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Hormonal regulation of Mg^{2+} transport and homeostasis in eukaryotic cells

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Introduction

The high concentrations of total and free magnesium (Mg_f^{2+}) within eukaryotic cells and its role as an essential co-factor or regulator for numerous cellular enzymes and functions surprisingly contrasts with the minimal knowledge of the mechanisms, genes and proteins that regulate Mg^{2+} homeostasis at the cellular and organismal level.

The reasons for this limited knowledge are both conceptual and methodological. Conceptually, the abundance of intracellular Mg^{2+} has led to the idea that no major changes in Mg^{2+} concentration occur or are required, as the cation is plentiful at all times for its co-factor role. It is therefore unlikely to operate as a 'on-off switch' for enzymes inside the cell as Ca^{2+} and other second messengers do. In turn, this point of view has limited interest in developing suitable techniques and methodological approaches to measure changes in cellular Mg^{2+} concentration accurately.

In the last 25 years, however, the concept that the intracellular content and/or concentration of Mg^{2+} is *static* has been revisited based on an increasing number of reports indicating that total cellular and plasma Mg^{2+} content can change significantly following varying hormonal and non-hormonal stimuli and consequently have a major physiological and/or pharmacological role in modulating cell and tissue functions. At the same time, to better detect changes in cellular Mg^{2+} concentration, technical approaches have been improved or developed. Although not all of them may work in every eukaryotic cell or tissue, we are now better equipped to detect and measure variations in cellular Mg^{2+} homeostasis and distribution.

The present review will attempt to provide a general framework to understand how cellular Mg^{2+} content and transport are regulated in eukaryotic cells, and how changes in cellular and plasma Mg^{2+} content contribute to a physiological regulation of intracellular and extracellular enzymes. We will emphasize mammalian cells since Mg^{2+} transport in *Arabidopsis* is reviewed by Shaul in this issue. In addition, Mg^{2+} homeostasis in the kidney is reviewed by Romero.

Cellular Mg^{2+} distribution

The majority of mammalian cells contain a total cellular Mg^{2+} concentration of between 14 and 20 mM (Romani & Scarpa 1992b), thus making Mg^{2+} the second most abundant cellular cation after potassium. Within the cell, Mg^{2+} appears to be evenly distributed among the nucleus, mitochondria and endo(sarco)plasmic reticulum. Electron probe X-ray microanalysis determinations on discrete portions of these organelles in skeletal (Somlyo *et al.* 1985) and smooth (Ziegler *et al.* 1992) muscle cells, cardiac myocytes (Shuman & Somlyo 1987), hepatocytes (Dalal *et al.* 1998) and HL60 proleukemia cells (Di Francesco *et al.* 1998) indicate a total Mg^{2+} content of 75 to 100 mmol kg dry weight, or 14 to 18 mM in any of these organelles. The majority of Mg^{2+} appears to be bound to phospholipids, proteins, chromatin, nucleic acids and nucleotides, especially ATP, the array of binding sites depending on the organelle. Thus, as with Ca^{2+}, only a small fraction of the Mg^{2+} content is actually *free* in the lumen of these structures or in the cytosol. Rutter *et al.* (1990) and Jung *et al.* (1987) have calculated a *free* Mg^{2+} concentration of 1.2 and 0.8 mM in the matrix of cardiac and liver mitochondria, respectively. Nuclear estimates of *free* Mg^{2+} are not known. An estimate of intrareticular *free* Mg^{2+} concentration is virtually impossible to obtain. The

relative affinity of the best Mg^{2+} selective dyes, Mag-Fura or Mag-Indo, is about 1.5 mM. This suffices for cytosolic estimation of free Mg^{2+} because cytosolic Ca^{2+} is < 1 μM. However, within the lumen of the endo(sarco)plasmic reticulum, the Ca^{2+} concentration is well above the K_m of the dyes for Ca^{2+} (50 μM), being on the order of 3–4 mM (Somlyo *et al.* 1985). Thus intralumenal free Mg^{2+} concentrations will not be accessible until a truly selective Mg^{2+} dye or probe is synthesized. It is to be hoped that the recent quinolizine carboxylic acids synthesized by Otten *et al.* have provided such a dye (2001).

The largest single pool of Mg^{2+} in most cells is in the cytosol, where Mg^{2+} mostly forms complexes with ATP, other phosphonucleotides and other energy substrates (Scarpa & Brinley 1981). ATP is about 5 mM in most cells. Since about 90% of total ATP is generally in the form of MgATP, ATP forms the single largest pool of bound Mg^{2+}. With the exception of calmodulin (Ohki *et al.* 1997) and troponin C (Wang *et al.* 1998) in skeletal muscle fibers, and possibly S100 protein (Ogoma *et al.* 1992), it is unknown whether other cytosolic proteins can bind substantial Mg^{2+} and therefore contribute to buffering (Corkey *et al.* 1986; Koss *et al.* 1993) the cation within the cell (see also the article by Grubbs, this issue). As a result of this binding and buffering activity, cytosolic *free* Mg^{2+} concentration has been estimated to range between 0.5 and 1 mM in the majority of the cell types examined by fluorescent indicator (Fatholahi *et al.* 2000; Harman *et al.* 1990; Raju *et al.* 1989; Tashiro & Konishi 1997), ^{31}P-NMR (Corkey *et al.* 1986; Mottet *et al.* 1997; Watanabe *et al.* 1998) or electrophysiological (Günther *et al.* 1995; Günzel & Galler 1991; Günzel & Schlue 1996; Hall *et al.* 1992; Hintz *et al.* 1999; Luthi *et al.* 1997; Zhang *et al.* 1997) techniques. Hence, considering that the concentration of Mg^{2+} in the plasma and extracellular fluid is approximately 1.2–1.4 mM (Quamme 1993), and that one-third of this is bound to circulating proteins or other biochemical moieties (Allouche *et al.* 1999; Quamme 1993), most cells are close to a *zero trans* condition with respect to *free* Mg^{2+} content across the cell membrane, while a small concentration gradient probably exists across the membranes of intracellular organelles and the cytosol.

