Magnesium Content and Net Fluxes in Squid Giant Axons

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ABSTRACT   The Mg content of axons freshly dissected from living specimens of the tropical squid *Doryteuthis plei* was determined by atomic absorption spectroscopy to be 4.2 ± 0.2 mmol/kg axoplasm. The axon's ability to maintain this physiological content of total intracellular Mg ($[Mg]_i$) was studied. Mg_i was shown to be a linear function of Mg_o when Mg_o of incubating fluid was varied between 0 and 250 mM. When $Mg_o = 15$ mM, Mg_i was found to be the same in incubated fibers as in fibers freshly dissected. Mg_i levels were unaffected by depolarization of the membrane by high K_o. Stimulation resulted in an extra influx of Mg of 0.05 pmol/(cm$^2 \cdot$impulse) when $Mg_o = 55$ mM. Mg_i was found to be a complicated function of the concentration of extracellular Na or Li (X_o), which was substituted for Tris. With 385 mM Li_o the Mg_i level was found to be 2.5-fold larger than the level observed with 385 mM Na_o after incubation for 3 h. The function relating Mg_o to X_o was qualitatively unaffected in axons poisoned with the mitochondrial uncoupler carbonyl cyanide, *p*-trifluoromethoxy-phenylhydraxone (FCCP) and the inhibitor of glycolysis, iodoacetic acid (IAA); the absolute levels of Mg_i, however, were some 30% higher in the poisoned axons at all $[X]_o$ explored. 2 h incubation of axons in a 333 mM Mg, 40 mM Li solution increased Mg_i 3.5-fold in control axons and 5-fold in poisoned axons. These Mg-loaded axons were able to recover physiological levels of Mg_i with a half-time of 3-5 h only if kept in a solution which contained Na (220 mM) regardless of whether the axons had been inhibited with FCCP + IAA. Therefore, it may be concluded that the physiological Mg_i concentration can be maintained by the Na electrochemical gradient, even when the axon is metabolically poisoned.

INTRODUCTION

The unidirectional fluxes of magnesium in both injected and dialyzed squid axons have been somewhat characterized as functions of the concentrations of external sodium $[Na]_o$, internal sodium $[Na]_i$, external magnesium $[Mg]_o$, internal magnesium $[Mg]_i$, and axoplasmic ATP (Baker and Crawford, 1972; DeWeer, 1976; Mullins et al., 1977; Mullins and Brinley, 1978). The number of studies, however, that attempt to describe the intracellular regulation of magnesium concentration has been limited because of the difficulty in obtaining and working with [28]Mg. Nevertheless, these studies have demonstrated

that the regulation of Mg is very similar to that of Ca (Baker, 1976). That is, the efflux of ^{28}Mg shows a dependence on external Na and is inhibited by internal Na whereas the influx of ^{28}Mg shows reverse behavior. With respect to ATP, both efflux and influx (Mullins and Brinley, 1978) are highly dependent on intracellular ATP concentration. This dependence of Mg efflux on ATP, however, can be eliminated either by increasing the Mg_i concentration or by decreasing the Na_i concentration (Mullins et al., 1977) indicating a catalytic role for ATP.

Although it is clear that there is a rather large inwardly directed electrochemical gradient for Mg (Baker and Crawford, 1972; DeWeer, 1976; Mullins et al., 1977), the magnitude of the chemical gradient based on free or ionized internal and external concentrations of magnesium ($[Mg^{++}]_i$ and $[Mg^{++}]_o$) has not been established. Various methods have indicated $[Mg^{++}]_i$ and $[Mg^{++}]_o$ to be about half that of the total content of Mg_i or Mg_o (Baker and Crawford, 1972; Brinley and Scarpa, 1975; DeWeer, 1976; Mullins et al., 1977). For the energy source that keeps $[Mg^{++}]_i$ lower than $[Mg^{++}]_o$ against an electrochemical gradient, three possibilities have been proposed; the inwardly directed Na electrochemical gradient, the hydrolysis of ATP, and a mechanism that combines both sources of energy.

In the experiments to be reported, spectrophotometric techniques were used to measure total Mg and Na content of axons, and hence net fluxes of Mg were studied as functions of the extracellular concentrations of Mg_o and various monovalent cations and the intracellular concentration of ATP. This method has been used in studying calcium regulation and the experiments reported in this paper are similar to those of Requena et al. (1979). In this manner the relative importance of the possible energy sources for the movement stated above was evaluated.

A preliminary report of some of these findings has been communicated to the Associación Venezolana para el Avance de la Ciencia (Caldwell-Violich and Requena, 1977) and to the Biophysical Society (Caldwell-Violich and Requena, 1979).

MATERIALS AND METHODS

Experimental Animals

Squid used in this work were live specimens of the tropical variety, *Doryteuthis plei*, obtained from the collecting station of the Instituto Venezolano de Investigaciones Cientificas (IVIC) at Mochima (Estado Sucre, Venezuela) and transported to the laboratory in Caracas for use.

