Simultaneous bidirectional magnesium ion flux measurements in single barnacle muscle cells by mass spectrometry

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ABSTRACT Stable isotopes of Mg were used to measure bidirectional magnesium ion fluxes in single barnacle giant muscle fibers immersed in Ca- and Na-free, isosmotic media. Measurements were made using a mass spectrometric technique, thermal ionization mass spectrometry (TIMS), in conjunction with atomic absorption spectroscopy. Kinetic relations based on a first-order model were developed that permit the determination of unidirectional rate coefficients for Mg influx, k_i, and efflux, k_e, in the same experiment from knowledge of initial conditions and the initial and final ratios of $^{26}Mg/^{24}Mg$ and $^{25}Mg/^{24}Mg$ in ambient solutions (i.e., by isotope dilution). Such determinations were made for three values of the external Mg ion concentration: 5, 25, and 60 mM. At the concentration $[Mg^{+2}]_o$ = 5 mM, k_i and k_e were about equal at a value of 0.01 min^{-1}. At the higher values of $[Mg^{+2}]_o$, the values of k_e increased along a curve suggesting saturation, whereas the values of k_i remained essentially constant. As could be expected on the basis of a constant k_i, the initial influx rate varied in direct linear proportion to $[Mg^{+2}]_o$, and was 11.8 pmol/cm^2s when $[Mg^{+2}]_o$ was 5 mM. However, the initial efflux rate appeared to increase nonlinearly with $[Mg^{+2}]_o$, varying from 13.4 pmol/cm^2s ($[Mg^{+2}]_o$ = 5 mM) to ~80 pmol/cm^2s ($[Mg^{+2}]_o$ = 60 mM). The results are consistent with a model that assumes Mg influx to be mainly an electrodiffusive inward leak with P_{Mg} = 0.07 cm/s and Mg efflux to be almost entirely by active transport processes. Where comparisons can be made, the rate coefficients determined from stable isotope measurements agree with those previously obtained using radioactive Mg. The rate coefficients can be used to correctly predict time-dependent changes in total fiber Mg content. The results support the conclusion that nonradioactive tracers can be used to measure ion fluxes and ion flux ratios in excitable cells; it is expected that this method will greatly assist in the study of Mg regulation in general.

INTRODUCTION

Regulation of Mg ions is important because Mg ions play a significant role in the regulation of some key physiological and biochemical processes in cells (Wong et al., 1985; Horie and Irisawa, 1987; Squire and Petersen, 1987; and Aikawa, 1981). Recently, recognition of the importance of Mg has been augmented by the discovery that Mg may play an important role in gating processes (Matsuda et al., 1987; Vandenberg, 1987*a* and *b*) and in the regulation of chloride fluxes and internal pH in barnacle muscle fibers (Russell and Brodwick, 1988). Unfortunately, this recognition has not been accompanied by increased performance of new research on Mg fluxes, which are of obvious importance to the control of Mg levels in cells. Much of the reason for this deficiency stems from difficulties in obtaining the only useful radioactive isotope of Mg, the relatively short-lived isotope ^{28}Mg available irregularly from only one supplier in this country. For this study, we circumvented these difficulties by using stable isotopes of Mg as tracers, applying them to the study of both efflux and influx (i.e., bidirectional fluxes) in the giant muscle fibers of the acorn barnacle. We chose these muscle fibers because of the ease of obtaining them and because some previous data on Mg fluxes and levels in these cells already exist, allowing for a better evaluation of the success of our approach.

The free or ionized Mg^{+2} concentration in muscle fibers of the barnacle has been measured directly and shown to be in the range 5–6 mM (Brinley et al., 1977); the total intracellular Mg level in blot-dried fibers has been measured to be in the range 9.6–12.1 mM (Page et al., 1971; Ashley and Ellory, 1972; Brinley et al., 1977). The Mg buffering ratio (i.e., $[Mg^{+2}]_{free}/[Mg^{+2}]_{total}$) is thus ~0.5. As the normal extracellular value of $[Mg^{+2}]$ is at least four times greater than that of intracellular $[Mg^{+2}]$ in barnacle muscle cells, the available data would indicate that Mg ions must be transported out of the cell by one or more active processes. There is some evidence that a Na-dependent Mg extrusion similar to Na/Ca

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exchange is present in barnacle muscle fibers (Vogel and Brinley, 1973; Chandler et al., 1986; Gonzalez-Serratos et al., 1988). In squid giant axons, Mg efflux is inhibited by metabolic poisoning with CN or DNP, by withdrawal of external Na, or by decreasing the internal level of ATP (Baker and Crawford, 1972; DeWeer, 1976; Mullins et al., 1977). The fact that declining levels of ATP in the fibers lead to reduced values of Mg efflux suggests the operation of an active mechanism for Mg ion extrusion. Whether or not a Mg pump is present is not settled by these observations, as the internal ATP level would be expected to regulate the rate of operation of a Na/Mg counter-transport process in much the same way as in the case of Na/Ca counter-transport (Baker and McNaughton, 1976; Blaustein, 1977). Potassium also appears to be involved in the regulation of Mg fluxes, perhaps through a counter-ion exchange process (Montes et al., 1988).

More work is required to determine the energy sources for Mg extrusion in excitable cells, and undoubtedly the improved ability to study the kinetics of Mg fluxes will be invaluable in properly characterizing the mechanisms—including a probably energy-dependent Mg transport system—responsible for the regulation of Mg levels in cells. Furthermore, the study of counter-fluxes involving Mg is scant, notwithstanding the interesting observations reported recently regarding the aforementioned role of Na and K in Mg transport in barnacle muscle fibers. Thus, it would be reasonable to expect that the rapidity of developments in this field could be significantly limited by problems concerning the availability of radioactive Mg.