Regardless of the lack of a significant chemical gradient, the net electrochemical gradient for Mg^{2+} is directed markedly inward because of the negative inside membrane potential. The equilibrium potential for intracellular *free* Mg^{2+} is generally around 50 mM in mammalian cells. Clearly, there must exist mechanisms to maintain a low intracellular free Mg^{2+} concentration and regulate Mg^{2+} homeostasis. The cellular distribution and disequilibrium of the Mg^{2+} electrochemical gradient leads to a series of questions about the ability of the eukaryotic cell to transport Mg^{2+} across biological membranes, the number and nature of the transport mechanisms involved, how these transporters are regulated, the nature of the intracellular sensor that determines Mg^{2+} homeostasis, and the physiological significance of Mg^{2+} movement. This review will attempt to summarize our current knowledge of these questions.

Mg^{2+} transport across biological membranes

In the absence of hormonal or metabolic stimuli, most eukaryotic cells do not significantly change total cellular Mg^{2+} content over several hours or days of incubation in the presence of a physiological extracellular Mg^{2+} concentration (Page & Polimeni 1972; Polimeni & Page 1973; Rogers & Mahan 1959). However, the turnover of Mg^{2+} within cells and tissues varies markedly. Using $^{28}Mg^{2+}$ several laboratories have measured Mg^{2+} turnover in isolated cells, tissues and intact animals. In general, results in isolated cells or cells lines have been compatible with those in the intact rat model, though exceptions exist. In the rat, intravenous injection of $^{28}Mg^{2+}$ followed by sampling of tissue specific activity of Mg^{2+} over several days have indicated that ventricular myocardium and kidney exchange total cellular Mg^{2+} fairly rapidly within 3 h (Rogers *et al.* 1960; Rogers 1961, 1965; Rogers & Mahan 1959). Adipocytes and a few other tissues turn over cellular Mg^{2+} even more rapidly, within 1 h. In contrast to these turnover rates, lymphocytes, brain and skeletal muscle only slowly turn over total cellular Mg^{2+}. Indeed, after 6 h, the relative specific activity of tissue to serum Mg^{2+} was only about 0.1. Lymphocytes turn over only 5–7% of total cellular Mg^{2+} after 48 h while, under identical conditions, Ca^{2+} turns over completely within 3 h (Grubbs *et al.* 1985). However, using the same approach, isolated cardiac ventricular myocytes (Polimeni & Page 1974) have been reported to have a slow turn-over of more than 72 h; the most obvious explanation for the difference in these results and those in the intact rat is the isolation procedure for the cells, though this has not been tested. Nonetheless, despite the evidence of rapid and complete turnover in some tissues, the relatively slow turnover in other

tissues has long been used to infer that cellular Mg^{2+} remains essentially unaltered irrespective of the physiological or metabolic conditions the cell encounters. Studies on the hormonal regulation of Mg^{2+} fluxes by Maguire and colleagues (Bird & Maguire 1978; Erdos & Maguire 1980, 1983; Grubbs *et al.* 1984; Maguire *et al.* 1984; Maguire & Erdos 1978, 1980) and by Romani & Scarpa (Keenan *et al.* 1995, 1996; Romani *et al.* 1991, 1992, 1993a; Romani & Scarpa 1990a, b) as well as reports by Günther & Vormann (Günther *et al.* 1984; Günther & Vormann 1985, 1987) that erythrocytes can exchange a significant amount of their cellular Mg^{2+} content for extracellular Na^+ have forced reconsideration of this viewpoint.

Na^+-dependent Mg^{2+} efflux

In the absence of external stimuli, erythrocytes loaded artificially with Mg^{2+} (Günther *et al.* 1984) attempt to restore their 'physiological' Mg^{2+} content by extruding excess Mg^{2+} into the extracellular space via a Na^+-dependent mechanism. The Mg^{2+} efflux activated under these experimental conditions is not affected by catecholamine or ouabain administration, but is inhibited by cyanate, iodo-acetate, or extracellular concentrations of Mg^{2+} or Mn^{2+} in excess of 3 mM. The specificity of Na^+ as counter-ion for intracellular Mg^{2+} is supported by the observation that lithium or other monovalent cations cannot effectively replace Na^+ to support Mg^{2+} extrusion. Further, amiloride, commonly used to inhibit Na^+ transport mechanisms, inhibits Mg^{2+} fluxes across the cell membrane (Günther *et al.* 1984).

The initial reports of Günther and Vormann on significant exchange of intracellular Mg^{2+} in the erythrocyte have been confirmed and expanded by other investigators (Feray & Garay 1986, 1988; Flatman & Lew 1980; Flatman & Smith 1990, Ludi & Schatzmann 1987; Schatzmann 1993). Feray & Garay (1988) have observed that other Na^+-transport inhibitors such as imipramine or quinidine also block Mg^{2+} transport, and that the energy status of the cell (Feray & Garay 1986) is important for Mg^{2+} efflux. In terms of physiological significance, it has been proposed that changes in intra-erythrocyte Mg^{2+} content may contribute to regulation of Na^+/K^+-ATPase activity (Flatman & Lew 1981), phosphoinositides turn-over (Muller *et al.* 1996), Na^+/H^+ exchange operation (Parker *et al.* 1989), K/Cl cotransporter (Flatman *et al.* 1996) and hemoglobin metabolism, either directly (Mulquiney & Kuchel 1997) or indirectly through Mg^{2+} binding to 3-phosphoglycerate (Günther *et al.* 1995). Moreover, as total and *free* Mg^{2+} concentrations appear to be in a linear relationship within the erythrocyte (Fujise *et al.* 1991), it is possible to estimate the *free* Mg^{2+} concentration and its variations based on total Mg^{2+} determination under varying experimental conditions. This in turn allows an evaluation of how variations in *free* Mg^{2+} concentration affect the operation of key enzymes in red blood cells.

