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Intracellular magnesium and magnesium buffering

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Abstract

The development of new techniques for measuring intracellular free Mg^{2+} during the 1980s has provided investigators with the tools needed to produce new insights into the regulation of cellular magnesium. Within the limits of this technology, it appears that all mammalian cells maintain free cytosolic Mg^{2+} levels within the fairly narrow range of 0.25–1 mM. While transport mechanisms and sequestration within cellular organelles will contribute to this regulation, it is binding of Mg^{2+} to an as yet poorly defined system of buffers that is largely responsible for determining the set point of this regulation. The lack of an adequately Mg^{2+}-selective ionophore remains an impediment to progress in this area.

Introduction

Although the physiological importance of magnesium has long been recognized, it has been difficult to establish a clear picture of how cells regulate the intracellular level of this divalent cation. An essential role for magnesium in mammals was first demonstrated 75 years ago (Leroy 1926). Subsequently, Kruse *et al.* (1932) demonstrated that a diet deficient in magnesium produced a characteristic set of symptoms in young rats that include hypersensitivity, hyperemia, skin lesions, irritability, and retarded growth. However, only in the last 25 years has substantial progress been made in understanding cellular regulation of magnesium, largely through the development of analytical techniques capable of measuring intracellular Mg^{2+} in cultured cells and intact tissues. This review will present the development of the current views on this issue as they have evolved in parallel with advances in the analytical tools for measuring Mg^{2+}.

Measuring cellular magnesium

The development of flame spectrometry and atomic absorption spectroscopy in the mid to late 1950s provided the first reliable analytical techniques for measuring the magnesium content of small amounts of biological material (Dawson & Heaton 1961; Thiers & Vallee 1957; Wacker & Vallee 1957). These and subsequent studies provided estimates of the magnesium content of various tissues that ranged from approximately 7–9 mmol kg wet weight in liver and striated muscle (Iyengar *et al.* 1978; Walser 1967) to 2 mM in erythrocytes (Seiler *et al.* 1966; Valberg *et al.* 1965). In contrast, human plasma magnesium content was found to be 0.89 ± 0.08 mM, of which roughly half was determined to be free (unbound or complexed) magnesium (Jackson & Meier 1968). Together these studies demonstrated that magnesium is not a micronutrient, but actually the second most abundant cation in the cells of soft tissues. More importantly, these studies demonstrated that the magnesium content of cells exceeds that of the plasma, providing evidence that cells concentrate and therefore are capable of regulating their magnesium content. These observations raised two important questions: What are the mechanisms responsible for regulating cellular magnesium content, and how much of the intracellular magnesium is bound versus free?

As early as 1961, Nanninga attempted to estimate the free Mg^{2+} of frog muscle utilizing an approach that has formed the basis of many subsequent studies (Nanninga 1961). Using literature values for the total cation content of this tissue and association constants for a number of potential chelating molecules, an ex-

pression was derived that provides an estimate of the free cation concentration. The precision of this estimate depends on the accuracy of the values for the pH, the identity and concentrations of all chelating species, the association constants for these chelators at the experimental pH and the total magnesium content. Since Mg^{2+} association constants for nucleotides in particular are subject to interference/competition from Ca^{2+}, H^+, K^+, and polyamines (Luthi *et al.* 1999), arriving at realistic values for these constants in a physiological milieu is not a trivial matter.

In a different approach, Rose (1968) utilized the Mg^{2+} dependence of the adenylate kinase reaction to derive an expression for the equilibrium constant of this reaction that depends only on the concentration of free Mg^{2+} ion. Applying this approach to human red blood cell extracts, free Mg^{2+} was estimated to be in the 0.1–0.2 mM range. It is interesting to note that 30 years later, this value is only a factor of two lower than current estimates (Flatman 1988; Fujise *et al.* 1991; Jelicks *et al.* 1989; Raftos *et al.* 1999).

