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INTRACELLULAR MAGNESIUM: TRANSPORT AND REGULATION IN EPITHELIAL SECRETORY CELLS

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1. ABSTRACT

The mechanisms of magnesium (Mg^{2+}) transport, the regulation of intracellular Mg^{2+} concentrations and the relationship between Mg^{2+} and Ca^{2+} signaling during the stimulus-secretion coupling process in pancreatic acinar cells and other secretory epithelia are reviewed in this article. Our results demonstrate the existence of a Na^{+}- and ATP-dependent transport system for Mg^{2+} extrusion from Mg^{2+}-loaded cells. Moreover, employing such different techniques as spectrofluorimetry and atomic absorbance spectroscopy to measure intracellular free magnesium concentration $[Mg^{2+}]_i$ from magfura-2-loaded acini and acinar cells and Mg^{2+} content in effluent samples from perfused pancreatic segments, respectively, confirm that secretagogues such as acetylcholine (ACh) and cholecystokinin-octapeptide (CCK-8) can evoke marked and significant extrusion of Mg^{2+} which is closely associated with the mobilization of intracellular calcium. These effects may be modulated by different mediators including cAMP, Protein Kinase C and nitric oxide/cGMP. This reduction in $[Mg^{2+}]_i$ seems to be a prerequisite for optimal generation and maintenance of the calcium signal and subsequently, the secretion of enzymes, since an increase in extracellular Mg^{2+} concentration, $[Mg^{2+}]_o$ and an increase in $[Mg^{2+}]_i$ inhibit secretagogue-induced secretory responses, an effect exerted through a derangement of the calcium signaling events. In conclusion, the evidence presented in this review strongly supports an important modulatory role of magnesium in the control of secretory epithelial cells function.

2. INTRODUCTION

The importance of extracellular and intracellular magnesium has become gradually recognized during the last century. At the present moment, pathologies as common as diabetes, hypertension and dyslipidaemia are associated with an altered metabolism of magnesium (1-3), and this divalent cation is even being considered as a potential tool in the prevention or co-adjuvant treatment of ventricular arrhythmias, coronary heart disease and cirrhosis of the liver (4-6), among others. Of the total magnesium body content, approximately 52% is found in bone, 46% in skeletal muscle and 2% in extracellular fluids (7). Serum magnesium levels, which amount to less than 1% of total magnesium, are controlled by the gastrointestinal tract and the kidneys. About 30-50% of dietary magnesium is absorbed in jejunum and ileum (8), although this is highly variable since absorption is inversely related to the intake (9). The composition of the diet affects intestinal magnesium absorption (10), and controversy exists whether or not vitamin D favors this process (10-12). On the other hand, the kidneys control serum magnesium through changes in the rate of its excretion. Reabsorption of magnesium occurs mainly in the loop of Henle and this is also where a number of major hormones act to modulate the magnesium excretion rate in final urine. The hormones are, calcitonin, glucagon, parathyroid hormone and antidiuretic hormone, all of which seem to favor magnesium reabsorption (13,14).

Magnesium is essentially an intracellular ion: approximately 98% of non-skeletal magnesium is in the

intracellular compartment. In fact, magnesium is the second most abundant intracellular divalent cation, exceeded only by potassium (8). Within cells, magnesium plays a vital role in unnumerable regulatory processes. It is involved in the synthesis of DNA and RNA as well as maintenance of their conformation (15). Possibly in relation to its capacity to form complexes with phospholipids, magnesium has demonstrated to affect membrane fluidity and permeability (16,17). Intracellular magnesium has been directly associated with processes as important as the secretion of hormones, including insulin (18) and prolactin (19). It must be said that many of the actions of magnesium are due to its recognized role as a cofactor of a wide range of enzymes. Thus, it is well known that magnesium activates virtually all the enzymes involved in the metabolism of phosphorylated compounds, as well as many enzymes in the glycolytic and tricarboxylic acid pathways (20). Mg^{2+} is necessary for optimal performance of the Na^+/K^+ -ATPase and calcium (Ca^{2+}) pumps (for review see 21). In addition, it has been shown to affect different transport systems in many tissues, such as the $Na^+/K^+/Cl^-$ and K^+/Cl^- co-transport, the Na^+/H^+ and Cl^-/ H^-CO_3 exchange (22), and several ion channels (23). We will show how the secretory activity of the exocrine pancreas and other secretory epithelia is indeed affected by intracellular magnesium levels. Thus, there is little doubt about the importance of this cation in cellular functions.