The purpose of this paper is to report on the successful application of thermal ionization mass spectrometry (TIMS) to the measurement of transmembrane Mg fluxes in barnacle muscle fibers; this paper makes available for the first time Mg influx rate constants and influx rates for isolated single muscle fibers. The use of stable isotope tracers permits determination of bidirectional fluxes for more than one ion species from samples taken from the same preparation, if so desired; this advantage is particularly attractive in the study of possible counter-ion fluxes. The application of stable isotopes to the measurement of Mg fluxes in isolated barnacle muscle fibers requires a mathematical model that lends itself to the calculation of kinetic constants from shifts in the ratios of stable Mg isotopes measured in the cells or their external medium. Stable isotopes have already been used successfully in other studies, for example, of Ca metabolism, including absorption, in humans (Yergey et al., 1980 and 1987), but such studies, including those also based on isotope dilution and TIMS, were not intended to address the determination of kinetic constants related to either transmembrane efflux or influx, nor were such studies performed on isolated cells. Existing models for radioactive tracer kinetics are not directly applicable to stable isotopic ratio kinetics, which necessitates the present mathematical treatment (see Appendix).

MATERIALS AND METHODS

Experimental set-up

For exchange of magnesium between the fibers and the ambient medium, isolated fibers were mounted vertically, two to a chamber, in specially designed lucite chambers containing ^{26}Mg-enriched medium in a well; a small stirring bar at the bottom of each well served to keep the medium homogeneous during the exchange process. The fibers were mounted, one apiece to each of two Teflon slides inserted in slots at the top of each chamber, by inserting the cut end of each fiber into a small notch cut into the edge of each slide, so that the fibers could be aligned along a common diameter of the well by moving the slides in and out of their respective slots and making them meet at the center of each chamber; the cut surfaces of the fibers were inserted flush to the top of the slides and sealed in petroleum jelly, thereupon to prevent evaporative losses. The fibers were kept surrounded by medium up to the bottom of the slides by capillary action when precisely 2.5 ml of media were instilled into the well of each chamber; thus, the volume of the well up to the slides was just greater than that of the medium plus fibers.

Experimental procedure

The mounted fibers were immersed in Na- and Ca-free medium made up of Tris (550–590 mM as needed to maintain 950–1,000 mosm), 10 mM K, and one of three different concentrations of Mg: 5, 25, or 60 mM; the pH of the media was 7.6 as maintained by the buffer. The Mg in the media was initially in excess of 99% ^{26}Mg, compared with the 14.2% that occurs naturally. A typical experiment consisted of two fibers that remained mounted in a chamber and immersed in 2.5 ml of medium for 15, 30, or 60 min before processing for analysis. At the indicated times, the media were removed from the wells and analyzed by atomic absorption spectroscopy for total Mg concentration and by mass spectrometry for isotope ratios; these assays were also performed on the media at time 0 to obtain baseline values of total Mg concentration and of isotope ratios.

Barnacle shell fragments bearing the still attached but otherwise prepared muscle fibers were first pretreated for 1.5–2 h in Na- and Ca-free medium (585 mM Tris buffer and 10 mM K) to significantly deplete the fibers of Na and Ca. At the start of the experimental runs, fibers were cut free from their shells one at a time and mounted in the chambers to which shortly thereafter were added the ^{26}Mg-enriched media. The fibers were maintained for the required durations in the chambers. After the required times had elapsed, they were removed and rinsed twice in isosmotic solution containing only Tris. The fibers were then placed on ultra-pure blotting paper and 2-mm segments were trimmed off (corresponding to the length of fiber embedded in the Teflon slides, i.e., the thickness of the slides). Next, the resulting length of each fiber was measured with a rule accurate to within 1 mm (or ~5% of total fiber length), taking special care not to stretch the fiber; this was followed by weighing of the blot-dried fibers. Finally, the fibers were hydrolyzed in platinum crucibles for a minimum of 1 h using 0.4 ml per fiber of 74–76% HNO_3, and the resulting hydrolysate diluted a minimum of 625 times before analysis of total Mg by atomic absorption spectroscopy.

Isotope ratios by thermal ionization spectrometry

Thermal ionization mass spectrometry (TIMS) was chosen for the determination of isotope ratios. This method yields very accurate and precise ratios in general and for Group IIA elements in particular. GC/MS analysis of volatile metal chelates, while excellent for the analysis of Se and Cr (Reamer and Viellon, 1981), has the drawback of significant sequential sample contamination problems ("memory") (Hachey et al., 1980). Whereas Mg isotope ratios have been determined by secondary ion mass spectrometry (SIMS) (Ramseyer et al., 1984), and isotope ratios of both Ca and Fe determined by fast atom bombardment (FAB), to date, TIMS has produced the best accuracy and precision. The TIMS for Mg was carried out using the silica gel technique (Barnes et al., 1973), in which a dilute suspension of finely ground silica gel is placed on the sample filament, dried, coated with the sample (5 μl) to be assayed, and then with dilute phosphoric acid, and finally dried again. This procedure is necessary for Mg, Zn, and several other metals of an ionization potential higher than that of Ca; the coating process reduces the effective ionization potential, through some matrix interaction not fully understood, so that ion signals of sufficient intensity are produced. The equipment used was a Finnigan-MAT THQ thermal ionization quadrupole mass spectrometer (Finnigan MAT, Bremen, FRG) equipped with a 13-sample filament turret, a liquid N_2 cold trap, and a data acquisition/analysis computer.