The Na^+-dependent Mg^{2+} extrusion mechanism, tentatively identified as a Na^+/Mg^{2+} exchanger, is not exclusively located in erythrocytes, as a similar Mg^{2+} transport mechanism has been observed in several other cell types or tissues. Cardiac myocytes (Romani *et al.* 1993c; Vormann & Günther 1987), hepatocytes (Romani *et al.* 1993b), ascites cells (Wolf *et al.* 1994b), freshly isolated spleen lymphocytes (Wolf *et al.* 1997), and sublingual mucosal acini (Zhang & Melvin 1996) all exhibit Na^+/Mg^{2+} exchange activity (see Romani & Scarpa 2000 for a list). Furthermore, while the original report (Günther *et al.* 1984) was obtained in erythrocytes artificially loaded with Mg^{2+}, subsequent observation with fluorescent indicators have shown that cytosolic *free* Mg^{2+} concentration is exquisitely sensitive to changes in extracellular Na^+ content. Removal of extracellular Na^+ results in a marked increase in cytosolic *free* Mg^{2+} (Handy *et al.* 1996; Tashiro & Konishi 1997) that returns towards basal level following the re-introduction of Na^+ in the incubation system. Both processes are blocked in the presence of amiloride or imipramine. These results suggest that under resting conditions (i.e., in the absence of any extracellular stimulus or ionic manipulation) the Na^+/Mg^{2+} exchanger operates at a basal rate to guarantee the physiological turn-over of Mg^{2+} across the cell membrane (Polimeni & Page 1974).

Despite increasing evidence for its operation in various cell types, in part because of the various experimental models and conditions utilized, controversy exists about stoichiometry and distribution of the Na^+/Mg^{2+} exchanger. Reports by Ludi and Schatzmann (Ludi & Schatzmann 1987), and Lew and Flatman (Flatman & Lew 1981) in human erythrocytes suggest that the exchanger operates with a $3Na^+_{in}$: 1 Mg^{2+}_{out}. Under different conditions a 1:1 ratio can be observed. In contrast, an electroneutral 2:1 exchange ratio has been reported by Günther & Vormann in chicken, turkey or human erythrocytes (Günther *et al.* 1984). Grubbs and Maguire (Grubbs *et al.* 1984) did not observe a Na^+-dependent Mg^{2+} extrusion in S49

lymphoma cells, while Wolf *et al.* (1997) have reported that freshly isolated rat spleen lymphocytes use Na^+ as counter-ion to extrude Mg^{2+}. A similar incongruence has also been observed in cardiac ventricular myocytes. The evidence provided by Handy *et al.* (1996), and Tashiro & Konishi (1997) for the operation of a Na^+/Mg^{2+} exchanger in cardiac ventricular myocytes contrasts with the lack of such an evidence in reports by Lieberman *et al.* (1992), and Murphy *et al.* (1991). While the difference in lymphocytes could be attributed to phenotypic modifications in immortalized *versus* freshly isolated cells, the reason for the discrepancy in cardiac ventricular myocytes is less apparent.

Differences have also been reported in terms of inhibition, in that amiloride effectively inhibits Mg^{2+} transport with an ED_{50} of 100–150 μM (Tashiro & Konishi 1997) in almost all the cell types in which it has been tested. In contrast, derivatives of amiloride (e.g., hexamethyl-amiloride) are effective only in certain cells types such as Erhlich ascites cells (Wolf *et al.* 1994a) but not hepatocytes (Cefaratti *et al.* 2000b) or leech neurons (Günzel & Schlue 1996). As amiloride derivatives inhibit the Na^+/H^+ exchanger with some selectivity, this exchanger may contribute to Mg^{2+} fluxes indirectly through changes in cellular pH and/or Na^+ content in some cell types but not others.

A recent report by Konishi et al. (Tashiro *et al.* 2000) suggests that cardiac myocytes artificially loaded with Mg^{2+} and over-expressing isoforms 1 or 3 of the Na^+/Ca^{2+} exchanger can extrude Mg^{2+} instead of Ca^{2+}. This possibility might explain some of the incongruities observed in cardiac myocytes. However, the time required for this mechanism to transport any significant amount of Mg^{2+} appears to be quite long (>45 min). It is thus likely that this observation has little physiological significance and is an artifact of overexpression.

Except for the few electrophysiological measurements of Mg^{2+} flux (Preston 1990, 1998; Shaul *et al.* 1999), current measurements of transport activity in all eukaryotic cells can determine only the movement of bulk Mg^{2+} into or out of a cell because of the unavailability of $^{28}Mg^{2+}$ as a tracer to follow unidirectional flux. Thus, necessarily, measurement of bulk Mg^{2+} movement represents the sum of the multiple Mg^{2+} transport processes in each cell. Most literature differences in stoichiometry and Na^+ dependence are probably due to measurement of different combinations of Mg^{2+} (and other) transport processes. The incongruities in current data also reflect the limited information we presently possess about the specific nature and number of Na^+/Mg^{2+} exchangers as well as the limited understanding of its operation and differential regulation in different cell types under varying experimental conditions. Specific data on individual transport systems must await cloning and expression or discovery of highly selective inhibitors.