3. TECHNIQUES FOR MEASURING INTRACELLULAR MAGNESIUM CONCENTRATION

For an understanding of intracellular magnesium homeostasis and its regulation, accurate measurements of total and free intracellular magnesium are necessary. Total (free plus bound) intracellular magnesium levels can be determined by atomic absorption spectrophotometry (AAS), inductively-coupled plasma atomic emission spectrophotometry, thin layer chromatography, and ion chromatography. All these methods require subjecting the sample to several destructive steps such as dry ashing, wet mineralization or acid extraction before measuring the concentration of the ion. As discussed in the review by Seiler (24), this implies that there is a source of sample contamination and the invasive nature may lead to errors in the examination of magnesium transport due to non-specific binding to cell membranes and components, as well as additional ion transport during sample preparation. On the other hand, several methods can be used to determine $[Mg^{2+}]_i$, including fluorescent dyes, magnesium-sensitive electrodes, nuclear magnetic resonance spectroscopy (NMRs) using the ^{31}P, ^{19}F and ^{25}Mg nuclei, metallochromic dyes, radiotracer methods, null-point for plasma permeabilization, and coupled assays using Mg^{2+}-dependent enzymes (citrate/isocitrate, glucokinase, etc). These analyses can be carried out in intact cells. The reference method for the measurement of total magnesium is AAS. It can be said, in fact, that until the advent of the commercially available atomic absorption spectrophotometer in the mid 1960s, the determination of total magnesium was difficult and not reproducible. We are going to focus in this section on the techniques used to measure $[Mg^{2+}]_i$, particularly in relation to practical aspects and applicability to biological materials. For more theoretical aspects, the reader can be referred to recent reviews.

3.1. Fluorescent probes

Along with Mg^{2+}-sensitive electrodes and NMR spectroscopy, fluorescent probes-based methods are the most widely used today to study $[Mg^{2+}]_i$ and its regulation. Fluorescence is a molecular process in which the absorption of radiation from an external source results in emission of electromagnetic radiation of longer wavelength (lower energy) (25). It has been shown to be a highly sensitive technique for detecting concentrations as low as 10^{-8} M in contrast to 10^{-4} M for NMR spectroscopy (26). Very few biomolecules contain fluorophores (molecular groups that fluoresce), thus synthetic fluorescent indicators are required for most biological applications. In order for a fluorescent probe to provide useful information about its environment, it is necessary that its fluorescent properties be altered in a suitable manner by the parameter to be measured. According to this, Tsien (27) synthesized in the early 1980s several compounds that underwent spectral changes upon Ca^{2+} binding. The initial Ca^{2+} probe, Fura-2, was later modified (28,29) to improve selectivity for Mg^{2+}, resulting in the Mg^{2+}-sensitive fluorescent probe Magfura-2 (Furaptra). The fluorescence- excitation maximum of Magfura (370 nm) is shifted to a lower wavelength (335 nm) upon Mg^{2+} binding without negligible shift in the emission maximum (510 nm)(29). Because of the shift in the excitation maximum, measurements can be carried out at the two different wavelengths to obtain signals which are proportional to magnesium-bound and magnesium-free indicator. The ratio of the fluorescence at the two wavelengths is directly related to the ratio of the two forms of the dye and, therefore, can be used to calculate $[Mg^{2+}]_i$ according to an equation (29). This equation includes the K_D value for magnesium as well as the fluorescence ratio values under saturating and Mg^{2+} free conditions, which are obtained in the calibration procedure. An important advantage of this ratio approach is that the $[Mg^{2+}]_i$ measurements are independent of the probe concentration, path length (to be considered in single cell analysis), and instrument-sensitivity parameters. Artifacts that are also eliminated include photo-bleaching, variable cell thickness and non-uniform indicator distribution within cells or among populations of cells (30). The method enables correction for cell autofluorescence by substracting the measurements observed with unloaded cells.

In order to measure $[Mg^{2+}]_i$ with Magfura-2, the dye has to be loaded into the cells. The most common procedure is based on the use of the acetoxymethyl ester (AM) form of the probe, which is membrane permeable. Once inside the cytosol, the ester is hydrolyzed by intracellular esterases releasing the anionic, Mg^{2+}-sensitive, indicator, which cannot leave the cell. After loading, excess extracellular probe is washed away (31).

Some problems may be encountered when using AM loading together with fluorescent probes (32,33). All of them can be assessed and usually prevented by the following means: i) Compartmentalization: The AM esters may accumulate in any membrane-enclosed structure within the cells. The extent of this phenomenon can be assessed by using membrane permeabilizing reagents such as digitonin and Triton-X 100, and can be reduced by lowering the temperature at which loading is performed. ii) Incomplete AM ester hydrolysis: Un-hydrolyzed esters may be present both extracellularly (incomplete removal) or intracellularly (low esterase activity, which depends on the cell type). Fluorescence quenching by Mn^{2+}, which only binds to the de-esterified probe, can be employed to quantify these effects. These two problems can be avoided if the anionic form of the probe is directly micro-injected, but this procedure is difficult and only suitable for single cell studies. iii) Leakage: Extrusion of the anionic indicators from cells may occur, and, in some cases, this is carried out by ion transporters (34). Reducing the temperature of the experiment can help to prevent it. In addition, the use of ion transporter inhibitors occasionally has been shown to be useful (34).