The landmark study from Richard Veech's lab (Veloso *et al.* 1973) addressed the intracellular distribution of magnesium using experimentally determined values for total cell magnesium and binding constants for operationally defined tissue fractions to calculate free magnesium concentrations in rat brain, liver, and kidney. The validity of these measurements was established by comparison with free Mg^{2+} values calculated from measuring Σcitrate/Σisocitrate concentrations in freeze-clamped tissue. This ratio reflects the equilibrium point of aconitate hydratase activity in the tissues and can be used to estimate free Mg^{2+} levels since this enzyme is very sensitive to magnesium concentrations. Not only does this study demonstrate that both experimental approaches provide comparable results, but it also shows that the free Mg^{2+} levels calculated for three tissues are very similar. However, the most important observation from this study is the realization that the free Mg^{2+} level of a cell is determined largely by the extensive buffering that occurs through binding to a variety of cellular constituents. While providing a benchmark for future studies, the techniques employed are ill suited to address the main question arising from this work: *Can intracellular Mg^{2+} levels be changed sufficiently by signaling systems for Mg^{2+} to function as a physiological regulator?*

To address this question experimentally required the development of new tools/techniques capable of monitoring changes in free cytosolic Mg^{2+}. Among the techniques developed and tested, three have emerged that are capable of providing multiple measurements from a single preparation, can be used with a variety of tissue/cell types, and produce reliable data: (1) ion-selective micro-electrodes, (2) fluorescent indicator dyes, and (3) ^{31}P-NMR spectrometry. While each has its limitations, these tools have provided the basis for much of what we have learned about the dynamics of cytosolic Mg^{2+} and magnesium buffering over the last 20 years.

Ion-selective microelectrodes

The first magnesium selective microelectrode was developed from a lipophilic ligand (ETH 1117) that exhibited ionophoric selectivity for Mg^{2+} over K^+, Na^+, and Ca^{2+}, utilizing standard microelectrode technology (Lanter *et al.* 1980). Unfortunately, the cytosolic Mg^{2+} values obtained with this electrode were quite variable (e.g., in frog skeletal muscle, 0.80 and 1.69 mM (Alvarez-Leefmans *et al.* 1986); 3.3 mM (Hess *et al.* 1982), 3.8 mM (Lopez *et al.* 1984)) and considerably higher than the values that were being obtained in muscle using ^{31}P-NMR spectrometry (Gupta & Moore 1980; Kushmerick *et al.* 1986). The major shortcoming of this particular electrode (which probably accounts for most of the variability) is that to obtain an accurate cytosolic Mg^{2+} value, simultaneous determination of $[K^+]_i$ is required in order to correct the interference from K^+ (Alvarez-Leefmans *et al.* 1986; Lanter *et al.* 1980). This problem was addressed by the Simon lab, resulting in a significantly improved Mg^{2+}-selective ionophore (ETH 5214) (Hu *et al.* 1989). Values for cytosolic Mg^{2+} in frog skeletal (0.93 mM), guinea pig (0.72 mM) and ferret (0.85 mM) ventricular muscle obtained using the ETH 5214 microelectrode are similar to what has been reported using both ^{31}P-NMR spectrometry and fluorescent indicator dyes (Blatter 1990; Buri *et al.* 1993; Buri & McGuigan 1990). More recently, magnesium selective ionophores have improved in both sensitivity and reliability. They are reviewed by Günzel elsewhere in this issue.

^{31}P-NMR spectrometry

Nuclear magnetic resonance spectrometry was first used to estimate free intracellular magnesium in 1977 by measuring the spin-spin relaxation time (T_2) of phosphocreatine in frog skeletal muscle (Cohen &