Total magnesium concentration of fibers and media by atomic absorption spectroscopy

Measurements of total Mg concentration of the fiber hydrolysates were made using a model 1100 flame atomic absorption spectrophotometer equipped with a deuterium arc background corrector (Perkin-Elmer Corp., Norwalk, CT). Flame was produced by combustion of an acetylene/air mixture (1.2 l/min:8.9 l/min); the slit setting was at 4 nm and the absorbance was measured at a wavelength of 285 nm. Standards and zero-point solutions were prepared containing the same concentration of HNO_3 as the fiber hydrolysates. Actual concentration of total Mg in the fibers was calculated from knowledge of the net dilution of the analyzed samples.

Barnacle muscle fibers

Culture of animals

Freshly gathered stocks of the acorn barnacle *Balanus nubilus* were supplied by Bio-Marine Enterprise (Seattle, WA). These were maintained in a 150-gallon aquarium containing a seawater substitute reconstituted from Instant Ocean (Aquarium Systems, Mentor, OH). The seawater was continuously aerated, filtered, and maintained at 11–13°C.

Preparation of muscle fibers

Muscle fibers from the rostral depressor muscles of the acorn barnacle *Balanus nubilus* were used in all of the experiments. Only muscle bundles from the same animal were used in a particular experiment. Most uncut fibers measure 2–3 cm in length and 1–1.5 mm maximum width at midlength (fibers are tapered and roughly triangular in cross-section). The fibers were carefully separated from each other starting from their free distal ends (opposite attachment to shell) by using surgical scissors and a thin glass probe to cut only through connective tissue in between the fibers, before being placed in calcium- and sodium-free seawater for 1.5–2.5 h to deplete them of calcium and sodium. This last step was undertaken to prevent spontaneous contraction of the fibers and any significant Na/Ca exchange that can take place, thereby circumventing some difficulties in interpreting results of the experiments, and to reduce fluctuations in Na that may originate from both Na/Ca exchange and the purposeful manipulation of external Mg levels itself, because there are reasons to believe that internal Na levels above a certain threshold value may significantly affect Mg fluxes (unpublished data). The procedures were carried out at room temperature (23–25°C).

Source of stable isotope ^{26}Mg

^{26}Mg was obtained from Oak Ridge National Research Laboratory, Oak Ridge, TN, in the form of the ^{26}Mg-enriched oxide (MgO); as supplied, the Mg used to make up the external media was 99.5% ^{26}Mg, 0.15% ^{25}Mg, and 0.35% ^{24}Mg. Before being added to the buffer-containing media, the MgO was dissolved in slightly more HCl than needed to fully convert all of the MgO into $MgCl_2$ and water.

Theory

The basic theory and relevant equations are outlined in the Appendix. First-order, time-independent rate constants for both influx and efflux were obtained from the raw data for each experiment and converted into initial flux rates. As the theory demonstrates, it was necessary to measure the isotope ratios only in the external medium to obtain rate constants for both efflux and influx, provided initial and final concentrations of total Mg in the fibers were determined by atomic absorption spectroscopy.

RESULTS

The raw isotope ratio data and other information necessary for calculating the rate constants and fluxes are presented in Table 1. The requisite data on fiber volume and length are presented as the combined dimensions of the two fibers responsible for every time point, and thus in effect referred to two fibers treated as a composite cylinder made up of the two fibers end-to-end. Correspondingly, two fibers were physically pooled to determine the initial concentration of Mg in the fibers ($[Mg^{+2}]_i$ plus bound Mg). The rate constants derived from the isotope ratio data, and the initial flux rates based on these rate constants and the volume and area of each of the fibers, are shown in Table 2.

Because the calculated rate constants and fluxes did not significantly depend on the duration of the experiments, as could be expected on the basis of the theoretical model, their values were averaged together according to external concentration of Mg, $[Mg^{+2}]_o$, to yield the results in Table 2. It can be seen that the rate constants for efflux, k_e, depended on $[Mg^{+2}]_o$. On the other hand, the rate constants for influx, k_i, did not change significantly with $[Mg^{+2}]_o$. From knowledge of the two rate

TABLE 1 Raw data, including isotope ratios, for experiments used to calculate rate constants and flux rates

Sample No.	Duration	$^{26}Mg/^{24}Mg$ (±% SD)	$^{25}Mg/^{24}Mg$ (±% SD)	Combined fiber volume	Combined fiber length	Initial $[Mg^{+2}]_o$	Initial fiber mg concentration
	min			*1 × 10⁻⁵ liters*	*cm*	*mM*	*mM*
In 5 mM Mg							
3B7	Initial	64.6 (3.4)	0.123 (3.3)	—	—	4.3	10.8
3B3	30	42.9 (9.4)	0.153 (3.6)	4.41	6.8	4.3	10.8
3B5	60	41.6 (9.0)	0.144 (6.0)	3.35	5.6	4.3	10.8
3A7	Initial	58.4 (4.6)	0.128 (5.3)	—	—	4.0	12.4
3A5	60	33.2 (—)	0.124 (—)	7.95	5.0	4.0	12.4
4B7	Initial	57.8 (6.6)	0.176 (11.2)	—	—	5.8	10.7
4B3	30	44.9 (4.1)	0.156 (4.0)	5.24	4.5	5.8	10.7
4B5	60	39.4 (0.4)	0.151 (0.9)	6.92	5.9	5.8	10.7
In 25 mM Mg							
4A8	Initial	168.8 (4.5)	0.204 (9.3)	—	—	25.9	9.9
4A2	15	150.0 (4.0)	0.223 (7.7)	4.91	5.1	25.9	9.9
4A6	60	156.0 (4.1)	0.187 (3.2)	4.41	5.4	25.9	9.9
4B8	Initial	155.2 (4.1)	0.214 (7.0)	—	—	25.4	10.7
4B2	15	110.2 (4.8)	0.197 (5.3)	5.48	5.1	25.4	10.7
4B4	30	113.2 (2.3)	0.192 (2.1)	4.23	4.3	25.4	10.7
5C7	Initial	240.3 (7.1)	0.249 (4.5)	—	—	24.1	9.4
5C3	30	192.5 (8.9)	0.215 (7.2)	1.56	4.8	24.1	9.4
5C5	60	183.2 (4.9)	0.240 (6.6)	1.49	4.4	24.1	9.4
In 60 mM Mg							
3A8	Initial	219.7 (7.9)	0.279 (22.3)	—	—	61.1	12.4
3A2	15	188.5 (5.6)	0.227 (8.8)	6.04	5.0	61.1	12.4
4A7	Initial	244.6 (8.1)	0.233 (4.9)	—	—	60.8	9.9
4A1	15	190.8 (2.3)	0.237 (3.2)	5.12	4.7	60.8	9.9
4A3	30	181.7 (6.9)	0.230 (9.8)	6.58	4.7	60.8	9.9
4A5	60	200.9 (5.3)	0.244 (3.7)	2.8	4.1	60.8	9.9