Solutions and Inhibitors

The Compositions of the external solutions used in these experiments are listed in Table I. The osmolarity of the solutions used (and squid's hemolymph) was determined by comparison of the dew points of a standard NaCl solution and the unknown samples using a commercial psychrometer (Wescor, Inc., Logan, Utah); solutions were adjusted to $1,030 \pm 10$ mosmol/kg and always were made SO_4^--free to avoid complexing Mg. Solutions with intermediate concentrations of electrolytes were made by mixing the stock solutions in the adequate proportions.

Carbonyl cyanide, *p*-trifluoromethoxy-phenylhydrazone (FCCP) (Pierce Chemical Co., Rockford, Ill.), inasmuch as it is insoluble in aqueous solution, was added to the external solution from a stock solution (2 mg/ml) made in dimethyl sulfoxide. Iodoacetate (IAA) was added from a stock solution (100 mM) neutralized with Tris base to pH 7.1. The tetrodotoxin (TTX) (Sigma Chemical Co., St. Louis, Mo.) used in these experiments was a gift of Dr. C. Sevcik (IVIC). TTX was added to external solutions immediately before their use from a stock solution of 100 μM which was stored at 5°C. All inorganic chemicals used were ProAnalysis grade (Merck, Darmstadt, West Germany); biochemical reagents used were of the best grade available from Sigma Chemical Co.

Collection of Hemolymph and Axoplasm

Hemolymph was collected from live squid by cardiac puncture. Samples of hemolymph (1–2 cm^3) were first diluted 1:10 and then frozen until analysis.

TABLE I
COMPOSITION OF SOLUTIONS

Solutions*	Na	Choline	Li	Tris	K	Mg	Ca	Cl
				mM				
Artificial seawater (Na)	440	—	—	10	10	50	10	580
0 Mg (Na)	400	115	—	5	8	—	35	598
80 Mg (Na)	400	—	—	5	8	80	35	643
250 Mg (Na)	175	—	—	10	10	250	10	715
200 K (Na)	200	—	—	10	200	100	10	630
50 Mg (Tris)	—	—	—	450	10	50	10	580
100 Mg (Tris)	—	—	—	410	10	100	10	640
250 Mg (200 Na)	200	—	—	10	—	250	3	716
100 Mg (Na)	385	—	—	10	10	100	10	625
100 Mg (Li)	—	—	385	10	10	100	10	625
100 Mg (Choline)	—	385	—	10	10	100	10	625
333 Mg, 40 Li	—	—	40	20	10	333	0.1	736
25 Mg (220 Na)	220	—	—	300	10	25	10	600
25 Mg (Tris)	—	—	—	525	10	25	10	605

* All solutions contained 0.1 mM (Na) EDTA.

The hindmost giant axon from the stellar ganglion isolated from freshly killed specimens was used. Axoplasm samples were obtained by extrusion from these axons. The axons were briefly rinsed (2–5 min) in isoosmolar sucrose solution, blotted on dry filter paper and cut at one end. The cut end was then laid on parafilm (American Can Company, Greenwich, Conn.) which had previously been cleaned with 50 mM EDTA; the rest of the axon rested on filter paper in order to absorb any extracellular fluid which might emerge during the extrusion process. The axoplasm was extruded from the cut end by gently pressing on the fiber with a finger wrapped in parafilm. Immediately after extrusion, the samples of axoplasm were aspirated into polyethylene tubes (PE 90) (Clay-Adams, Div. of Becton, Dickinson & Co., Parsippany, N.J.). The mass of the axoplasm was obtained by reweighing the tube after removal of the axoplasm sample. The samples were digested and sonicated for 4 h in 300 μl of 50% HNO$_3$ 1 N/ 50% HClO$_4$ 1 N. After diluting the samples for chemical analysis they were sonicated another hour. Samples analyzed for chloride were digested in 300 μl of 1 N HNO$_3$, but otherwise the digestion routine was identical for all samples.

Analytical Methods

Cl⁻ concentrations were determined by two methods, one titrimetric, the other conductrimetric (Zanders, 1975); sulfate concentrations were measured simultaneously in the second technique. PO_4^- concentrations were assayed using the molybdenum blue colorimetric test with the King modification (Fiske and Subbarow, 1925; King, 1932).

Na and K concentrations were measured using a flame photometer (Zeiss PMQ II, Oberkochen, West Germany), and the following wavelengths and slits: for Na, 598 nm, 0.02 mm; and for K, 769 nm and 0.65 mm. Due to the high sensitivity of the apparatus, it was found unnecessary to add K to the Na standards to eliminate its interference in the Na measurement.

Mg, Ca, and Li concentrations were determined using a model 303 Perkin-Elmer atomic absorption spectrophotometer (Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.). Wavelengths of 286, 212, and 335 nm and slits of 3, 1, and 1 mm were used for Mg, Ca, and Li determinations, respectively. Acid-prepared La_2O_3 (Koch Light, Colnbrook, Buckinghamshire, England) was added to all samples and standards in order to avoid interference by inorganic phosphorus.