Burt 1977). This approach, which yielded a value for free magnesium of 4.4 mM, has proven to be inadequate, since T_2 values are generally believed to be sensitive to intracellular factors other than Mg^{2+} binding that cannot be readily taken into account (Gupta & Moore 1980). Measuring the ^{31}P resonance peaks of ATP has provided a more reliable method for determining cytosolic Mg^{2+} values, based on the peak shifts that occur when Mg^{2+} binds to ATP (Cohn & Hughes 1962). Gupta *et al.* (1978) first used this approach to measure a free magnesium level in human red blood cells of 0.25 mM and subsequently of 0.6 mM in frog skeletal muscle (Gupta & Moore 1980). Since this technique is based on measuring the Mg-ATP complex at equilibrium, the accuracy of the free Mg^{2+} determination depends on the equilibrium constant used and on factors that influence this equilibrium, including pH, temperature, and ionic strength (Luthi *et al.* 1999). Thus, the difficulty of measuring or estimating the K_{app} for this equilibrium with a high degree of certainty imposes a limit on the confidence of the free Mg^{2+} values obtained by this method. Furthermore, the sensitivity of the β-ATP peak shift to change in free Mg^{2+} is greatest between 3×10^{-3} and 1×10^{-5} M magnesium (Kushmerick *et al.* 1986), which means that for most mammalian cells this technique will be relatively insensitive to physiological changes in cytosolic free Mg^{2+}. This shortcoming has been addressed by a new formalism for calculating free $[Mg^{2+}]$ from ^{31}P NMR spectra based on the β/α peak height ratio of ATP instead of the commonly used β-α peak chemical shift (Clarke *et al.* 1996). The peak height ratio provides a much more sensitive measure of free cytosolic $[Mg^{2+}]$ as demonstrated by the 2.5-fold change in ratio observed as free $[Mg^{2+}]$ was varied from 0.25 to 1.5 mM.

Another potential limitation of this technique concerns the instrumentation needed to collect the spectra: the length of time needed to acquire a spectrum of sufficient resolution for analysis varies inversely with the field strength of the magnet. A modern instrument will permit an investigator to acquire usable spectra in seconds or less (Clarke *et al.* 1996), whereas older less powerful instruments may require 1 to 2 h of acquisition to produce comparable spectra (Gupta & Moore 1980). Longer acquisition times present another problem, specifically that of maintaining the metabolic stability of the animal/tissue/cell preparation during the measurement (Fatholahi *et al.* 2000).

Despite these caveats, ^{31}P-NMR remains a useful tool for estimating free Mg^{2+} non-invasively in isolated tissue and is particularly well-suited for studies in which intracellular Mg^{2+} is likely to reach millimolar levels.

Fluorescent indicator dyes

The first report of a fluorescent indicator to be used for measuring magnesium levels in serum and urine appeared in 1959 (Schachter 1959). Upon binding Mg^{2+}, 8-hydroxyquinoline was shown to fluoresce maximally at 530 nm when excited by light at 420 nm at a pH of 6.5. The difference in fluorescence observed at pH 3.5 (where fluorescence is Mg-independent) from that observed at pH 6.5 was found to be linearly and directly proportional to magnesium concentrations between 0.025 and 0.2 mEq/l (or 0.05 to 0.4 mM). While useful for measuring Mg^{2+} in these fluids, interference from cellular constituents has precluded its use in measuring intracellular Mg^{2+} levels.

Thirty years elapsed before the next significant advance in the development of a fluorescent Mg^{2+} indicator. Using the approach pioneered by Tsien (1980), a compound designated FURAPTRA was designed and synthesized which possesses several properties that permit it to function as an intracellular indicator for free Mg^{2+} (Raju *et al.* 1989). This compound is based on the 'second generation' Ca^{2+} indicator, fura-2, (Grynkiewicz *et al.* 1985) and subsequently was marketed under the name, mag-fura-2. The usefulness of this compound to function as an intracellular indicator for cytosolic $[Mg^{2+}]$ depends on: (1) the ability to load cells with the compound to permit measurement and (2) the affinity of the compound for Mg^{2+} being appropriate for measuring changes in intracellular $[Mg^{2+}]$. Additionally, the indicator needs to be relatively free of interference from other cations and changes in pH. Cell loading with mag-fura-2 is generally accomplished by incubating with the acetoxymethylester derivative of the indicator, which diffuses into the cell where cytosolic esterases cleave the acetoxymethyl groups. The concentration of the anionic free mag-fura-2 in the cytosol will continue to increase as long as the ester form is present outside the cell, with the rate determined by the esterase activity level of the cell. However, some cells express an organic anion transporter capable of rapidly exporting the free mag-fura-2, which can be blocked by sulfinpyrazone to maintain adequate indicator levels over the course of an experiment (Di Virgilio *et al.* 1989; Grubbs *et al.* 1991).