Na^+-independent Mg^{2+} efflux

Under conditions in which no extracellular Na^+ is available to be counter-transported for cellular Mg^{2+} because of the presence of Na^+-transport inhibitors (Günther *et al.* 1990; Günther & Vormann 1990a) or the replacement of external Na^+ with other monovalent cations (Feray & Garay 1987, Günther & Vormann 1990b; Romani *et al.* 1992, 1993b; Grubbs & Maguire, unpublished observations), cellular Mg^{2+} can still be extruded from the cells, through an apparently Na^+-independent Mg^{2+} transport mechanism. Several divalent cations have been reported to support Mg^{2+} extrusion through this mechanism in various cell types. Günther and Vormann (Günther *et al.* 1990; Günther & Vormann 1987) have observed that Mg^{2+} efflux from loaded erythrocytes is modulated by the extracellular concentration of divalent cations like Mn^{2+} or Mg^{2+}, and Feray and Garay (Feray & Garay 1987) have indicated that extracellular Mn^{2+} can be exchanged for intracellular Mg^{2+} with a 1:1 stoichiometry. Under similar experimental conditions, extracellular Ca^{2+}, Sr^{2+} (Cefaratti *et al.* 2000b; Romani *et al.* 1993c), and even anions like HCO_3^- or Cl^- (Günther & Vormann 1990a) have been observed to support Mg^{2+} extrusion (Günther 1996; Günther & Vormann 1990a; Maguire *et al.* 1984; Romani *et al.* 1993c). This alternative Mg^{2+} transport mechanism becomes active in the presence of a low extracellular Na^+ concentration, and is inhibited by high concentrations of external Na^+, ATP depletion, or by the presence of quinidine (Cefaratti *et al.* 1998; Günther 1993). Since ATP represents the main buffer for Mg^{2+} in the cytosol, the inhibition of the Na^+-independent Mg^{2+} extrusion mechanism following ATP depletion would be consistent with an extrusion of Mg^{2+} via a mechanism requiring ATP hydrolysis or, alternatively, via a transporter that utilizes ATP for phosphorylation and activation (Günther & Vormann 1990a; Maguire *et al.* 1984). Mg^{2+}:Mg^{2+} exchange has been reported by Günther and Vormann (Günther & Vormann 1987) in erythrocytes and Maguire and coworkers (Maguire *et al.* 1984) in perfused rat heart. It is possible that a

Mg^{2+}_{in}:Mg^{2+}_{out} exchange is the final result of two transport mechanisms operating in sequence and utilizing the electrochemical gradient of another (intermediate) cation to move Mg^{2+} in and out of the cell. Günther (1996) has suggested that a Na^+/Mg^{2+} exchanger can accommodate Mg^{2+}, and eventually another divalent cation, at the extracellular site and thereby activate the transporter. This hypothesis is supported by kinetic evaluation of the transporter activity using ^{24}Mg and ^{28}Mg radioisotopes on opposite sides of the cell membrane (Günther & Vormann 1987), which has provided a K_m value similar to that calculated for the Na^+/Mg^{2+} exchanger (Günther & Vormann 1985) and is consistent with activation of a Na^+-independent Mg^{2+} extrusion mechanism only when extracellular Na^+ concentration is low (Cefaratti *et al.* 1998; Günther 1993). Nonetheless, the physiological significance of such a mechanism is unclear since exposure to very low extracellular Na^+ should not occur in the normally functioning organism.

Mg^{2+} transport in purified plasma membranes

Our understanding of Mg^{2+} transport mechanisms in mammalian cells has benefited recently by the utilization of purified plasma membrane vesicles from specific tissues like liver (Cefaratti *et al.* 1998, 2000b), heart (Cefaratti *et al.* 2000a), or brush border cells from ileum (Juttner & Ebel 1998), duodenum and jejunum (Baillien *et al.* 1995; Baillien & Cogneau 1995).

Studies with purified liver plasma membrane vesicle fractions have provided evidence for the presence of three distinct Mg^{2+} transporters. A Na^+/Mg^{2+} exchanger able to operate in either direction based upon the relative cation gradients can be observed in vesicles from the hepatocyte basolateral membrane (Cefaratti *et al.* 1998, 2000b). This transporter is highly Na^+ selective, operates with a K_m for Na^+ <20 mM, and is specifically inhibited by imipramine but not by amiloride (Cefaratti *et al.* 2000b) or amiloride derivatives (Cefaratti *et al.* 1998).

The apical hepatocyte membrane contains two unidirectional Mg^{2+} extrusion mechanisms activated by Na+ and Ca^{2+}, respectively (Cefaratti *et al.* 2000b). The apical Na^+/Mg^{2+} exchanger is also Na^+-selective, has a K_m similar to that calculated for the basolateral exchanger and is inhibited by amiloride or imipramine. The Ca^{2+}/Mg^{2+} exchanger is also inhibited by amiloride or imipramine (Cefaratti *et al.* 2000b) and is distinguished from the other transporter by its activation by Ca^{2+} or other divalent cations (Cefaratti *et al.* 1998) with a K_m of $\approx 50\ \mu$M.

These vesicular systems transport Mg^{2+} under *zero trans* conditions (for example 20 mM Mg^{2+} inside and outside the vesicles), an indication that the driving force for Mg^{2+} extrusion is provided by the counter-transported cation (Cefaratti *et al.* 1998). None of the three transporters appears to require intravesicular ATP, although ATPγS inhibits their transport activity to some extent (Cefaratti *et al.* 1998). Furthermore, the three transporters are all inhibited by *in vitro* treatment with alkaline phosphatase (Cefaratti *et al.* 2001), possibly implying their regulation via phosphorylation/dephosphorylation. Assessment of transporter stoichiometry indicates that the Ca^{2+}/Mg^{2+} exchanger operates electroneutrally (1 Ca^{2+}_{in}:1 Mg^{2+}_{out}) while the two Na^+/Mg^{2+} exchangers operate electrogenically (1 Na^+_{in}:1 Mg^{2+}_{out}) (Cefaratti *et al.* 2000c). A preliminary report suggests that both a Na^+- and a Ca^{2+}-dependent Mg^{2+} transport mechanism also operate in sarcolemmal vesicles (Cefaratti *et al.* 2000a) with properties that closely resemble those of the transporters present in liver plasma membranes.