TABLE II

COMPOSITION OF THE HEMOLYMPH AND AXOPLASM OF *D. plei* AND THAT
OF THE ARTIFICIAL SEAWATER IN WHICH THEY LIVE

	Cl	SO₄	PO₄	K	Mg	Na	Ca	Osmolarity mosM/kg	pH
Hemolymph, mM	566±12 (13)	10±1 (10)	0.0048±0.0006 (3)	15.5±0.4 (9)	57±1.0 (7)	461±28 (9)	10.3±0.6 (5)	1,041±6 (9)	7.2
Axoplasm, mmol/kg	110±13 (12)	—	—	411±19 (12)	4.2±0.2 (18)	27±2 (31)	0.07±0.01* (8)	—	—
Artificial seawater, mM	542	32	—	11	48	414	7.5	1,015	7.9

Values in parentheses are numbers of experiments.
* Requena et al., 1977.

The concentrations determined for the electrolytes in hemolymph and in axoplasm samples from the squid *D. plei* are listed in Table II. Throughout the text values for concentration (mmol/kg of axoplasm) of a given ion will be presented as mean ±SEM of the number (n) of axons tested. The figure presented for [Ca] of the axoplasm was obtained by Requena et al. (1977) for the same species. Axoplasmic chloride, potassium, and sodium were measured in the same samples. The value reported here for chloride is similar to those that have been reported in the literature for *Loligo* (108 mmol/kg, Keynes, 1963; 119 mmol/kg, Russell, 1976). For potassium, the concentration found for *D. plei* was higher than usually observed although comparable values have been reported (Deffner, 1961; Hinke, 1961). For sodium the value reported is one-half to one-third of that usually reported; for Ca_i it is one-eighth. As suggested by Requena et al. (1977) these discrepancies most probably are due to the unusually small delay between decapitation and extrusion of the axoplasm (≈15 min) in the experiments with *D. plei*. During this brief interval there is not sufficient time for the electrochemical gradients for Na and Ca to change significantly. In reference to the hemolymph concentrations of the electrolytes, these are similar to values that have been reported for *Loligo*.

Table II also lists the electrolyte composition of the artificial seawater (ASW, Instant Ocean, Aquarium Systems, Eastlake, Ohio) in which the squid lived at the Centro de Biofísica y Bioquímica for some 12–36 h prior to use.

ATP Levels in the Axoplasm

The inhibitor combination 10 μM FCCP + 1 mM IAA was used in this work when it was necessary to reduce intracellular ATP levels. The efficiency of this method was investigated by comparing the effect on axoplasmic Na concentrations of incubation of axons with FCCP + IAA with the effect of incubation for a similar time period with 1 mM ouabain, which is known to completely stop the sodium pump within minutes of application (Caldwell and Keynes, 1959). In Table III are listed $[Na]_i$ after inhibition with FCCP + IAA, with these two compounds individually, or with ouabain. From the increments in Na_i one can calculate net sodium fluxes, assuming that the increases occur linearly with time. It was found that inhibition of the Na pump with ouabain or with the combination of poisons of metabolims resulted in similar Na_i gains equivalent to net influxes of this cation of 74 pmol/cm^2·s in the case of FCCP + IAA inhibition, and 62 pmol/cm^2·s in that of ouabain. Table III also shows that, although FCCP by itself may induce a much larger gain in Na_i than IAA could, for practical purposes, the combination of both poisons is more effective. The

TABLE III

EFFECT OF INHIBITORS ON $[Mg]_i$ AND $[Na]_i$ IN AXONS INCUBATED FOR 3 h

	Condition				
	Control	FCCP, 2 μg/ml; IAA, 1 mM	FCCP, 2 μg/ml	IAA, 1mM	Ouabain, 1 mM
Intracellular magnesium, *mmol/kg*	5.4±0.2 (6)	7.6±0.3 (6)	7.0±0.4 (5)	6.9±0.2 (5)	6.9±0.4 (4)
Intracellular sodium, *mmol/kg*	46±2 (6)	126±6 (6)	116±10 (5)	62±3 (5)	113±6 (4)

Values in parentheses are number of experiments.

K_m of the sodium pump for ATP in *Loligo* axons is of the order of 35 μM (Brinley and Mullins, 1968). If the K_m for ATP of the Na pump is similar for the species *D. plei*, the fact that the Na_i increment produced by FCCP + IAA is analogous to that observed with ouabain suggests that the inhibitor combination reduces $[ATP]_i$ to concentrations much less than 35 μM ATP and with a time scale of inhibition similar to that of ouabain. This conclusion has been confirmed by Requena et al. (1979), who measured ATP_i in *D. plei* axons poisoned with the inhibitor combination FCCP + IAA or with each poison individually using the firefly flash method. It was found that, after 1 h of incubation with the two inhibitors, $[ATP]_i$ had decreased from a control concentration of 1.5 ± 0.1 mM to a concentration of 30 μM; IAA alone reduced ATP to 0.9 mM in a similar period.

