IONIZED MAGNESIUM CONCENTRATION IN AXOPLASM OF DIALYZED SQUID AXONS

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1. Introduction

Magnesium ion is a significant constituent of cell cytoplasm and participates in many intracellular enzymatic reactions. Although total cell magnesium can be rather easily determined by atomic absorption spectroscopy, the free magnesium is more difficult to measure and is much less than the total because of magnesium binding to cellular organelles or ionic constituents. Moreover, since magnesium is strongly bound to the phosphonucleotides involved in energy metabolism the concentration of the free form might be expected to vary with the state of metabolism of the cell, as for example might occur following metabolic poisoning. Despite the ubiquitous participation of magnesium in cellular reactions, very little is known about concentration of the ionized form under physiological conditions. This report describes the application of a dual wavelength microspectrometry to permit nondestructive measurement of ionized magnesium in single, isolated squid giant axons.

The formation of the magnesium complex of the dye, Eriochrome Blue SE, [3-(5-chloro-2-hydroxyphenyl)azo]-4,5-dihydroxy-2,7-naphthalene disulfonic acid] is accompanied by a significant shift in spectrum compared with the unbound dye. This dye also binds calcium, but it is possible by appropriate selection of wavelengths, to obtain difference spectra which are almost insensitive to changes in ionized calcium [1].

2. Materials and methods

Although in principle it is possible to calculate the ionized magnesium concentration either from the three parameters of K_m, dye concentration and pathlength or from a calibration curve relating absorbance change to ionized magnesium concentration, in these preliminary experiments a null point method was used. We determined the internal ionized magnesium by noting that concentration of magnesium which, when dialyzed into the axoplasm produced no change in the observed differential absorbance. Because the dye binds magnesium very weakly, (K_m = 25 mM in our solutions) the ionized magnesium was reduced less than 5% by the concentrations of Eriochrome Blue which we used (approx. 500 μM). Axons were isolated by the usual methods from living specimens of *Loligo pealei*, supplied by the Marine Biological Laboratory, Woods Hole, Massachusetts during May, 1974. The procedures for dialyzing giant axons has been described previously [2]. The microinjection apparatus was essentially that previously described [3] modified by positioning the injector so that it moved in a horizontal plane. The external bathing solution contained (mM): K:9, Na: 439; Mg:21; Ca:8; Cl:502; EDTA:0.1; TES:4 (TES = N-Tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid). The internal dialysis solution contained (mM) : K:293; Na:34; aspartate; 179; isethionate; 145; glycine: 235; BES:8.5 (BES = N, *N*-bis (2 hydroxy-

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methyl)-2-aminoethane sulfonic acid). Eriochrome B SE was obtained from Baker Chemical Co. and twice recrystallized before use. Fresh solutions of the dye were prepared for each experiment.

3. Results and discussion

The absorbance changes were measured in situ at four wavelengths simultaneously as illustrated in fig. 1. The system consisted of a lamp; four interference filters having 40-50% transmittance and 1 nm half band width (Omega Optical Co., Brattleboro, Vt.) equally spaced in a rotating wheel which is driven by compressed air at a frequency of 20–5000 Hz; a bridge with two miniscule optical fibers (0.3 × 3 mm) which can be positioned in the dialysis chamber on either side of the squid axon; a photomultiplier tube and sensitive electronics which made possible time sharing detection of four pulses of light individually or their difference, and a multichannel pen recorder. Some details of light modulation, electronic circuit and synchronization have been described and referenced recently [4]. With this instrumentation it was possible to maintain continuous readout of the changes in absorbance of Eriochrome blue inside the axon a) at 566 nm (an isobestic point for the absorbance of Eriochrome blue vs. Eriochrome blue plus Mg^{++}); b) at 566–550 nm, where $[Mg^{++}]$ increase produces an increase of absorption of Eriochrome blue and c) at 566–592 nm where increase of $[Mg^{++}]$ produces a decrease of absorbance of the dye.

Under these conditions, the readout at the isobestic point (566 nm) was measured as indication of the changes in concentration of Eriochrome blue inside the axons. The differential readout at two closely adjacent wavelengths permits specific measurements of the absorption of the dye with no, or minimal interference by non-specific changes, such as swelling or changes in refractivity indexes. Furthermore, the simultaneous readout of three sets of wavelengths pairs, selected in close areas of spectrum where the same changes in Mg^{++} concentration produce differential absorbance changes in opposite direction, reveals occurrences of

