffering agent when the free-calcium concentration in axoplasm is in the range of several hundred nM. At this level of ionized calcium, the membrane pump may be as effective or more effective than mitochondria as a calcium sequestering mechanism, since the membrane either in the presence or absence of ATP has been shown to be sensitive to free-calcium concentration as low as 10 nM [7,8,14].

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