Samples are grouped according to the same initial conditions (fibers taken from the same animals, irrespective of duration of the experiment); requisite information on fiber dimensions and internal and external Mg concentrations are provided. (Percent standard deviation [% SD] is given for each isotope ratio; —, not determined or not necessary.)

constants, k_i and k_e, it is possible to calculate curves for the internal Mg concentration in the fibers as a function of time and for each of the three values of external Mg. Such predictive curves can be fitted against the data points for internal Mg concentration as in Fig. 1; the data in this figure were normalized to unity for initial values of internal Mg concentration, because this practice reduces error due to variability among the initial properties of the individual fibers. The data points, each representing the average from three separate experiments, were obtained by using atomic absorption spectroscopy to measure total Mg concentration in fibers incubated in different $[Mg^{+2}]_o$ for the indicated times. The curves, on the other hand, were generated from Eq. 10. Insertion into Eq. 10 of the appropriate calculated values for the rate constants, and properly normalized, empirically determined concentra-

TABLE 2 Flux rates (at t = 0) and flux rate constants for Mg in barnacle muscle fibers, according to external concentration of Mg

$[Mg^{+2}]_o$	Initial efflux rate (±SEM)	Initial influx rate (±SEM)	k_e (±SEM)	k_i (±SEM)	Sample size
mM	*pmol/cm²s*	*pmol/cm²s*	*10⁻³ × min⁻¹*	*10⁻³ × min⁻¹*	
5	13.4 (±1.5)	11.8 (±3.8)	9.5 (±1.6)	9.6 (±4.0)	5
25	53.7 (±18.1)	68.7 (±19.6)	46.0 (±22)	12.3 (±3.9)	6
60	80.43 (±17.62)	144.37 (±24.8)	49.1 (±14)	7.6 (±1.1)	4

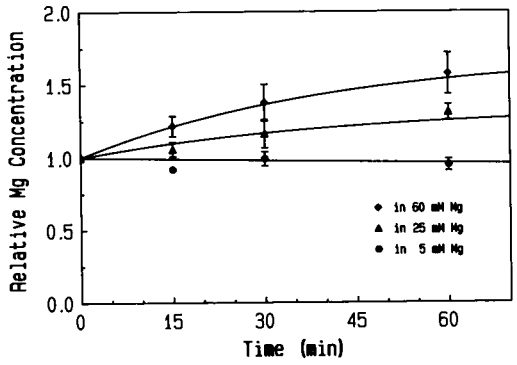


FIGURE 1 Total Mg content per fiber volume normalized to 1.0 at $t = 0$ is plotted against the duration of immersion in solutions of different Mg ion concentration. Each point is plotted as the average of three observations (plus/minus standard error of mean). The curves drawn through the points are calculated from Eq. 10 using average values for k_i and k_e obtained from isotopic exchange experiments performed at the given concentrations.

tion terms gives rise to the three curves in Fig. 1 that describe the net uptake of Mg by the fibers: for $[Mg^{+2}]_o$ = 5 mM, $C_i(t) = 0.160e^{-0.0048t} + 0.840$; for 25 mM, $C_i(t) = -0.339e^{-0.230t} + 1.339$; and for 60 mM, $C_i(t) = -0.700e^{-0.245t} + 1.700$.

Fig. 2 shows that Mg efflux is activated by external Mg. It also shows the relative constancy of the values of the rate constants for Mg influx in the range of $[Mg^{+2}]_o$ used in this study. Computer-generated, nonlinear regression curves were tested on a sigmoidal kinetic model for different plausible values of the Hill coefficient requiring only that the minimum value of k_e be kept at 0; this last boundary condition seemed appropriate, because it was

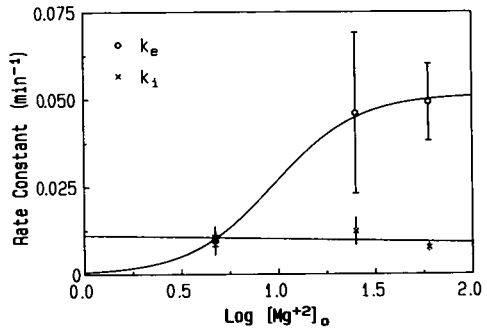


FIGURE 2 Average values (plus/minus standard error of mean) of the rate coefficients k_i and k_e are plotted against the logarithm of $[Mg^{+2}]_o$ with $[Mg^{+2}]_o$ in millimolar. The line drawn through the points for k_i is a linear least-squares regression line. The curve drawn through the points for k_e is a computer-assisted best fit curve (least squares fit) for a sigmoid relationship under the stipulation that k_e pass through the origin at $[Mg^{+2}]_o = 0$ and that the Hill coefficient = 2.