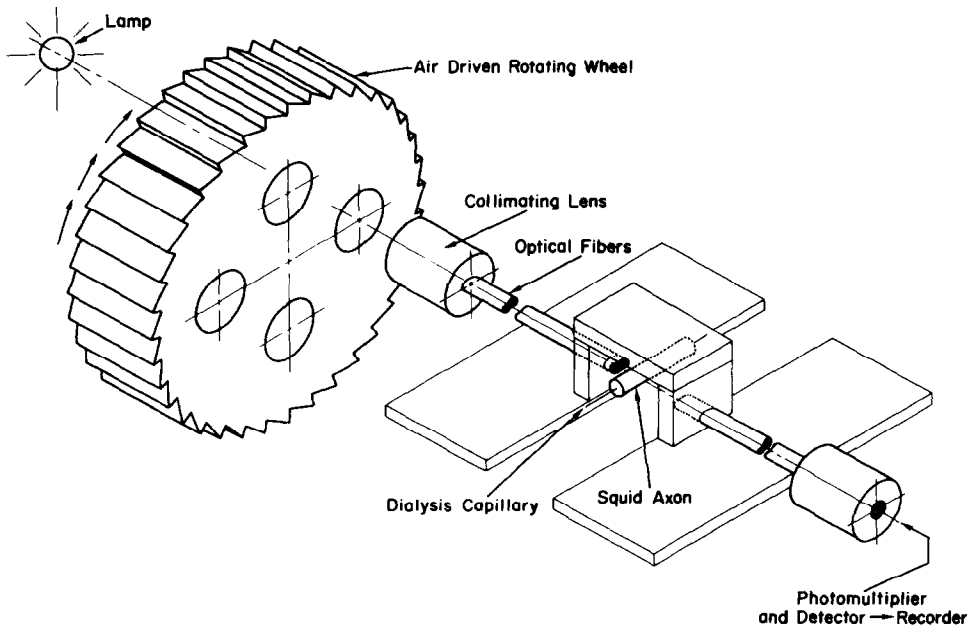

Fig.1. Schematic diagram (not to scale) of optical system. Light passing through the rotating wheel is collimated and then directed to the axon through flexible light pipes. Transmitted light passing through the axon is collected by similar fiber optics and directed to phototube. Area of beam is 0.4 × 4 mm and the distance between light pipes is about 2 mm. Mean path length through axoplasm averaged 400 microns.

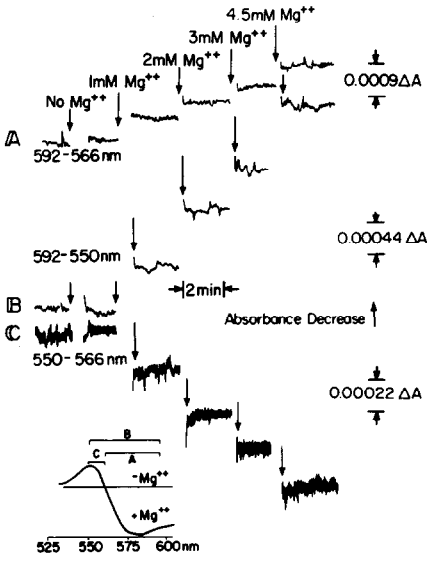

Fig.2. In vitro calibration curve showing absorbance change vs. magnesium concentration at three pairs of wavelengths selected to minimize calcium interference. Dye concentration 0.5 mM in dialysis solution, contained in glass tube with inside diameter 500 microns.

possible artifacts and make easier the interpretation of the data obtained.

The sensitivity of the system is illustrated by the calibration curve shown in fig.2. For this calibration, the axon was replaced by a glass capillary whose inside diameter was approximately equal to the diameter of the axon. The capillary was then flushed with solutions containing a constant concentration of dye but varying magnesium concentrations and the differential absorbance obtained of each concentration. The absorbance change when plotted against magnesium concentration gave a satisfactory linear relation.

The response to dialysis of the axon with various concentrations of magnesium is shown in fig.3. After microinjecting Eriochrome B into the axoplasm, and waiting for at least 15 to 20 minutes for diffusional equilibration, a base line signal was obtained as shown in the figure. At the vertical arrow, dialysis with medium containing 5 mM of magnesium was begun. The dialysis solution evidently contained a higher ionized magnesium concentration than did the axoplasm because all three differential absorbances deflected in the direction of greater magnesium binding to dye reflecting diffusion of magnesium from the capillary into the axoplasm. After several minutes of dialysis, the concentration was changed to medium containing 10 mM of magnesium and a further change in absorbance was found. The final procedure in the experiment was to replace the dialysis media with one containing no ionized magnesium. In this case all three differential absorbance changes reversed direction indicating that ionized magnesium was being washed out of the axoplasm by the dialysis.