RESULTS

External Magnesium Concentration

A large part of the total Mg present in the hemolymph is bound to sulfate and protein (Mullins et al., 1977). An approximation of the concentration of free or ionized Mg in the extracellular fluid was obtained in this work by determining the level of extracellular Mg that did not result in a change in

Mg_i in axons incubated 3 h in solutions containing this concentration of Mg but not containing compounds that would complex the cation. This technique has been successfully used with Ca (Requena et al., 1979). As shown in Fig. 1, a straight line can be used to describe the relationship between $[Mg]_i$ and $[Mg]_o$ up to a concentration of 250 mM Mg_o. This relationship held even though the concentration of Ca in the incubation solutions varied between 3 and 35 mM and that of Na between 175 and 400 mM.[1] However, in the range of 0–80 mM Mg_o, Ca_o and Na_o were kept constant. From this relationship, $[Mg]_i$ vs. $[Mg]_o$, it was found that only when $[Mg]_o$ was ~15, or in any case below 25 mM, the concentration of intracellular magnesium was found to be the same in incubated fibers as in freshly dissected.
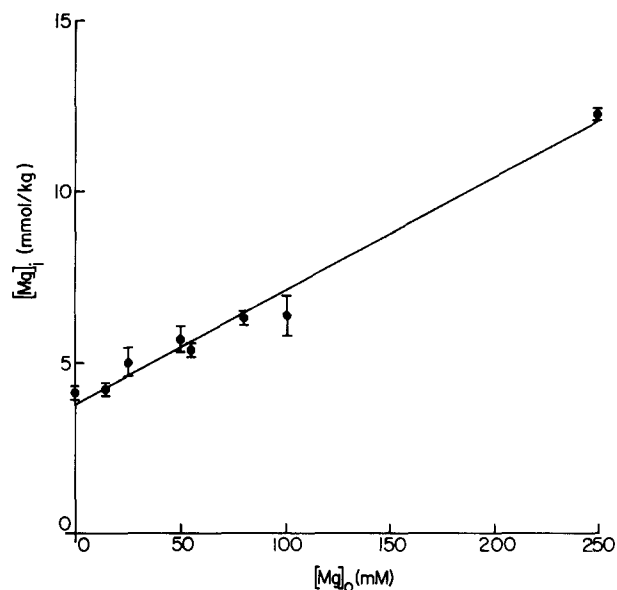

FIGURE 1.   The effect on the intracellular concentration of Mg (mmol/kg) of the extracellular concentration of Mg (mM) in control axons incubated for 3 h. Mg_o was replaced isosmotically with choline. The relation represents the entire range of $[Mg]_o$ studied, 0-250 mM. In the range of $[Mg]_o$, 0-80 mM, $[Ca]_o$ and $[Na]_o$ were kept constant at Ca_o 35 mM and 400 mM while at 100 mM Mg_o; $Ca_o = 10$ mM; $Na_o = 385$ mM and at 250 mM Mg_o; $Ca_o = 3$ mM; $Na_o = 175$ mM. Points represent mean ± SEM, each point corresponds to about six to eight axons tested. The straight line was obtained by the least squares method.

Membrane Depolarization and Electrical Stimulation

Membrane depolarization, produced by incubation of axons in seawater containing 150 mM K, does not have an appreciable effect on ^{28}Mg efflux (DeWeer, 1976). Experiments were done to determine the effect on total intracellular magnesium levels of exposure of axons to high K seawater. Axons

[1] 35 mM Ca was required to avoid spontaneous firing of the axons when the Mg concentration in the ASW was low; it was necessary to reduce $[Na]_o$ in order to maintain osmolarity when $[Mg]_o$ was high (see legend to Fig. 1).

incubated for 3 h in seawater containing 100 mM Mg_o, 200 mM Na_o, and 10, 100, or 200 mM K_o showed a magnesium content of 6.1 ± 0.4 ($n = 9$), 6.5 ± 1.2 ($n = 5$), and 6.8 ± 0.6 ($n = 10$) mmol/kg respectively, indicating that there does not seem to be a significant effect of K-induced membrane depolarization on $[Mg]_i$.

As in the case of other ions whose electrochemical gradients are inwardly directed, an extra influx of Mg has been observed with electrical stimulation. The value reported for this extra influx varies between 0.007 pmol/cm² per impulse (Baker and Crawford, 1972) and 0.07 pmol/cm² per impulse (Rojas and Taylor, 1975) when $[Mg]_o$ = 55 mM. In the present work axons were stimulated at a rate of 100 impulses/s for varying times in a solution containing 200 mM Na and 250 mM Mg. The Na concentration in the bathing media was kept low in order to minimize loading of this ion by the axons, while the Mg concentrations was made abnormally high so that a larger increase in Mg_i would occur with each impulse.

Because of the high concentration of extracellular Mg used in the experiments with stimulated axons, a correction must be made for the amount of Mg that would have leaked in even without stimulation. For this purpose, analysis of intracellular Mg and Na content were carried out in two sets of seven axons each: one set at zero time and the other set after 25 min of incubation in the stimulation solution, a period equivalent to 150,000 impulses at 100 impulses/s. The values obtained were 5.0 ± 2 mmol/kg for Mg and 24 ± 2 mmol/kg for Na at the onset of the period and 7.1 ± 5 mmol/kg and 28 ± 5 mmol/kg for Mg and Na, respectively, at the end of the period. It appears therefore, that for the case of Mg a correction equivalent to 1.4 μM/kg must be made for each second of stimulation to account for the entry of Mg not associated with the conduction of the nervous impulse. For the case of Na no such correction appears necessary.

