KINETIC MEASUREMENT OF Ca^{2+} TRANSPORT BY MITOCHONDRIA IN SITU

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1. Introduction

Mitochondria isolated from all mammalian tissues accumulate Ca^{2+} in a process which requires energy. Reports have appeared in the literature describing in vitro study of Ca^{2+} transport and Ca^{2+} interaction by mitochondria, as well as kinetics and energy requirements and the effect of transported Ca^{2+} on the metabolic functions of the cell. Only isolated reports have dealt with the ability of mitochondria to accumulate calcium in situ [1]. In this report we present quantitative information regarding the calcium buffering capacity of mitochondria of squid axons studied in situ, and some qualitative information regarding the metabolic requirements for in situ function.

2. Materials and methods

Pulsed, multiwavelength, differential absorption spectrophotometry using Arsenazo III as an indicator of ionized Ca^{2+} was used to provide continuous measurement of the free calcium level in isolated squid axons as described previously [2].

The axon was microinjected with sufficient Arsenazo III dye to give a final concentration of about 500 μM. High intensity light from a tungsten iodine halide lamp was directed through the axons by a glass fiber optic light pipe and the transmitted light collected by a similar light pipe positioned on the other side of the axon. The light from the lamp was converted into pulses of monochromatic light by passage through 8 interference filters mounted in a rotating wheel driven by compressed air at a frequency of about 1000 Hz. The signal from these pulses was stored electronically and displayed either as individual or differential absorbances on a multipen recorder. The absorbance was usually monitored at the isosbestic point (570 nm) and at the wavelength pairs 675–685 nm or 660–685 nm, where increments in ionized Ca^{2+} result in a specific change in ΔA. Absorbance changes were converted into changes in ionized calcium by in vitro calibrations using salt solutions with a composition similar to the axoplasm, in a glass capillary of the same size as the squid axon, positioned in the dialysis chamber [2]. Experiments were performed during May and June, 1976, using living specimens of *Loligo pealei* obtained from the Marine Biological Laboratory,

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Woods Hole, Mass. Arsenazo III was purchased through Sigma Chemical Co. (St Louis, Mo.) and purified as described previously. FCCP (carbonyl cyanide 4-trifluoromethoxyphenylhydrazone) was a gift of Dr Heytler, Du Pont Co., Wilmington, Del. Apyrase (adenosine 5′-triphosphatase and adenosine 5′-diphosphatase) was obtained from Sigma and calcium contamination removed by passage through a Chelex-100 column.

3. Results and discussion

Figure 1 shows the results of an experiment where two axons of comparable size were microinjected with similar concentrations of Arsenazo III and the absorbance changes monitored in situ. A known calcium load was introduced into both axons by repetitive stimulation of the fiber at 100 impulses/s while immersed in 112 mM calcium seawater. Previous work has demonstrated [3] and we have verified, that stimulation of axons in 112 mM seawater at this rate, increases the calcium content of axons by approx. 50 μmol calcium/kg axoplasm/min stimulation. Therefore the duration of stimulation can be converted directly into calcium load introduced into the axon as shown in fig.1.

The virtually flat trace in the figure shows the response of a normal axon to such stimulation. Experiments of this sort, as well as those in which calcium was introduced by microinjection or soaking in sodium-free solutions (which is known to increase the total calcium content [4]) show that an intact axon can buffer about 99.5% of an imposed calcium load for total loads between 50 μM and 2500 μM. (The actual increase in ionized calcium was about 0.6 nm/μM imposed load.) In contrast, axons treated with FCCP, which effectively uncouples oxidative phosphorylation in situ, are much less able to buffer calcium.

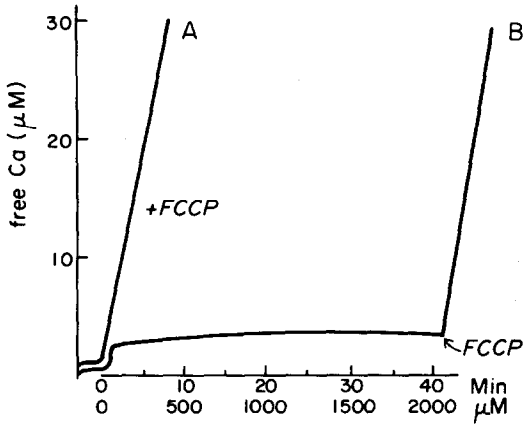

Fig.1. Two experiments showing the effect of stimulation upon ionized calcium. Increase of ionized calcium during stimulation in FCCP is about 6% of the entering load. In absence of FCCP rise of ionized calcium amounts to approximately 0.6 nM/μmol calcium load.

This reduced buffering capacity can be seen not only when FCCP is added simultaneously with the onset of stimulation (in this case the ability of the axon to sequester entering calcium is affected, see left trace, fig.1) but also when FCCP is added after stimulation (in this case calcium is released from the internal stores, see right trace, fig.1). Because of the documented in vitro capacity of mitochondria to sequester calcium, it is reasonable to suppose that the marked change in buffering capacity demonstrated by these experiments was due to mitochondria.