Unfortunately it was not possible to continue dialysis until the ionized magnesium reached diffusional equilibration, because the dialysis capillary was not entirely impermeable to the dye. As indicated by curve D, in fig.3, which measured the absolute isobestic absorbance at 566 nm, there was a slow but continuous loss of dye from the axoplasm during dialysis which counted to about 3–4% during the course of the experiment.

The initial deflections were measured for 24 different axons, in which the initial magnesium concentration in the dialysate ranged from 0 to 10 mM. Eight representative experiments are collected in fig.4 which shows the initial absorbance change measured differentially at 592–565 nm. There was essentially no initial deflection when the ionized magnesium in the dialysate

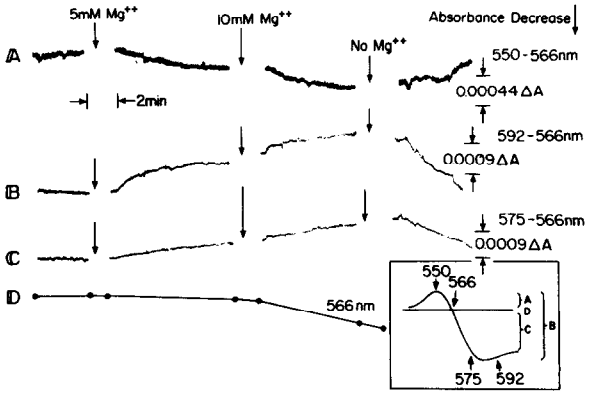

Fig.3. Absorbance change at three pairs of wavelengths, and at the isobestic point during dialysis of squid axon. Dialysis interrupted for about one minute at the times indicated by arrows to change the internal magnesium concentration. The first two solution replacements (5 and 10 mM) produced changes in absorbance reflecting increased free magnesium in the axoplasm. The third change (to 0 magnesium) reduced the free magnesium concentration in the axoplasm.

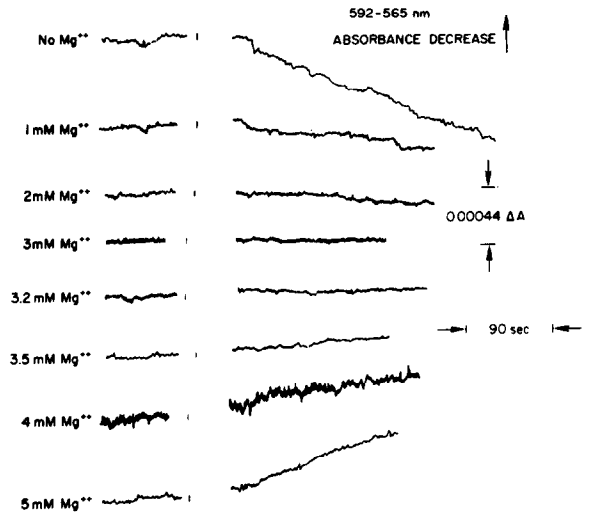

Fig.4. Collected difference spectra (592–565 nm) from 24 experiments similar to the one illustrated in fig.3 showing the direction of deflection during initial dialysis with solutions containing various concentration of magnesium. The initial concentration producing no deflection is between 3.0 and 3.5 mM.

ranged from 3 to 3.5 mM, whereas an initial deflection was obvious when the initial ionized magnesium was outside this range.

The results indicate that the level of ionized magnesium in the axoplasm of fresh squid axons is equivalent to that present in our dialysis solution containing between 3.0 and 3.5 mM of magnesium.

One reservation about the present method of estimating internal free magnesium must be mentioned. The null point method actually compares the magnesium activity in the fluid inside the dialysis capillary and a thin rim of axoplasm around the outside of the capillary. If the free magnesium concentration in this rim were different from that in the rest of the axoplasm, as might occur if there were sources or sinks for free magnesium in the axoplasm, then the mean concentration might be quite different from the value given by the null point method. There is no direct experimental evidence on distribution of magnesium within axoplasm, but a large gradient seems unlikely. The diffusion coefficient of magnesium is about 2×10^{-6} $cm^2 sec^{-1}$ [5] and if a local disturbance of the magnesium concentration were to occur, as for example during insertion of the capillary, the resulting gradient, distributed over distances comparable to the axon radius, would be dissipated with a time constant of 2–3 min.

The present results indicate that the ionized magnesium in squid axons is 3.0–3.5 mM. This result is in close agreement with indirect results [5] estimated on the basis of mobility and diffusion measurements that between one quarter and one half of the total axoplasm magnesium was ionized. It is somewhat lower than a recent estimate of 4 mM for the free magnesium determined by noting the effect of various concentrations of injected magnesium upon the sodium pump [6].

Acknowledgements

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