In Fig. 2, the increases of cellular Mg and Na which are only due to the passage of nervous impulses are presented as a function of number of impulses conducted. It can be seen that the stimulation related influx of Mg seems to saturate at high numbers of impulses while for sodium the stimulation-related influx is linear throughout with respect to the number of impulses and has a value of 31 fmol/(cm²·impulse·mM Na_o) which is somewhat larger than that found by Villegas et al. (1966) for the same species. The additional influx of Mg caused by stimulation was calculated to vary between 1.20 and 0.67 fmol/(cm²·impulse·mM Mg_o) or ~0.05 fmol/(cm²·impulse) when $[Mg_o$ = 55 mM.

External Monovalent Cation and ATP_i Levels

The effect on $[Mg]_i$ of substituting Tris, Tris-Li for external Na was investigated. Fig. 3 A and B gives the axoplasmic Mg contents of axons that had been incubated 3 h in 100 mM Mg-ASW with varying concentrations of Na or Li substituted by Tris. Fig. 3 A represents results with control axons and Fig. 3 B axons inhibited with FCCP + IAA. Clearly the relationship between $[Mg]_i$ and $[X]_o$ (Na or Li) is the same in the two cases; however, total internal magnesium is 30–40% higher in poisoned axons. The curves relating $[Mg]_i$ and $[X]_o$ are biphasic. In control axons, in the range of 0–200 mM X_o,

[Mg]$_o$ varies from 7.1 ± 0.5 mmol/kg ($n = 7$) to a peak value of 9.4 ± 1.0 ($n = 8$) mmol/kg Mg$_i$ at 40 mM X$_o$, falling to ~6.6 ± 0.4 ($n = 6$) mol/kg Mg$_i$ at 200 mM X$_o$. In the range of 200-385 mM X$_o$, however, the relation is dependent on whether the principal extracellular cation is Na or Li. With the former, [Mg]$_i$ no longer varies with respect to [Na]$_o$, remaining constant at ~6.2 mmol/kg Mg$_i$ up to 385 mM Na$_o$. When the principal external cation is Li, the concentration of intracellular Mg increases continuously after 200 mM reaching a concentration of 14.5 mmol/kg at 385 mM Li$_o$.
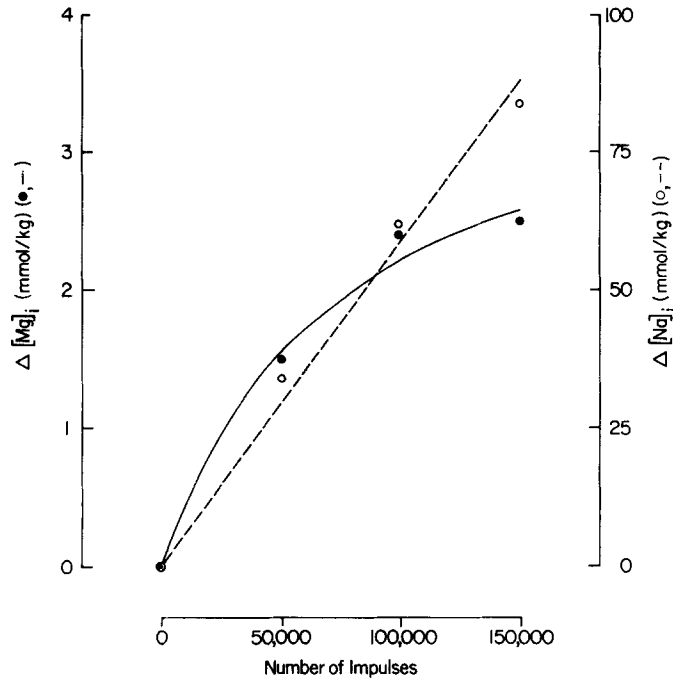

FIGURE 2. The effect of the number of impulses conducted on Mg and Na gains (mmol/kg) in control axons. The axons were stimulated at a rate of 100 impulses/s in 250 mM Mg (200 mM Na) ASW. The increases in [Mg]$_i$ and [Na]$_i$ have been corrected for passive gains of the cations and represent concentration increases as a result of stimulation only. (●—●) Mg; (O---O) Na. The curves were obtained by the least squares method.

In Fig. 3 C the intracellular concentration of the main extracellular cation [X]$_i$ as a function of the concentration of the same cation in the extracellular fluid is shown for control axons; in Fig. 3 D [Na]$_i$ as a function of [Na]$_o$ (poisoned axons only) is presented. It is apparent that although the axon is capable of maintaining a low [Na]$_i$ (≃25 mmol/kg) under control conditions, this ability is lost when axons are incubated in solutions containing FCCP + IAA, whereupon [Na]$_i$ increases linearly with rising [Na]$_o$. In the case of Li, control axons appear to be incapable of extruding this cation against an electrochemical gradient as [Li]$_i$ also increases linearly with [Li]$_o$.