By using cell permeant and impermeant Mag-Fura, Juttner and Ebel (1998) have observed a saturable Mg^{2+} uptake mechanism activated exclusively when the intracellular concentration of Na^+ is larger than the extracellular one. In the complete absence of extracellular Na^+, however, this pathway does not operate. Furthermore, this Mg^{2+} uptake mechanism is not reversible, and is not inhibited by amiloride analogs (DMA or EIPA) or Ca^{2+} channel inhibitors. The transporter is electroneutral and has a calculated K_m of 16 mM for Na^+, similar to the K_m reported by other investigators for the Na^+/Mg^{2+} exchanger in various experimental models (Cefaratti *et al.* 1998, 2000a; Günther 1996; Günther & Vormann 1985; Maguire *et al.* 1984; Tashiro & Konishi 1997). However, the physiological significance of such a system is again questionable since mammalian cells should never see a condition where intracellular Na^+ is greater than extracellular Na^+.

Mg^{2+} uptake mechanisms also operate in plasma membrane vesicles isolated from brush border cells of rat duodenum and jejunum (Baillien *et al.* 1995; Juttner & Ebel 1998). In duodenal cells a single Mg^{2+} uptake mechanism has been observed with a K_m of 0.8 mM, whereas two distinct transporters with K_m's of 0.15 and 2.4 mM appear to operate in rat jejunal cells. Transport of Mg^{2+} is stimulated by an inwardly

directed negative membrane potential (Baillien *et al.* 1995), is not affected by Ca^{2+}-channel blockers, but is inhibited by amiloride to a variable extent depending on the extravesicular concentration of Mg^{2+} (Juttner & Ebel 1998).

The physiological significance of multiple Mg^{2+} transporters in liver or brush border membranes is not clear. However, since it is now well documented that other cations and anions are transported via multiple and differentially regulated transporter systems, which all contribute to overall homeostasis under various conditions, it should be no surprise that multiple transporters are also present for Mg^{2+}.

Hormonal regulation of Mg^{2+} homeostasis

Hormonal regulation of Mg^{2+} transport was first observed by Elliot and Rizack (1974) in adipocytes, although the specificity of transport was not determined. Extensive characterization of hormonal modulation of Mg^{2+} fluxes was reported by Maguire and colleagues in S49 lymphoma cells as well as primary lymphocytes and other cell types (Erdos & Maguire 1983; Grubbs *et al.* 1984, 1985; Maguire 1982; Maguire & Erdos 1978, 1980) using $^{28}Mg^{2+}$ to specifically monitor influx vs. efflux. Influx of Mg^{2+} in S49 lymphoma cells was specifically inhibited by β-adrenergic agonists and PGE_1 and stimulated by inhibitors of protein kinase C. Efflux of Mg^{2+} was unaltered by any of these agents. Moreover, the availability of clonal derivatives of S49 cells lacking protein kinase A or adenylyl cyclase showed that the β-adrenergic receptor mediated inhibition of Mg^{2+} influx was *not* controlled through cyclic AMP and protein kinase A but apparently mediated by a signal transduction pathway independent from that classically activated by β-receptors (Maguire & Erdos 1980). These observations appear to be the first demonstration of a single receptor being coupled to more than one downstream signal transduction pathway, a situation now relatively common. The influx of Mg^{2+} in S49 lymphoma cells is not dependent on extracellular Na^+ nor, surprisingly, is it dependent on membrane potential (Grubbs & Maguire, unpublished observations). Moreover, as already noted, turnover of total cellular Mg^{2+} in S49 lymphoma cells and primary lymphocytes is extremely slow, taking more than 40 h in comparison to Ca^{2+} turnover which is complete in < 3 h. Such observations are entirely compatible with the unique chemistry of Mg^{2+} and the highly unusual nature of cloned Mg^{2+} transport systems from prokaryotes (see reviews by Kehres & Maguire and Maguire & Cowan in this issue).

Vormann and Günther (Günther *et al.* 1991), Jakob *et al.* (1989), and Romani and Scarpa (1990a, b) have reported that perfused heart and liver extrude a considerable amount of cellular Mg^{2+} into the perfusate, in a dose-dependent fashion, within a few minutes following the administration of α- or β-adrenergic agonists. In these studies extracellular Mg^{2+} was measured by atomic absorption spectrometry under conditions of low extracellular Mg^{2+}. Since only net mass change in the extracellular medium can be determined by this approach, it is generally unclear whether such changes reflect an increased efflux of Mg^{2+} or a cessation of influx with or without a change in efflux. While control experiments suggest in most cases that this hormonal regulation of flux is an increase of the rate of efflux, an effect of the various hormonal manipulations on influx cannot be excluded. Regardless, these groups have quite clearly demonstrated that massive movement of Mg^{2+} occurs in some tissues after hormonal stimulation, with as much as 15% of total intracellular Mg^{2+} being lost within 15–30 min in several different tissues. The observation of such massive Mg^{2+} movement is in distinct contrast to hormonally induced movement of other cations which rarely involve movement of such a high percentage of intracellular cation over short periods of time. Such movement of Mg^{2+} is not an artifact of tissue isolation of experimental manipulation since infusion of isoproterenol or other catecholamine to anesthetized rats results in a dose-dependent increase in circulating Mg^{2+} level of $\sim$ 15–20% from a basal level of $\approx$ 0.7 mM to more than 0.9 mM within 10 min of agonist infusion (Günther & Vormann 1992b; Keenan *et al.* 1995). Increases in serum Mg^{2+} can persist for more than 90 min and are prevented by propranolol or other selective blockers of β-adrenergic receptor in both cardiac and liver cells.