Although the K_D values ranging from 4.6 to 22.3 mM for Mg^{2+} binding to mag-fura-2 at 37 °C have been reported (Buri *et al.* 1993; Tashiro & Konishi 1997; Westerblad & Allen 1992), most labs report a value of 1.5 ± 0.1 mM (Murphy 1993). The different values in some cases reflect experimental differences (i.e., ionic strength, temperature, pH) in how the data were collected. The K_D of mag-fura-2 for magnesium is highly temperature dependent but relatively insensitive to changes in pH (Lattanzio & Bartschat 1991). It should be noted that two of the groups reporting higher K_D values for mag-fura-2 used *in vivo* calibration approaches in contrast to the *in vitro* approach used by most (see below).

It is also important to note that mag-fura-2 has a higher affinity for Ca^{2+} ($K_D \approx 50$ μM; (Murphy 1993)) than for Mg^{2+}. Thus, only the fact that the intracellular resting free Ca^{2+} concentration in most cells is in the nanomolar range permits mag-fura-2 to be used as a Mg^{2+} indicator. In cells where Ca^{2+} transients rise into the micromolar range or in organelles that store Ca^{2+} such as the sarcoplasmic reticulum, the response of fura-2 would be saturated, leading some investigators to use mag-fura-2 to measure calcium (Konishi *et al.* 1991; Sugiyama & Goldman 1995). Given the possibility that smaller increases in cytosolic Ca^{2+} may interfere with mag-fura-2 measurements of cytosolic Mg^{2+} (Hurley *et al.* 1992), it is advisable that fura-2 and mag-fura-2 experiments be run in parallel to determine directly the importance of this potential concern.

Other documented potential sources of interference include polyamines (Gunther *et al.* 1994) and cytosolic viscosity (Grubbs *et al.* 1991). Polyamines, such as putrescine and spermine, are capable of reducing the response of mag-fura-2 to changes in $[Mg^{2+}]$. Thus, to the extent that polyamine concentrations vary during the course of an experiment, mag-fura-2 measurements will be altered either by a direct effect of the polyamine on the indicator or by displacing Mg^{2+} bound to ATP and other nucleotides. Similarly, changes in cell volume during an experiment will produce viscosity changes that will alter mag-fura-2 responses.

Dye calibration

The widely used formalism developed for calibrating fura-2 measurements (Grynkiewicz *et al.* 1985) also works well for *in vitro* calibration of mag-fura-2 signals (Raju *et al.* 1989). Since Mg^{2+} binding to mag-fura-2 causes a shift in the emission spectrum from 335 to 370 nm, the best sensitivity with respect to change in free Mg^{2+} is achieved by obtaining the ratio, R, of fluorescence emissions at these two wavelengths. This experimental ratio is then normalized to limiting values for the indicator obtained at zero (R_{min}) and saturating (R_{max}) Mg^{2+} concentrations and to the fluorescence intensities observed at 370 nm under these same conditions. Most studies in the literature have used this calculation to calibrate *in vivo* measurements with the indicator despite the obvious question of how relevant this calibration is to the behavior of mag-fura-2 in a cell (Grubbs & Walter 1994; Koss *et al.* 1993; Quamme & Rabkin 1990; Raju *et al.* 1989; Schachter *et al.* 1990).