The above series of experiments was also performed, for control axons only,

in the presence of 1 mM La_o^{3+} (LaCl$_3$), an inhibitor of Mg efflux (DeWeer, 1976). The results of these experiments are summarized in Table IV where it can be observed that the presence of La^{+++} in the incubating media rendered the intracellular concentration of Mg virtually independent of the concentra-
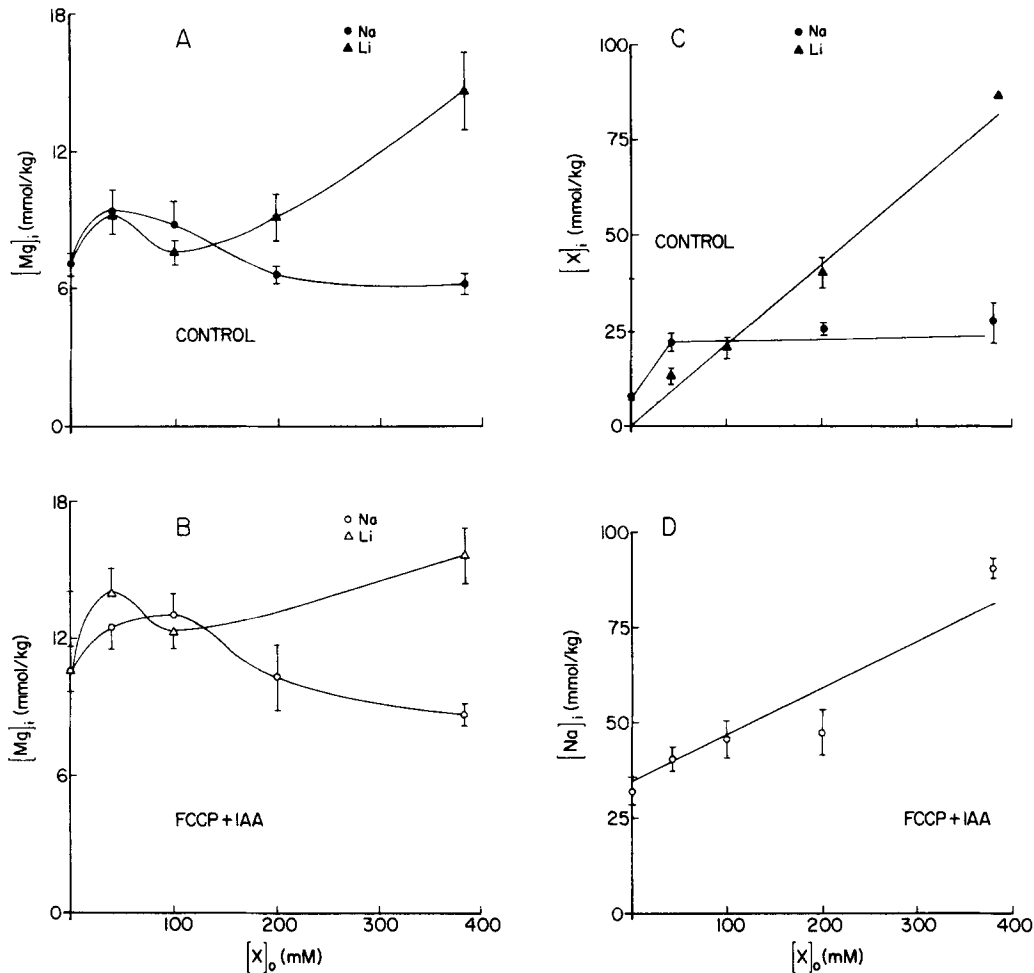

FIGURE 3. The effect on the intracellular Mg, Na, or Li concentration (mmol/kg) of the concentration (mM) of the main extracellular monovalent cation (X_o) in control and metabolically poisoned axons incubated for 3 h. [Mg]$_i$ is plotted as a function of [X]$_o$ (X = Na or Li) in control axons (A) and in axons inhibited with FCCP + IAA (B). [X]$_i$ is plotted as a function of [X]$_o$ in control axons (C). [Na]$_i$ is plotted as a function of [Na]$_o$ in poisoned axons (D). Filled symbols represent control axons and open symbols inhibited (FCCP + IAA) axons. The circles refer to X = Na and the triangles to X = Li. Na and Li were replaced isosmotically by Tris. Points represent mean ± SEM. In (C) for the dependence of [Li]$_i$ on [Li]$_o$, a straight line was obtained by the least squares method. For Na, the points were joined by a curve drawn by eye. In (D) the line connecting the points obtained from inhibited axons was obtained by the least squares methods. Each experimental point represents six to eight axons tested.

tion or nature of the principal extracellular monovalent cation. Substitution of choline for Na_o resulted in an axoplasmic Mg content of 9.1 ± 0.4 mmol/kg ($n = 8$) in the presence of 1 mM La_o^{+++}, whereas in control axons, in the absence of La^{+++}, similar treatment resulted in Mg_i of 8.4 ± 0.8 mmol/kg axoplasm and Na_i of 12 ± 1 mmol/kg.