Mg^{2+} extrusion

The initial evidence of a β-adrenergic receptor induced Mg^{2+} extrusion from heart and liver cells has been largely confirmed in several other tissues or cell types (see Table I in Romani & Scarpa 2000 for a list), including erythrocytes (Matsuura *et al.* 1993), thymocytes (Günther & Vormann 1992a), primary lymphocytes (Wolf *et al.* 1997), Erhlich ascites cells (Wolf *et al.* 1996), and sublingual acini (Zhang & Melvin

1992), although one group (Altschuld *et al.* 1994) has been unable to observe it in perfused hearts or isolated myocytes under experimental conditions similar to those reported by several other groups (Howarth *et al.* 1994; Romani & Scarpa 1990a; Vormann & Günther 1987).

In the same cells or tissues, a Mg^{2+} extrusion comparable to that induced by isoproterenol or catecholamine can be elicited by the administration of the cell permeant cyclic-AMP analogs 8-Cl-cyclic AMP, 8-Br-cyclic AMP or dibutyryl-cyclic AMP or by forskolin (Fagan & Romani 2001; Günther & Vormann 1985, 1992a; Howarth *et al.* 1994; Matsuura *et al.* 1993; Romani & Scarpa 1990a, b; Romani *et al.* 2000; Wolf *et al.* 1997), while the administration of R_p-cyclic AMP isomer, a stable inhibitor of adenylyl cyclase, fully prevents Mg^{2+} extrusion (Wolf *et al.* 1997). In keeping with a role of cyclic AMP in mediating Mg^{2+} extrusion, pretreatment of perfused heart (Romani *et al.* 2000) or liver (Keenan *et al.* 1996) with insulin prevents the Mg^{2+} extrusion elicited by isoproterenol or cell permeant cyclic AMP analogs, while having no affect on extrusion elicited by the α_1-adrenergic agonist phenylephrine (Keenan *et al.* 1996). Taken together, these results suggest that cyclic AMP acts as second messenger to activate the Mg^{2+} extrusion pathway (Günther & Vormann 1992a). In line with this hypothesis, arachidonic acid, and either PGE_1 or PGE_2 which also increase cellular cyclic AMP, can induce Mg^{2+} extrusion from primary lymphocytes or Erhlich ascites cells (Wolf *et al.* 1996, 1997). Experiments with insulin (Keenan *et al.* 1996), and the report by Jakob *et al.* (1989) suggest, however, that Mg^{2+} extrusion can be also elicited though a signaling pathway activated by α_1-adrenergic receptors and not mediated by cyclic AMP. By investigating in detail the α_1-adrenergic receptor signaling pathway in perfused livers and isolated hepatocytes, Fagan & Romani (2001) have observed that the amount of Mg^{2+} extruded from the liver following catecholamine administration (3.4 μmol/8 min) correspond to the amounts mobilized by β- (1.1 μmol/8 min) and α_1-adrenergic agonist (2.2 μmol/8 min) together. A similar conclusion can be deduced from the data published by Keenan *et al.* (1996). Moreover, Fagan & Romani (2001) have observed that the α_1-adrenergic receptor-mediated Mg^{2+} extrusion requires the activation of intracellular Ca^{2+} signaling and an increase in cytosolic Ca^{2+} concentration. As already noted however, it is not clear in some experimental conditions whether the hormonal regulation is selective for influx vs. efflux pathways. If it is assumed that at least some of the observations in liver, heart and other tissues represent an actual inhibition of influx without a change in efflux, significant cellular loss of Mg^{2+} would be observed and the observations would be compatible with experiments in S49 lymphoma cells where β-receptor activation inhibits influx but not efflux. Nonetheless, the number of cell types in which hormonal influences on Mg^{2+} transport have been observed strongly suggests that both influx and efflux pathways can be affected depending on cell type and receptor involved.

In the majority of these experimental models, Mg^{2+} extrusion is a time-dependent process that reaches its maximum within 5–8 min after agonist administration, declining towards basal level thereafter, irrespective of the agonist used, its dose, and its persistence in the perfusion medium (Fagan & Romani 2000, 2001; Günther & Vormann 1992a, b; Keenan *et al.* 1996; Matsuura *et al.* 1993). Based upon the dose of agonist administered, the total amount of Mg^{2+} mobilized from cardiac or liver cells into the perfusate accounts for 500–800 μmol, a figure that is equivalent if not larger than the cytosolic *free* Mg^{2+} content within these cells (Romani & Scarpa 1992a, 2000). Moreover, an organ stimulated repeatedly by submaximal doses of agonist releases a diminishing amount of Mg^{2+} with each stimulation (Romani & Scarpa 1990a). Hence, these results can reasonably be interpreted as a progressing depletion of the intracellular store(s) from which Mg^{2+} is mobilized. In this respect, reports from this laboratory (Romani *et al.* 1991, 1993c) and from Zhang and Melvin (1992) suggest that a considerable amount of the extruded Mg^{2+} is mobilized from mitochondria, as inhibitors of mitochondrial respiration affect the ability of cardiac myocytes, liver cells or sublingual mucous acini to extrude Mg^{2+}. Whether stimulation of α_1- and β-adrenergic receptors mobilizes Mg^{2+} from a common pool is currently unknown. Based upon the estimate of Mg^{2+} extruded into the perfusate following selective α_1- or β- vs. mixed adrenergic receptor stimulation, it would appear that the two adrenergic signaling pathway mobilize Mg^{2+} from two distinct cellular pools (Fagan & Romani 2000).