Calibrating the fluorescence of mag-fura-2 in biological material remains problematic due to the difficulty encountered using the available divalent cation ionophores, 4-Br-A23187, and ionomycin, to equilibrate intracellular indicators with extracellular calibrating solutions (Raju *et al.* 1989) (A23187 cannot be used for calibration due to intrinsic fluorescence when excited at 339 nm (Deber *et al.* 1985)). Ionomycin is much more efficient as a transporter of Ca^{2+} than of Mg^{2+} while the A23187 family moves both Mg^{2+} and Ca^{2+} (Reed & Lardy 1972). In addition, these ionophores also move 2 protons for every divalent cation, thus markedly changing intracellular pH from the values at which the experimental measurements were taken. Finally, the amount of Mg^{2+} needed to saturate mag-fura-2 is large – 40 to 60 mM (Grubbs *et al.* 1991; Westerblad & Allen 1992). Therefore, using Br-A23187 to equilibrate cytosolic mag-fura-2 with an external buffer containing a saturating $[Mg^{2+}]$ has been shown to require from 5 to 14 h (Tashiro & Konishi 1997), clearly limiting both the appeal and the applicability of this approach. In a different approach to saturating intracellular mag-fura-2, 0.5 M $MgCl_2$ was pressure injected into mouse skeletal muscle fiber until no further increase in R_{max} was seen; R_{min} was established by pressure injecting 0.5 M K_2EDTA until no further decrease in R was seen (Westerblad & Allen 1992). However, noticeable swelling of the fiber was observed which raises concerns about the effect of the changing environment of the indicator on the apparent K_D for Mg^{2+}.

In summary, at the present time both approaches to calibrating mag-fura-2 are unsatisfactory for entirely different reasons. The *in vitro* approach will readily yield calibration parameters, but it remains uncertain whether this approach adequately represents

Table 1.

Tissue	$^{19}F/^{31}P$ NMR	ISE (ETH 5214)	Furaptra/mag-fura-2
Skeletal muscle			
Mouse			0.78 mM (Westerblad & Allen 1992)
Frog	0.6 mM (Gupta & Moore 1980)	0.93 mM (Blatter 1990)	
Rat (SHR)		1.3 mM (Gunzel & Galler 1991)	
(WKY)			
			0.42 mM
			0.56 mM (Ng *et al.* 1992)
Cardiac myocyte			
Chicken	0.56 mM (Rotevan *et al.* 1989)		0.51 mM (Murphy *et al.* 1989a)
Rat			0.48 mM (Quamme & Rabkin 1990)
	1.02 mM (Amano *et al.* 2000)		1.1 mM (Koss *et al.* 1993)
Guinea pig	0.78 mM (Headrick & Willis 1989)	0.72 mM (Buri *et al.* 1993)	0.67 mM (Hongo *et al.* 1994)
	0.85 mM (Murphy *et al.* 1989b)		0.82 mM (Handy *et al.* 1996)
Smooth muscle cells			
A7r5 cells			0.31 mM (Schachter *et al.* 1990)
A10			0.74 mM (Tashiro & Konishi 1997)
Tenia cecum	0.3–0.4 mM		
(Guinea Pig)	(Nakayama & Nomura 1995)		0.52 nM (Quamme *et al.* 1993)
Rabbit			
Rat (SHR)	0.54 mM (Kopp *et al.* 1990)		0.98 mM (Tashiro & Konishi 1997)
(WKY)			
	0.40 mM		
	(Kushmerick *et al.* 1986)		
			0.41 mM
			0.62 mM [62]
Neuronal cell			
Human	0.99 mM (Vink *et al.* 1988)		0.68 mM (Gotoh *et al.* 1999)
Exocrine cell			
Rat			0.35 mM (Zhang & Melvin 1992)
Lymphocyte			0.29 mM (Delva *et al.* 1996)
			0.19 mM (Ng *et al.* 1991)
Red blood cell			
Human	0.25 mM (Levy *et al.* 1988)		
	0.25 mM (Gupta *et al.* 1978)		
Hepatocyte	0.7 mM (Malloy *et al.* 1986)		0.59 mM (Raju *et al.* 1989)
	0.46 mM (Cohen 1983)		0.33 mM[a] (Gaussin *et al.* 1997)

[a]Value calculated from measurement of 0.25 μmol g of cells, assuming that 75% of the weight of the cell is water.