Magnesium Extrusion by Loaded Axons

It was necessary to increase the levels of intracellular Mg in a loading solution so that a net efflux of Mg could be reliably measured while investigating the energy source for Mg pumping in a recovery solution. Axons were incubated in a loading solution containing a very high Mg concentration (333 mM), a low concentration of Li (40 mM), and no Na. After 2 h incubation, Mg_i was

TABLE IV

$[Mg]_i$ AND $[Na]_i$ IN AXONS AFTER 3-h INCUBATION IN SOLUTION CONTAINING 1 mM La^{+++}, 100 mM Mg, AND VARYING CONCENTRATION OF NA_o OR Li_o (X_o) SUBSTITUTED FOR TRIS

		$[X]_o$, mM				
	X_o	0	40	100	200	385
Intracellular magne-	Na	9.0±0.9	10.7±0.8	8.8±0.2	9.3±0.4	9.2±0.5
sium, mmol/kg		(8)	(6)	(8)	(5)	(8)
	Li	9.0±0.9	11.3±0.6	9.2±0.3	9.8±0.5	9.3±0.4
		(8)	(6)	(8)	(6)	(6)
Intracellular sodium,	Na	18±2	20±2	21±2	28±1	46±2
mmol/kg		(8)	(6)	(7)	(5)	(8)
	Li	18±2	18±1	17±2	15±2	18±2
		(8)	(5)	(8)	(4)	(6)

Values in parentheses are number of experiments.

15.2 ± 0.9 mmol/kg ($n = 11$), an increase of 11 mmol/kg of Mg over the control value of 4.2 mM in control axons. In axons that were inhibited with FCCP + IAA during the 2nd h of incubation, Mg_i was 20.1 ± 1.1 mmol/kg ($n = 6$); this represents a gain of 15.8 mmol/kg Mg.

Axons were allowed to recover under the following experimental conditions: in the presence and absence of Na in the incubation media and with and without the inhibitors FCCP + IAA. Recovery solutions contained 25 mM Mg_o, a concentration chosen so that axons would be extruding Mg against a chemical gradient and yet Mg_i^+ would be close to its physiological value. A reduced Na concentration (220 mM) was used in the recovery solution containing Na, in order to minimize Na_i increases during the recovery period in those axons inhibited with FCCP + IAA. This precaution was taken so that Na_i would be similar in both control and poisoned axons throughout the experiment. This Na_o concentration, however, is sufficient to ensure that axons do not gain Mg_i in time (see Fig. 3 A). Loading and recovery solutions also contained TTX^2 and Tris as Na substitute.

[2] Preliminary experiments in which axons were loaded in high-Mg seawater and allowed to unload for 2 h in 50 mM Mg ASW resulted in very large gains in Na_i, the equivalent of an

Fig. 4 summarizes the result obtained. It can be seen that only those axons allowed to recover in 220 mM Na are capable of extruding the Mg load, and this capability is unaffected by the presence of FCCP + IAA in the incubation medium. However, those axons allowed to recover in 0 Na not only fail to extrude Mg but continue to gain Mg_i during the recovery period.
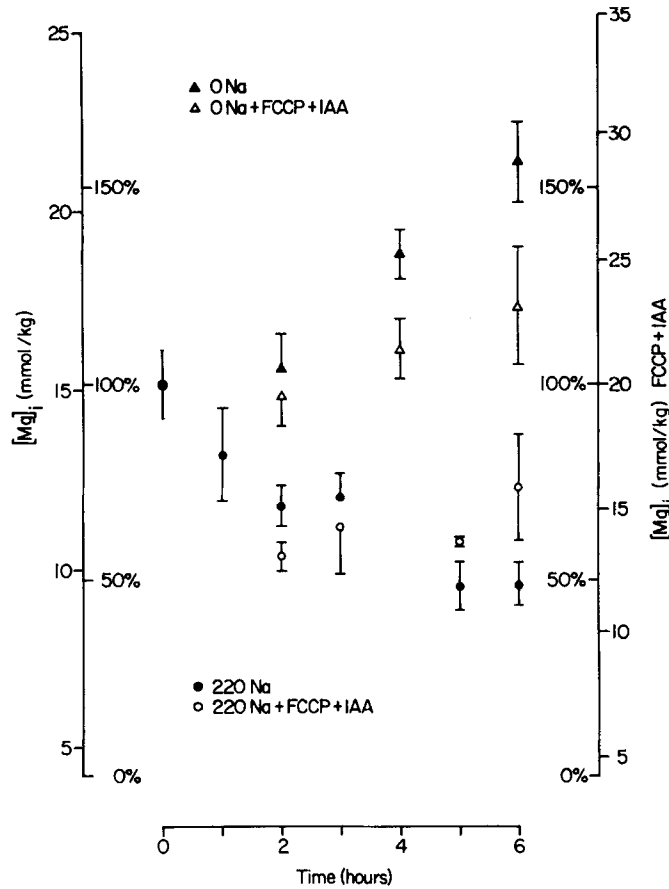