Interestingly, under conditions where a large amount of Mg^{2+} is extruded from perfused hearts or isolated cardiac myocytes, cytosolic free Mg^{2+} changes relatively little (Fatholahi *et al.* 2000). This raises the question of whether Mg^{2+} extruded from an intracellular organelle is transported through the

cytosol to the plasma membrane bound to some specific cytosolic moiety (e.g., ATP or other phosphonucleotides), or whether a 'preferential' transport route across the cytosol exists, so that fluorescent indicators or ^{31}P-NMR technique cannot detect changes in cytosolic free Mg^{2+} (Fatholahi *et al.* 2000).

Consistent with its major role in regulating Mg^{2+} fluxes discussed above, the putative Na+/Mg^{2+} exchanger appears to be the predominant pathway involved in the Mg^{2+} extrusion mediated by cyclic AMP in liver and heart. Under conditions in which no extra-cellular Na^+ is available to support the operation of the exchanger (Romani *et al.* 1993b, c), or the transport of Na^+ across the cell membrane is inhibited by amiloride (Vormann & Günther 1987), Mg^{2+} extrusion does not occur, irrespective of the dose of agonist utilized and the time of agonist administration. Romani *et al.* (1993b, c) have observed that the amount of Mg^{2+} mobilized from cardiac or liver cells is proportional to the amount of Na+ or Ca^{2+} present in the extracellular compartment, suggesting that hormonal stimuli activate transport mechanisms similar to those observed to operate in liver plasma membranes (Cefaratti *et al.* 1998, 2000b) or sarcolemma vesicles (Cefaratti *et al.* 2000a). Moreover, Fagan and Romani (2000) have observed that in the absence of extracellular Ca^{2+}, isoproterenol activation of β-adrenergic receptors induces Mg^{2+} extrusion normally, being specifically affected by the removal of extracellular Na^+. When epinephrine or norepinephrine are used to stimulated Mg^{2+} efflux (i.e., when both α_1- and β-adrenergic receptors are activated), the amount of Mg^{2+} extruded in the absence of extracellular Ca^{2+} corresponds to the amount extruded through the Na^+-dependent pathway following isoproterenol administration. Conversely, when extracellular Na^+ is removed, the amount of Mg^{2+} extruded by catecholamine stimulation is reduced to a mere 10% of that occurring in the presence of a physiological concentration of Na^+ and Ca^{2+}. Lastly, phenylephrine-induced Mg^{2+} extrusion requires both extracellular Na^+ and Ca^{2+}, being almost completely abolished in the absence of either of these cations (Fagan & Romani 2000). These data, and the evidence that Ca^{2+}-signaling is involved in mediating the α_1-adrenergic receptor induced Mg^{2+} extrusion (Fagan & Romani 2001), would suggest that the Na^+/Mg^{2+} exchanger is the primary pathway involved in Mg^{2+} transport, and that it can be activated independently or simultaneously by cyclic AMP (via β-adrenergic receptors) or Ca^{2+}/calmodulin (via α_1-adrenergic receptors) (Fagan & Romani 2000; Keenan *et al.* 1996). The Ca^{2+}/Mg^{2+} exchange mechanism evident in apical membrane vesicles from hepatocytes (Cefaratti *et al.* 2000b) would contribute to Mg^{2+} extrusion only slightly under physiological conditions, since the apical membrane surface accounts for only $\approx 10\%$ of the total plasma membrane surface (Hubbard *et al.* 1994). However, this pathway might become predominant under conditions in which the cyclic AMP-signaling pathway is inhibited (Keenan *et al.* 1996).

Counter-regulation of Mg^{2+} extrusion and Mg^{2+} accumulation

In physiological terms, it is reasonable to envision that as there are hormones stimulating Mg^{2+} extrusion, so other hormones or agents must operate in eukaryotic organisms to promote Mg^{2+} accumulation and the maintenance of Mg^{2+} homeostasis. Consistent with a role of cyclic AMP in mediating Mg^{2+} extrusion in some cell types, hormones or agents that decrease cyclic AMP production within cells can prevent Mg^{2+} extrusion, and in some cases induce Mg^{2+} accumulation. Agents acting at muscarinic cholinergic receptors like carbachol, phorbol derivatives or diacyl-glycerol analogs, or hormones like vasopressin or insulin have been reported to induce Mg^{2+} accumulation in several cell types or tissues, including cardiac myocytes (Romani *et al.* 1992, 1993c, 2000), hepatocytes (Romani *et al.* 1992, 1993b), S49 lymphoma cells (Grubbs & Maguire 1986), lymphocytes (Rijkers & Griffioen 1993), epithelial cells of the thick ascending limb of the Henle's loop (Dai *et al.* 1999; Dai & Quamme 1991), or platelets (Takaya *et al.* 1998). A more complete listing can be found in reference (Romani & Scarpa 2000). The common denominator to these agents or hormones is that they decrease cyclic AMP level (carbachol or insulin), or activate a protein kinase C pathway (phorbol derivatives, diacylglycerol analogs, vasopressin, or insulin) which, in turn, decreases cyclic AMP in many tissues.

Hence, we can envision a scenario in which an increase in cellular cyclic AMP or Ca^{2+} level results in an extrusion of Mg^{2+} from the cell into the extracellular space, whereas a decrease in cyclic AMP content or the activation of protein kinase C signaling pathway results in an accumulation of Mg^{2+} within the same tissue. The switch between Mg^{2+} accumulation and release is very rapid, as cells accumulating Mg^{2+} under influence of a phorbol ester revert to an extrusion as soon as a stimulatory agent is added, and *vice*

versa (Romani *et al.* 1993b). The amount of Mg^{2+} extruded from the cell is usually similar to the amount accumulated by the cell, suggesting that there exists a dynamic pool into which Mg^{2+} can be accumulated, or from which Mg^{2+} can be rapidly extruded by proper stimuli.