the behavior of the indicator in the cytosol. The *in vivo* approach is hampered by the inadequacies of the available ionophores needed to establish the calibration endpoints and the uncertainty of how these heroic changes in cytosolic free Mg^{2+} will themselves alter the ionic strength, viscosity, biochemical equilibria, and ultimately the fluorescence response of the indicator. Until better ionophores for Mg^{2+} become available for *in vivo* calibration, the *in vitro* calibration at least provides a means for standardizing measurements between laboratories with respect to differences in instrumentation optics. If investigators would report values obtained for the calibration parameters (R_{min}, R_{max}, S ratio), it would also help to determine

sources of variability and perhaps provide a means to eventually reconcile these differences.

Despite these caveats, the values obtained for cytosolic free Mg^{2+} using mag-fura-2 are remarkably consistent with values obtained in the same tissues using both the ion-selective microelectrode and ^{31}P NMR spectrometry (Table 1).

With few exceptions (notably red blood cells and perhaps lymphocytes), the values for free cytosolic Mg^{2+} fall in a narrow range of 0.5–1.0 mM. As noted by others (Tashiro & Konishi 1997), free Mg^{2+} values reported for muscle cells tend to be slightly higher than values for non-muscle cells. When these free Mg^{2+} values are compared to the total magnesium content of these cells, which ranges between 10–20 times more than the free concentration, the obvious question arises: How do mammalian cells maintain their free Mg^{2+} concentrations within this narrow range?

Intracellular magnesium distribution

There are two possible explanations for the large difference between the free and total Mg^{2+} content of cells. Given the clear evidence that Ca^{2+} is actively sequestered by the sarcoplasmic reticulum in muscle cells and by the endoplasmic reticulum in non-muscle cells, the possibility that Mg^{2+} might be similarly sequestered must be considered. Interestingly, electron probe microanalysis of cultured cells and thin sections of flash-frozen tissue has provided no evidence supporting the existence of Mg^{2+} sequestration in any organelle or region of a cell (Di Francesco *et al.* 1998; Somlyo *et al.* 1979). In the absence of sequestration, binding of Mg^{2+} to high affinity sites within the cell provides the most likely explanation for reconciling these data. In most if not all cells, adenine nucleotides, ATP in particular, constitute the major binding element for cellular Mg^{2+} by virtue of their relatively high affinity for the cation and millimolar abundance (Koss *et al.* 1993; Polimeni & Page 1973). Other cell-type specific physiological chelators of Mg^{2+} include creatine phosphate in muscle (Gupta & Moore 1980), and 2,3 bisphosphogylcerate (DPG) and hemoglobin in red blood cells (Raftos *et al.* 1999). In these cell types, Mg^{2+} binding to ATP and these other chelators accounts for approximately 75% of the total magnesium content of the cells, the remaining Mg^{2+} being either free or bound to other unidentified cellular constituents. Identifying and characterizing these other Mg^{2+} binding constituents may prove to be a difficult challenge, but will ultimately provide the key for understanding how cytosolic Mg^{2+} levels are so tightly regulated.

Magnesium buffering

The idea that cellular buffering is important in determining the level of free cytosolic Mg^{2+} was first proposed by Veloso and colleagues (Veloso *et al.* 1973). These investigators demonstrated that rat liver has a large pool of magnesium binding sites with dissociation constants that range from 0.66 to 2 mM and that these sites are only about 37% saturated under normal physiological conditions. Subsequently, utilizing the unique physiology of the human red blood cell, Flatman and Lew were able to characterize the magnesium buffering system of these cells into kinetically distinct components (Flatman & Lew 1980). The data were fitted to an equation that describes magnesium buffering as a series of mass action interactions between the cation and binding sites that differ in concentration and affinity for Mg^{2+}. The best fit was achieved with three classes of binding sites: a low capacity, high-affinity class thought to correspond to binding sites on cell proteins, and two classes that corresponded reasonably well with the concentrations and Mg^{2+}-binding affinities of ATP and 2,3 DPG. This model was recently refined to incorporate hemoglobin and a 2:1 binding stochiometry for Mg_2BPG and Mg_2ATP, and now provides an excellent fit for experimental data under a variety of conditions in these cells (Raftos *et al.* 1999).