FIGURE 4. Time-course for loss of a given load of intracellular Mg under different experimental conditions. $[Mg]_i$ is plotted as a function of time for axons, control or metabolically poisoned, whose $[Mg]_i$ had been increased by incubation in a 333 mM Mg, 40 mM Li solution and allowed to recover in the absence and presence of 220 mM Na. 100% of load refers to $[Mg]_i$ at 0 time; 15.2 mmol/kg in control axons and 20.0 mmol/kg in inhibited axons. 0% of load refers to $[Mg]_i$ in axons freshly dissected, 4.2 mmol/kg. Filled symbols and the left axis refer to control axons while open symbols and the right axis refer to inhibited axons. Circles represent axons recovered in 25 mM Mg, 220 mM Na (Tris) ASW; triangles represent axons incubated in 25 mM Mg, 0 Na (Tris) ASW. Points represent mean ± SEM of about six to eight axons tested.

influx of 150 pmol $Na/cm^2 \cdot s$. This influx could be blocked by TTX in the incubation fluids (100 nM in the loading solution and 300 nM in the recovery solutions).

Control axons recovering in 220 mM Na-ASW lose half the Mg load applied in 5 h. This extrusion rate is equivalent to an average net magnesium efflux of 3.2 pmol/cm^2·s. Poisoned axons seem to lose Mg with a faster extrusion rate. This could be due to a decreased electrochemical gradient for Mg against which poisoned axons must extrude as in the case of these axons: (a) [Mg]$_i$ at the beginning of the recovery period is 20.0 mmol/kg as compared to 15.2 mmol/kg Mg$_i$ in control axons ([Mg]$_o$ = 25 mM); and (b) the ratio [Mg]$_o$/[Mg]$_i$ is larger in poisoned axons due to the lack of ATP and ADP to which a substantial portion of intracellular Mg is normally bound.

DISCUSSION

The present work clearly shows that a net efflux of Mg from axons is possible only in the presence of extracellular Na. Reducing internal ATP levels has no effect on this process since axons incubated with the metabolic inhibitors FCCP + IAA and allowed to recover in 220 mM Na artificial seawater maintain their ability to extrude Mg against an electrochemical gradient. This provides strong evidence in favor of a Na:Mg-coupling process that provides the energy for [Mg] regulation. It is important also that the dependence of the levels of intracellular Mg on the concentration of the main extracellular cation do not differ but quantitatively in FCCP + IAA-poisoned axons in which ATP levels have been presumably decreased to ~35 μM, a value <10% of the reported K_m of ^{28}Mg efflux for ATP (Mullins et al., 1977). This fact suggests that low ATP level do not have a preferential effect on the energy-requiring process, Mg efflux, but rather a nonspecific effect on the rate of the transport-mediated movements. Thus, the role of ATP in Mg regulation seems to be a catalytic one.

In contrast to Ca efflux which has been shown to be voltage dependent (Mullins and Brinley, 1975), experiments carried out by DeWeer (1976) with ^{28}Mg efflux and the present experiments with total axoplasmic Mg content clearly indicate that there is no effect of membrane depolarization on Mg fluxes, since treatment of axons with high-K seawaters did not produce an increase in intracellular concentration of Mg. This implies that Mg movements, both influx and efflux, are an electroneutral exchange. For Mg efflux, therefore, a 2:1 Na:Mg-coupling ratio is indicated. Such stoichiometry, for a Na concentration ratio across the membrane of ~10, would give a value for Mg$_o$/Mg$_i$ of 100. The system appears therefore capable of operating under conditions far from equilibrium.

The curves obtained plotting Mg$_i$ against the extracellular concentration of Na or Li deserve some comment. First, it is apparent that in control axons an extracellular concentration of Na >200 mM is capable of maintaining Mg influx and efflux in balance. For Li it is also evident that this concentration range, above 200 mM Li$_o$, is not adequate. It appears, therefore, that in this respect there is a substantial difference between Na$_o$ and Li$_o$, Na$_o$ being more effective in maintaining Mg fluxes in balance. The increase in Mg influx observed in 385 mM Li seawater seems to be an enhancement of the influx mediated by the transport mechanism responsible for the regulation of intracellular Mg and not a nonspecific increment in Mg flux due to the absence of

Na from the extracellular space. This conclusion is supported by the observation that externally applied La^{3+} abolishes the biphasic dependency of Mg_i on the concentration of the main extracellular cation and that substitution of Na_o by Tris or choline (385 mM) do not produce such a dramatic increase in Mg_i. On the contrary, these cations are "as effective as" Na_o in maintaining a low Mg_i. The apparent contradiction in the equal ability of 385 mM of Tris, choline, or Na_o to maintain a similar Mg_i concentration on the one hand, and the strict requirement for Na_o to produce a net extrusion of Mg by the transport mechanism on the other hand, suggest that in the presence of 385 mM Tris or choline, Mg influx has to be negliable and comparable to the greatly reduced 28 Mg efflux observed under those conditions by DeWeer (1976) in *Loligo* axons.

The results presented indicate, therefore, that intact squid axons require for the maintenance of normal levels of intracellular Mg, or recovery after a Mg load, the presence of at least 220 mM Na in the extracellular fluid. Since in metabolically poisoned axons, in which ATP is virtually absent, the effect outlined above is also observed, one concludes that the extrusion of Mg against its electrochemical gradient is supported by the free energy stored in the Na electrochemical gradient.

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