These results, and several others obtained with various mitochondrial inhibitors, clearly indicate that the mitochondria of squid axons are a powerful Ca^{2+} buffering system which can maintain the axoplasmic Ca^{2+} at low levels (3–5 μM) in spite of the substantial Ca^{2+} loading into the axoplasm. On the other hand, the mitochondria seem to be unable to accumulate effectively Ca^{2+} (even when the axoplasmic Ca^{2+} load was reduced by decreasing extracellular Ca^{2+} or the frequency of stimulation) if the concentration of ionized Ca^{2+} in the axoplasm is below 2–3 μM, although this concentration is roughly two orders of magnitude higher than physiological. These results are in line with kinetic data available on mitochondrial Ca^{2+} uptake in vitro and with the proposed physiological role of mitochondria in controlling intracellular Ca^{2+} homeostasis [5].

These data also suggest that two distinct mechanisms are operative in controlling the intracellular Ca^{2+} homeostasis of squid axons: the axolemmal Ca^{2+} pump and the energy-dependent Ca^{2+} transport by mitochondria. The Ca^{2+} pump should effectively control the level of axoplasmic ionized Ca^{2+} during limited Ca^{2+} loading, when the axoplasmic ionized Ca^{2+} concentrations remain confined to the nM levels. On the other hand, the mitochondrial Ca^{2+} uptake seems to be the more powerful Ca^{2+} buffering system in axoplasm

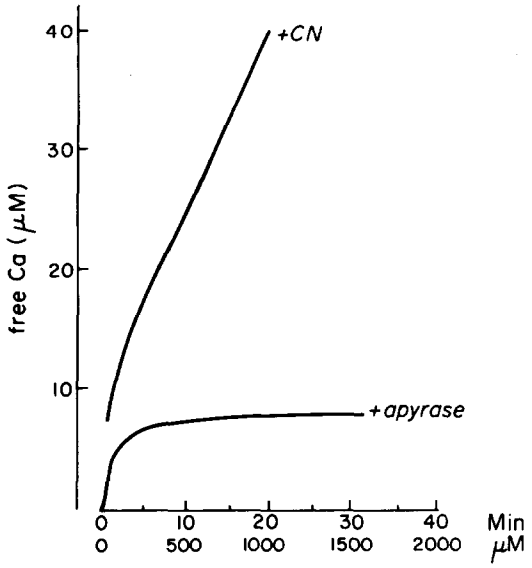

Fig.2. Composite of two experiments showing the effect of apyrase or cyanide on ionized calcium during stimulation. Cyanide causes rise in ionized calcium comparable to that seen in the presence of FCCP (compare fig.1).

when physiological or pathological events result in a massive Ca^{2+} load producing concentrations of ionized Ca^{2+} in the axoplasm much greater than resting.

Figure 2 provides qualitative evidence regarding the metabolic requirements for in situ mitochondrial calcium buffering. Apyrase was injected to give a final concentration in axoplasm of 0.1–0.3 unit/μl. The injection of this enzyme which breaks down axoplasmic ATP [6], leaves essentially unaffected the capacity of the axon to buffer large calcium loads. In contrast, exposure of the axon to 2 mM cyanide seawater simultaneously with the onset of stimulation immediately impairs the ability of the axon to buffer calcium, although it is known that the ATP concentration and the ATP/ADP ratio are essentially unaffected for the first 10–15 min after exposure to CN^- [7]. The conclusion is that ATP is neither necessary nor sufficient for in situ calcium buffering, whereas coupled oxidation of substrates is both necessary and sufficient.

The technique described in this communication is also suitable for estimating the releasable calcium present in mitochondria in situ at rest. Mitochondria freshly isolated from mammalian tissues are generally stated to contain 5–20 nmol calcium/mg protein (corresponding to 10 mM Ca if calcium were free in the mitochondrial matrix). This endogenous calcium is not releasable by EGTA or EDTA but is rapidly released in the medium to various extents, upon addition of uncouplers or calcium ionophores. However, the amount of calcium present in isolated mitochondria is not necessarily related to the amount present in the mitochondria in vivo, since it is obvious that mitochondria can either accumulate more calcium from the homogenate during cell disruption, or release endogenous calcium during the various stages of the preparation, or both.

Calcium content of mitochondria in situ of freshly dissected axons was estimated in the following way. Freshly dissected axons were positioned in the measuring chamber containing Ca^{2+}-free seawater, and microinjected with Arsenazo III. The application of maximally effective concentrations of the uncoupler FCCP causes a prompt increase in the axoplasmic Ca^{2+} from the resting levels of 20–50 nM to approx. 200 nM (mean of six fresh axons).

Since only about 6% of the calcium released by mitochondria appears as free calcium (see fig.1), the total calcium released from fresh axons into axoplasm after FCCP treatment is (200–50) nM × 1/0.06 = 2.5 μM. Since the volume fraction of mitochondria in axoplasm is about 1%, the actual concentration of releasable calcium in the mitochondria would be about 0.25 mM, or a few percent of the calcium content of freshly isolated mitochondria.

Acknowledgements

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