not unreasonable to assume that the component of k_e attributable to activation by $[Mg^{+2}]_o$ was much larger than that resulting from electrodiffusion (see Discussion). A coefficient of 2 was the lowest integral coefficient that forced a curve through the tightly barred 5-mM point and still fit the data (other integral coefficients >2 could also force passage through the very "tight" point); the EC_{50} was 9.5 mM and the maximum or saturation value of k_e was 0.0513 (the abscissa in Fig. 2 was plotted on a log scale because the computer program that was used required that this transformation be made before performing the nonlinear regressions). The values for k_i were fitted to a simple linear regression, assuming near-constancy of the values of k_i as a function of $[Mg^{+2}]_o$; the slight slope of the line was not statistically significant. An interesting feature of Fig. 2 is the intersection of the activation curve for k_e with the curve for k_i. The point of intersection, which coincides with an almost perfect super-position of empirically determined "tight" data for k_i and k_e at the same values of $[Mg^{+2}]_o$ (i.e., 5 mM), would correspond to a steady-state condition of internal Mg if $[Mg^{+2}]_o = [Mg^{+2}]_i$. In fact, the latter is nearly true, for the concentration of ionized Mg in a typical fiber freshly removed from a barnacle is ~5.5 mM, close to the point of intersection.

An analysis of the reproducibility of the measurement of the isotope ratios revealed that significant flux data can be generated for the fibers at intervals as close as 2 min apart (depending on ratio of Mg inside to outside the cell). This level of resolution should permit determination, using only a modest number of replicates, not only of fluxes and their first-order rate constants, but also of the kinetic order of more complicated fluxes by employing computer-assisted curve-fitting. However, it must be kept in mind that the true surface area of a barnacle muscle fiber is up to 15 times its apparent area, and that this advantage doubtless played an important part in the rapid resolution of the fluxes. In other preparations, such as in giant squid axon, measurements may have to be made farther apart, conceivably up to 30 min apart.

DISCUSSION

The data presented here show that the technique of mass spectrometry, supplemented with a standard analytical technique for measuring total Mg concentration, can be successfully applied to the determination of magnesium ion bidirectional fluxes across the membrane of the barnacle giant muscle fiber. The application of this method is based on the treatment of the isolated barnacle giant muscle fiber as a single well-mixed intracellular compartment in contact with extracellular fluid. Though this model is a relatively simple one, it provides a remark-

able amount of consistency in the results we have obtained using mass spectrometry to measure fluxes and using atomic absorption methodology to follow net changes in the total magnesium content of muscle fibers.

The first order flux kinetics applied to the ratios of stable magnesium isotopes measured by mass spectrometry (see Materials and Methods) permits the rate coefficients for both efflux, k_e, and influx, k_i, to be measured in one and the same experiment. The rate coefficients have been determined at three values of $[Mg^{+2}]_o$ (Table 2) and show a relative constancy for k_i over the concentration range of $[Mg^{+2}]_o$ studied and in contrast, a marked dependence of the value of k_e on $[Mg^{+2}]_o$. The value of k_e increases with increasing $[Mg^{+2}]_o$ in a manner consistent with an activating effect of $[Mg^{+2}]_o$ on a process transporting Mg ions in the outward direction. According to this hypothesis, the activation would be Michaelis-Menten-like in that saturation is approached at higher values of $[Mg^{+2}]_o$; the tentative value of EC_{50} based on this description is ~10 mM. Though more data would be required to establish the exact shape and behavior of the activation curve, particularly at lower values of $[Mg^{+2}]_o$, the data are nonetheless consistent with a sigmoidal activation with a Hill coefficient of at least 2.

The value of k_i is nearly independent of $[Mg^{+2}]_o$, therefore suggesting a mechanism for Mg influx that differs from that for efflux. The precise analysis of the measured rate coefficients in terms of actual membrane processes is impossible at present because so little is known about the mechanisms by which Mg ions cross the cell membrane. On purely thermodynamic grounds it seems clear that an outwardly directed Mg pump must be present in the membrane. Supporting this view is the strong dependence of Mg efflux on metabolic factors (Baker and Crawford, 1972; DeWeer, 1976; Mullins et al., 1977). The fact that the present experiments were performed in Na-free media rules out the operation of one Mg transport process, namely, Na/Mg exchange. As measurements of total Mg content of fibers stored in Na-free media did not indicate a gain of Mg under these conditions, another Mg pump must be present that works in the absence of $[Na^+]_o$. Our data suggest that the rate coefficient k_e refers to this Mg extrusion process. The process yielding the rate coefficient k_i and moving Mg ions into the muscle fibers could be electrodiffusional in nature. The independence principle applied to the constant-field equation for ion flux predicts a constant rate coefficient for any ionic influx occurring at constant membrane potential (Sjodin, 1959). Measurements of the membrane potential, E_m, of several single barnacle muscle fibers at the three values of $[Mg^{2+}]_o$ used, over the time intervals during which isotopic fluxes were measured, indicated that E_m was stable over these intervals to within 2 mV. The electrical requirement for constancy of the rate constant for Mg influx is thus met, to within experimental errors, in these experiments.