Defining the Mg^{2+} buffering systems in other cell types has proven to be more difficult due to the complexity of the biology, physiology, and biochemistry of these cells relative to the red cell. The first characterization of Mg^{2+} buffering in muscle cells defined Mg^{2+} buffering power as the observed change in $[Mg^{2+}]_i$ relative to the amount of Mg^{2+} added to the cell by pressure injection (Westerblad & Allen 1992): Buffer power $= \Delta[Mg^{2+}]_i/\Delta\Sigma[Mg^{2+}]_i$.

This equation is analogous to the buffering power equation for weak acids and bases first described by Koppel & Spiro (1914) (translated to English: Roos & Boron 1980), the differences being that this equation is not logarithmic and is the inverse of the Koppel and Spiro function.

Koss *et al.* (Koss *et al.* 1993) subsequently developed a similar expression to define a magnesium buffering coefficient, B_{Mg}, to characterize Mg^{2+}

buffering in cultured chick cardiomyocytes. In this study the metabolic inhibitors iodoacetate and NaCN were used to deplete ATP levels, liberating into the cytosol Mg^{2+} that had been bound to nucleotide. The amount of Mg^{2+} released was calculated and compared to the change in cytoplasmic free Mg^{2+} measured with mag-fura-2 to determine a value for B_{Mg}. Values ranging from 1.4 to 5.4 were obtained depending on assay conditions, indicating that Mg^{2+} is weakly but effectively buffered. Thus, when 2.7 mM was liberated, cytosolic free Mg^{2+} increased only from 1.1 mM to 2.7 mM over a period of 10 min. Under physiological conditions, i.e, in the presence of ATP, energy dependent efflux mechanisms would presumably have reduced this increase by exporting 'excess' Mg^{2+} from the cells.

In a recent review addressing multiple issues related to the equilibrium between Mg and ATP, Luthi and colleagues (Luthi *et al.* 1999) also provided an interesting comparison of the formalisms that have or can be applied to describing Mg^{2+} buffering. Each of the equations considered has offsetting advantages and limitations, such that none emerges with a clear consensus as the best approach. Ultimately, the choice will depend on whether the goal is to characterize intracellular Mg^{2+} buffering *per se* or cellular 'muffling' of Mg^{2+}. The concept of 'muffling', first proposed by Thomas (1991), was introduced by Luthi *et al.* to differentiate physiochemical Mg^{2+} buffering from the broader cell biological imperative to regulate cytosolic ionic levels by more than buffering, i.e., including potential transport and sequestration mechanisms. These transport systems (reviewed elsewhere in this issue) obviously play an important role in regulating cytosolic Mg^{2+} levels. As noted above, the approach used by Koss *et al.* (1993) entails depletion of cellular ATP which would be unsuitable to determining 'muffling'.

A final point regarding Mg^{2+} buffering concerns the physiological role of ATP in this process. Under normal physiological conditions, the ATP level in most cells appears to be relatively constant and, with an affinity for the cation that is roughly a hundred-fold less than the cytosolic free concentration, is essentially saturated with Mg^{2+} (88–90% $MgATP^{2-}$ by NMR measurements). Given this stability and saturation, ATP functions more as a 'sink' rather than a true buffer for Mg^{2+}, i.e, it has very little capacity to absorb additional Mg^{2+} entering the cell or released from other sources. However, ATP could play an important role as the source of Mg^{2+} in a scenario in which bursts of metabolic activity, perhaps initiated by signaling events, induce transient decreases in [ATP], which would transiently increase [Mg^{2+}] levels. Mg^{2+} efflux pathways would be activated to return cytosolic Mg^{2+} back to resting levels. Technologically, it may be difficult to demonstrate rapid and transient changes in ATP, but this scenario could help explain some interesting recent findings in cardiac tissue (Fatholahi *et al.* 2000; Keenan *et al.* 1995).

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