It remains unclear whether Mg^{2+} enters the cells through the reverse operation of the Na^+/Mg^{2+} exchanger, as potentially suggested by experiments in liver plasma membrane vesicles (Cefaratti *et al.* 1998, 2000b), or a distinct transport mechanism, as suggested by Quamme and coworkers (Quamme & Rabkin 1990). The latter group has observed that the incubation of cardiac and renal cells in a medium devoid of Mg^{2+} results in the marked decrease of cytosolic *free* Mg^{2+} from $\approx$0.5 to $\approx$0.3 mM. The transfer of these cells into a medium containing a physiological concentration of extracellular Mg^{2+} results in a rapid restoration of cytosolic *free* Mg^{2+} level to 0.5 mM (Quamme & Dai 1990; Quamme & Rabkin 1990). The rate of Mg^{2+} entry is accelerated by several hormones (Dai *et al.* 1997), including insulin (Dai *et al.* 1999), and is inhibited by the Ca^{2+}-channel inhibitor verapamil (Quamme & Rabkin 1990) or by the removal of extracellular K^+ (Dai *et al.* 1997). Based on these results, Quamme and co-workers have proposed that Mg^{2+} enters the cell through a channel (Quamme & Dai 1990; Quamme & Rabkin 1990). Whether specific Mg^{2+} channels exist is an open question in mammalian cells although a Mg^{2+} channel has been reported in *Paramecium* (Preston 1990, 1998) and both the *Arabidopsis* AtMHX Mg^{2+} exchanger (Shaul *et al.* 1999) and the *S. typhimurium* CorA Mg^{2+} influx system (Smith & Maguire 1998) have some characteristics of channels. Lifton and colleagues have reported that mutations in a claudin protein, named Paracellin-1 and localized to the thick ascending limb of the kidney tubule, result in Mg^{2+} wasting (Simon *et al.* 1999). They have suggested that this protein may be a tight junction channel for Mg^{2+}.

Effects of intracellular and/or extracellular Ca^{2+} on Mg^{2+} influx have been observed by Romani *et al.* (1993b). They demonstrated that nifedipine can inhibit Mg^{2+} transport in liver cells and that vasopressin-induced Mg^{2+} accumulation in hepatocytes is prevented by pre-treatment with thapsigargin. These results suggest that blockade of Ca^{2+} channels might indirectly influence Mg^{2+} transport (Romani *et al.* 1993b). The dependence of Mg^{2+} accumulation on extracellular K^+ (Dai *et al.* 1997) or Na^+ (Romani *et al.* 1993b) concentration in some but not all tissues and cells would suggest that changes in membrane potential can play a key role in regulating the operation of the Mg^{2+} entry mechanism for some systems. Again, the available data indicate that multiple Mg^{2+} transport mechanisms exist and numerous issues are outstanding. For example, it is unclear why the stimulation of α_1-adrenergic receptors elicits Mg^{2+} extrusion from liver cells while stimulation of vasopressin receptors in the same cells elicits a Mg^{2+} accumulation since both receptors induce phospholipase C activation and generation of inositol phosphates and diacylglycerol.

Physiological significance

The lack of adequate technical approaches has precluded many experiments with Mg^{2+} that have been feasible with other cations, and the identity and properties of individual Mg^{2+} transporters at the molecular levels lags far behind that of other cations. Nonetheless, from the initial viewpoint of Mg^{2+} as a stable intracellular cation, unchanging in its concentration, we have come a long way to the notion that significant amounts of Mg^{2+} are transported across cell membranes following metabolic and hormonal stimuli. Moreover, unlike other cations, hormonal modulation of intracellular Mg^{2+} in most cells examined moves a very large mass of Mg^{2+} in a short period of time. Why do so many cells respond to hormonal stimuli by transporting such a large amount of Mg^{2+} across the plasma membrane? What is the physiological significance of this massive Mg^{2+} flux in eukaryotic cells and the acute change in circulating Mg^{2+} content?

More than a decade ago one of us (Grubbs & Maguire 1987; Maguire 1990) proposed that Mg^{2+} acted as a *long-term or chronic regulator* for cellular enzymes and functions, in opposition to a short-term, acute regulation by agents such as Ca^{2+}, based on the hormonal modulation of Mg^{2+} fluxes and in many cells types the quite sustained nature of the effect of the hormone. In many cell types clear evidence for changes in *free* Mg^{2+} concentration within the cytosol or in the lumen of selective cellular organelles following hormonal or metabolic stimuli has not been obtained or even determined. Yet, the hypothesis that Mg^{2+} is regulatory cation is strongly supported by the growing list of hormones or agents that can modulated cellular Mg^{2+} content and transport across the plasma membrane, and by the very large number of enzymes whose activity is modulated by changes

in Mg^{2+} concentration (see ref. Romani & Scarpa 1992b for a partial list). In many cases, such Mg^{2+} regulation occurs over very small ranges of Mg^{2+} concentration. If we consider that Mg^{2+} affects the operation of key enzymes like adenylyl cyclase (Bird & Maguire 1978; Cech *et al.* 1980; Maguire 1984), glycogen phosphorylase (Gaussin *et al.* 1997), or α-ketoglutarate dehydrogenase (Panov & Scarpa 1996), that it modulates the amount of catecholamine being dismissed into the circulation (Stanbury 1948), coronary vasodilation (DiPette *et al.* 1987; Teragawa *et al.* 2001), and responsiveness of β_2-adrenergic receptors in the respiratory airways (Rolla *et al.* 1994; Rolla & Bucca 1991), we can then picture the hormone-induced changes in serum and cellular Mg^{2+} content as a signal to regulate energy production and its delivery and utilization in tissues under specific metabolic conditions. In short, the sum of these observations strongly support the hypothesis that Mg^{2+} can indeed act as a regulatory cation at the systemic level and within the cell.

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