It is of interest to calculate the value of the magnesium permeability coefficient that would be consistent with our measured value of k_i on this basis. The rate coefficient of ~0.01 min^{-1} for k_i translates to $P_{Mg} = 1.1 \times 10^{-6}$ cm/s using cylindrical geometry for the barnacle muscle fiber and an average fiber radius of 600 μm. The barnacle giant muscle fiber has a system of surface clefts and sarcolemmal infoldings, however, which gives a much higher surface area than that calculated for a simple cylinder. The factor by which surface area is increased is ~15 times (Ashley and Ellory, 1972), and the value of P_{Mg} taking the true surface area into account amounts to a value of 0.07×10^{-6} cm/s. This is near the value of 0.05×10^{-6} cm/s for P_{Na} in squid giant axons (Sjodin, 1984) and is consistent with the low magnesium ion permeability believed to apply to vertebrate muscle fibers (Flatman, 1984). Because our results for influx of Mg ions indicate a nearly linear rise of the value of influx with increasing $[Mg^{+2}]_o$, as demanded by the electrodiffusion equation, given the reasonable value of P_{Mg}, there is nothing in our work so far that is contrary to the notion that Mg influx in barnacle muscle fibers is a passive diffusive process. However, caution must be exercised in arriving at such a conclusion as a portion of Mg influx may be due to a component of Na/Mg exchange, which is apparent in squid giant axons as an influence of $[ATP]_i$ and $[Na^+]_i$ on Mg influx (Mullins and Brinley, 1978). In addition, the presence of other ions either inside or outside of the cell could have an influence on P_{Mg} and so also affect Mg influx.

It might be argued that, for as yet unknown reasons, the mass spectrometry method gives spurious values for ionic flux. A strong counter-argument is that the isotopic fluxes of Mg are in good agreement with our own measured values of net total Mg flux determined from direct atomic absorption analytical measurements. Further argument that our mass spectrometric Mg fluxes are accurate to within usual experimental error is that the measured values for the rate coefficients are in agreement with the radioisotopic Mg flux measurements of others. For example, for similar conditions, Vogel and Brinley (1973) reported a value of 13 pmol/cm^{2}s for Mg efflux in barnacle muscle fibers, which compares favorably with our value (Table 2) of 13.4 pmol/cm^{2}s. Ashley and Ellory (1972) reported a range of 6–12 pmol/cm^{2}s for Mg efflux in barnacle muscle fibers. Reported magnesium flux rates in squid giant axons are lower than these rates and usually given as 2 or 3 pmol/cm^{2}s (range, 0.6–4.4) (Mullins et al., 1977; Mullins and Brinley, 1978). Flux rates in barnacle muscle fibers as reported here and in the rest of the literature may be inordinately high because of the extensive infolding of the surface membrane (cleft system) of

the fibers. Using the surface membrane area estimates of Ashley and Ellory (1972), barnacle fluxes can be divided by ~15 to give approximately true surface membrane fluxes. Applying this correction brings the values of the Mg fluxes in barnacle muscle down to ~1 pmol/cm^{2}s, which is much closer to the values measured in squid giant axons for which there is no such problem. No such corrections are needed for rate constants, because these do not depend explicitly on the surface area of the membrane. The rate constant for ^{28}Mg efflux in heart muscle has been reported to be 3.8×10^{-3}min^{-1} (Polimeni and Page, 1973), and in frog skeletal muscle the rate coefficient for external ^{28}Mg exchanging with intrafibrillar Mg has been found to be 8.5×10^{-3}min^{-1} (Gilbert, 1960). These values compare favorably with our present values of $\sim 9 \times 10^{-3}$min^{-1} for similar values of $[Mg^{+2}]_o$ (5 mM in the present study, 8 mM in work of Vogel and Brinley [1973], and 2 mM in work of Gilbert [1960]).

At higher values of $[Mg^{+2}]_o$, our measured values for k_e increased while those for k_i remained about the same. Polimeni and Page (1973) and Vogel and Brinley (1973) also found the rate coefficient for ^{28}Mg efflux to rise when $[Mg^{+2}]_o$ was raised. These authors attributed this behavior to Mg/Mg exchange. We do not attribute the observed increases in k_e with increase in $[Mg^{+2}]_o$ primarily to Mg/Mg exchange because there is excellent agreement of our stable isotopic flux data with the net flux data from measurements made by atomic absorption analyses. The agreement of these separate sets of data by different methods is obtained by applying a simple first-order flux model that assumes no special Mg/Mg exchanges. In other words, Mg/Mg exchange does not to any significant extent change the value of $[Mg^{2+}]_i$, and we have measured the changes in $[Mg^{2+}]_i$ that occur and found them consistent with the isotopic flux imbalances we have measured.

Concerning the action of $[Mg^{+2}]_o$ on the rate coefficient for Mg efflux, it should be noted that there is no general agreement among investigators in this area as to just what the effect is, even in a single preparation such as the barnacle muscle fiber. An ~50% reduction in the magnitude of Mg efflux has been reported in barnacle muscle fibers when Mg ions are removed from the external medium (Vogel and Brinley, 1973). Other workers report either no effect, slight increases, or slight decreases when $[Mg^{+2}]_o$ is reduced to nominally zero (Ashley and Ellory, 1972). In the squid giant axon, the action of $[Mg^{+2}]_o$ on Mg efflux depends upon the composition of the external medium. In the presence of external Na ions, Mg-free solutions increase Mg efflux, whereas in the absence of external Na ions Mg-free solutions decrease the Mg efflux (DeWeer, 1976). The inhibiting effect of $[Mg^{+2}]_o$ on Mg efflux in the presence of external Na can be partially overcome by raising the value of $[Na^+]_o$, suggesting that external Mg ions competitively inhibit Na/Mg exchange. The activating effect of $[Mg^{+2}]_o$ on Mg efflux in Na-free media was also observed in our work on barnacle muscle fibers. It is tempting to conjecture that, in the absence of Na/Mg exchange, external Mg ions may activate Mg efflux at sites normally used by Na/Mg exchange. It is clear that this area merits further study.

The behavior of the measured rate coefficients, k_e and k_i, at low values of $[Mg^{+2}]_o$ is of interest. The rate curves intersect at $[Mg^{+2}]_o = 5$ mM suggesting that at the usual value of $[Mg^{+2}]_i$ equal to ~5 mM, flux balance occurs and a steady-state ensues. Below a value of 5 mM for $[Mg^{+2}]_o$ we have no measurements. However, Ashley and Ellory (1972) present the rate coefficient for the zero $[Mg^{+2}]_o$ case in Na-free media at $\sim 0.48 \times 10^{-3}$min^{-1}. In Fig. 2 this point falls at ~5% of the 5 mM $[Mg^{+2}]_o$ value and thus occurs just above the origin, indicating a decidedly sigmoidal character to the activation curve. A kinetic treatment of the activation curve in Fig. 2 by computer-assisted analysis yielded a Hill coefficient of at least 2.0 as the most likely one for the data. As the value of $[Mg^{+2}]_o$ is raised in the range of 5 to ~40 mM, the steep rise in the value of k_e causes Mg efflux to keep in close pace with Mg influx. This agrees with our observation that it is very difficult to get the fibers to gain total Mg in this range of $[Mg^{+2}]_o$. Only by increasing $[Mg^{+2}]_o$ to values beyond ~40 mM can considerable net gains of Mg be induced.

In summary, the mass spectroscopic method of flux measurement gives values of Mg flux in barnacle muscle fibers that agree with those obtained using radioactive isotopes. Influx and efflux rate coefficients are obtained in a single experiment and, because mutual interference between isotopes is virtually absent, more than one ion can be measured simultaneously. It is our intent to continue to apply the present method in seeking answers to the many questions that yet remain concerning magnesium regulation in muscle fibers and possibly other large cells. In particular, it will be of interest to see how Na and K concentrations inside and outside the fibers combine to regulate the Mg ion fluxes.

APPENDIX

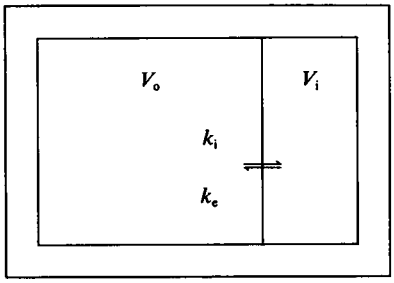
Theoretical model

Mass spectrometry allows measurement of bidirectional fluxes and rate constants

Our starting kinetic model is based on a single intracellular compartment of volume V_i separated from an extracellular compartment of volume V_o by a single plasma membrane. Both compartments are assumed to be well mixed and to contain the three stable isotopes of Mg (^{24}Mg, ^{25}Mg, and ^{26}Mg) in varying amounts and relative proportions. The method for flux measurement is based upon placing a highly purified stable Mg isotope, in practice ^{26}Mg, in the outside compartment

and measuring the rate at which the ratio of the concentration of that isotope to that of the others in the compartment changes. From the rate at which a new isotopic equilibrium is approached, and knowledge of the total Mg concentration in the fiber and medium at the beginning and end of the experiment, the membrane rate constants for both inward and outward Mg ion movement can be determined. It is assumed that the rate coefficients are the same for all of the Mg isotopes.

The kinetic relations are summarized below for ^{26}Mg and ^{24}Mg. The following symbols apply:



V_i Intracellular volume
V_o Extracellular volume
k_i Rate coefficient for Mg influx
k_e Rate coefficient for Mg efflux
C_i^n Variable total inside concentration of isotope n
C_o^n Variable total outside concentration of isotope n
$C_{I,i}^n$ Initial total inside concentration of isotope n
$C_{II,i}^n$ Final total inside concentration of isotope n
$C_{I,o}^n$ Initial outside concentration of isotope n
$C_{II,o}^n$ Final outside concentration of isotope n
r Ratio of intracellular ionized Mg concentration to total Mg concentration for all isotopes.

It should be noted that some of the intracellular Mg is in chemically bound form so that the "buffering ratio," r, assumed constant here on the basis of experimental evidence, must be used to get ionized Mg values. It is assumed that all extracellular Mg is freely ionized.

The basic system differential equations are:

$$\frac{dC_i^n}{dt} = k_i C_o^n - k_e r C_i^n \tag{1}$$

$$\frac{dC_o^n}{dt} = -\frac{V_i}{V_o} k_i C_o^n + \frac{V_i}{V_o} k_e r C_i^n. \tag{2}$$

These equations can be integrated for each isotope used for the initial and final boundary conditions holding in each experiment. As total Mg ion concentrations are always measured by atomic absorption spectrometric analysis, the measurement of any two of the three Mg isotopes suffices to give complete information. Equations will be developed for the two isotopes ^{26}Mg and ^{24}Mg. It is convenient to define two quantities that can be calculated for each experiment: the total amount, in moles, of the two key isotopes present in the entire system after specification of the initial external solution composition (in addition to the ratio ^{26}Mg/^{24}Mg, the requisite calculation requires measurement of one of the ratios, ^{26}Mg/^{25}Mg or ^{25}Mg/^{24}Mg outside of the cell). These amounts are designated T^{26} and T^{24}. Conservation of mass requires that these quantities remain constant throughout the experiment. The quantities, T^n, can be used to relate concentrations in the extracellular and intracellular compartments as follows:

$$C_i^{26} = \frac{T^{26} - C_o^{26} V_o}{V_i} \tag{3}$$

$$C_i^{24} = \frac{T^{24} - C_o^{24} V_o}{V_i}. \tag{4}$$

Eqs. 1 and 2 can be solved for initial and final boundary conditions by standard methods. As it is more convenient to take advantage of mass spectrometric measurements made on the outside media (see next section), Eq. 2 is solved for the concentration of ^{24}Mg and ^{26}Mg and the resulting solutions are used to calculate the ratio k_i/k_e. One obtains for ^{26}Mg and ^{24}Mg:

$$\frac{a C_{II,o}^{26} + b}{a C_{I,o}^{26} + b} = e^{(V_i/V_o)a\Delta t} \tag{5}$$

and

$$\frac{a C_{II,o}^{24} + c}{a C_{I,o}^{24} + c} = e^{(V_i/V_o)a\Delta t}, \tag{6}$$

where $a = -(k_i + k_e r V_o/V_i)$, $b = k_e r T^{26}/V_i$, $c = k_e r T^{24}/V_i$, and Δt is the elapsed time between initial and final conditions. Equating Eqs. 5 and 6 and solving for k_i/k_e yields:

$$\frac{k_i}{k_e} = r \frac{V_o}{V_i}\left\{\frac{T^{26}(C_{II,o}^{24} - C_{I,o}^{24}) + T^{24}(C_{I,o}^{26} - C_{II,o}^{26})}{V_o(C_{I,o}^{26}C_{II,o}^{24} - C_{I,o}^{24}C_{II,o}^{26})} - 1\right\}. \tag{7}$$

In addition, solving Eq. 5 for k_e yields:

$$k_e = \frac{1}{\left[\frac{V_i}{V_o}\left(\frac{k_i}{k_e}\right) + r\right]\Delta t} \ln\left\{\frac{\frac{k_i}{k_e} + r\frac{V_o}{V_i}\left(1 - \frac{T^{26}}{V_o C_{I,o}^{26}}\right)}{\frac{k_i}{k_e} + r\frac{V_o}{V_i}\left(1 - \frac{T^{26}}{V_o C_{II,o}^{26}}\right)}\right\}. \tag{8}$$

As the ratio k_i/k_e is known from Eq. 7, the value of k_e (and, hence, k_i) is obtained from Eq. 8. Thus the measurement of final values of the concentration of ^{26}Mg and ^{24}Mg in the extracellular solution, along with known initial values, completely determines the values of the rate coefficients k_i and k_e from Eqs. 7 and 8.

It is evident that the measurement of the two Mg isotopes, ^{24}Mg and ^{26}Mg, versus time in contact with the external medium provides values of both Mg influx and Mg efflux in the same experiment. This is true in general for any substance for which at least two stable isotopes exist and at least one is available in enriched form. The mass spectrometric method for flux measurement is thus ideally suited for measurements of flux ratios in cells. Because interferences in the measurement of the isotopic ratios are virtually absent, there is no reason why the method cannot be applied simultaneously to the measurement of the ionic flux ratios of different ionic species in one and the same experiment.

Once the two rate coefficients k_i and k_e have been determined for Mg ions using Eqs. 7 and 8, the change in total analytical Mg within the fibers can be predicted. One simply applies Eq. 1 to the total Mg concentrations due to all isotopes present, C_i^T.

$$\frac{dC_i^T}{dt} = k_i C_o^T - k_e r C_i^T. \tag{9}$$

As the external volume is usually ~50 times greater than the intracellular volume, the total Mg ion concentration externally is approximately constant even though its isotopic composition changes during the experiment due to exchange of isotopes. If Eq. 9 is solved for the case of constant C_o^T, the following relation is obtained for $C_i^T(t)$:

$$C_i^T(t) = \frac{k_i}{k_e} r^{-1} C_o^T (1 - e^{-k_e r \Delta t}) + C_{I,i}^T e^{-k_e r \Delta t}. \tag{10}$$

Because the two rate coefficients are both known from the isotope ratio measurements, and both C_o^T and $C_{I,i}^T$ are known, $C_{II,i}^T$ can be calculated for any value of Δt desired. The predicted value of $C_{II,i}^T$ can then be compared with the value determined directly by the analytical atomic absorption method.

Important considerations: choice between external and internal compartments

There may be circumstances for which it will be desirable to measure isotope ratios in the cell as opposed to outside the cell (for example, when the volume of the outside medium is considerably larger than the volume of the cell), but in general small external volumes can be used to avoid some of the difficulties of using the shifts in internal isotope ratios to find the bidirectional fluxes. For example, it may be difficult to directly measure the initial or final isotope ratio in a cell, a quantity which must be known to calculate the fluxes. In the case of the initial cellular isotope ratios, a natural distribution of isotopes can be assumed, but only in experiments in which preloading with label has not occurred, because in the latter case the amount of fiber that can be sacrificed at the beginning of the experiment is limited. In the case of final isotope ratios in cells, the processing of the cells needed to perform quantitative analysis of the cellular samples may result in undesirable shifts in isotope ratios caused not by transmembrane fluxes but rather by very small amounts of contaminating species possessing a natural isotope distribution, although in principle these shifts can be calculated empirically and corrected for. Using only the outside medium, however, the exact isotope ratios can be determined because the starting isotope ratio of the medium that a cell is placed in can be measured accurately, even after the original isotope distribution has been shifted somewhat by impurities in the solutes (such as buffer and salts) that were used to make up the medium (this effect is evident in the data in Table 1, where the initial isotope ratios in all of the external media should have been about the same but clearly were not); furthermore, the final isotope ratio in the external medium is also measured directly without the intervention of additional impurities.

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