We have used Magfura-2 AM in order to study the changes in $[Mg^{2+}]_i$ in the exocrine pancreas. Although it has been widely employed in many different cell types such as pancreatic beta cells (35), heart cells (28,36), platelets (37), and sublingual acinar cells (38,39), no studies have included a protocol for pancreatic tissue. A suitable method for loading pancreatic acinar cells was developed in our laboratory. The protocol adopted, which is described in detail elsewhere (40), enables sufficient dye to be loaded into cells to give fluorescence readings. Loading times are short and no detectable compartmentalization is evident. From our studies using dual wavelength fluorimetry with Magfura-2, it would appear that resting $[Mg^{2+}]_i$ in both rat and mouse pancreatic acinar cells is about 0.8 mM (41-46), which is very similar to the value found by other authors with the same technique in pancreatic beta cells (35). Accordingly, using this approach, $[Mg^{2+}]_i$ between 0.9 and 1 mM have been reported in heart tissue as measured with Mg^{2+}-selective microelectrodes (47-49) as well as in skeletal muscle as determined by ^{31}P NMRs (50). We observed a lower $[Mg^{2+}]_i$ (around 0.5 mM) in Magfura-loaded rat parietal cells (51), and studies with this probe give basal $[Mg^{2+}]_i$ values of approximately 0.25 mM for human platelets (37), 0.30-0.35 mM for human lymphocytes (52) and sublingual acinar cells (38,39), and 0.5 mM for renal epithelial cells and isolated hepatocytes (29,53).

The affinity of Magfura-2 for Ca^{2+} is not very different from that for Mg^{2+} (29) and several authors have indicated the existence of a clear interference from this source (54,55). However, given the very low intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$) in most cells, caution should be taken only when manipulation involves marked changes in $[Ca^{2+}]_i$, as it will be demonstrated in a later section of this chapter.

Some of the advantages of Magfura-2 in the analysis of $[Mg^{2+}]_i$ are: i) Ease of use: No special skills or instrumentation are required. ii) Non-disruptive loading procedure that can be used with small or fragile cells which are not amenable to micro-injection. iii) Applicability to either cell suspension (40, 41) or single cell studies (46, 51). In addition, different approaches can be undertaken, including perfusion of the tissue (28, 46) or the employment of static-volume cuvettes (42, 43). iv). Very good temporal resolution also makes it useful for monitoring rapid changes in $[Mg^{2+}]_i$. v). The low Magfura-2 concentrations (as low as 2 μM) needed to obtain fluorescence signals of sufficient intensity rules out toxicity problems. vi) Changes in extracellular pH (5.5 to 7.4), temperature (22-37 degrees C) and either NaCl or KCl concentrations do not affect the emission spectrum of the probe (29, 56-58).

3.2. Magnesium-selective microelectrodes

Magnesium-selective microelectrodes constitute a suitable mean for the assessment of $[Mg^{2+}]_i$. A big advantage is that they provide a direct measurement, as opposed to the indirect ones based on ^{31}P or ^{19}F NMR spectroscopy as well as fluorescent or optical probes. The main disadvantages arise from (c) the need for large, non-fragile cells, given the invasive nature of the procedure (59-61), and (b) depending on the cell type and from the response times (30 seconds), but can make this method useless in dealing with preparations such as beating hearts or contracting muscles (62). In addition, it is very important to consider the selectivity of Mg^{2+}-sensitive microelectrodes for Mg^{2+} in relation to other ions. Such selecting depends on the carrier chosen. Several neutral carriers have been introduced for Mg^{2+}-sensitive microelectrodes. ETH 1117 has been shown to be more sensitive to Ca^{2+} than to Mg^{2+} (at mM Ca^{2+} concentrations). Moreover, the selectivity of ETH 1117 for Mg^{2+} over K^+ and over Na^+ is only 24- and 12-fold greater, respectivey (63). Considering the intracellular environment, the interference from Ca^{2+} can be tolerated, but this is not the case with K^+, whose intracellular concentration is more than 100 times greater than that of intracellular Mg^{2+}. Another carrier, ETH 5214 incorporates marked improvements in its selectivity over Na^+ and K^+. Selectivity against Ca^{2+} is still limited, although McGuigan et al. (61) demonstrate that changes in cytosolic Ca^{2+} which can occur physiologically do not affect the response of the microelectrode. This ionophore, however, has been shown to be quite sensitive to some of the Na^+ substitutes more commonly used in studies on Na^+-dependency on Mg^{2+} transport systems, such as TMA (tetramethylammonium chloride) and choline (64). Finally, ETH 7025 has been one of the last sensors to be introduced. Moreover, it presents certain problems in relation to its use for plasma or serum samples (62, 65-68), but evidence (64, 69) indicates that it is a promising tool to improve our knowledge on intracellular magnesium homeostasis provided that the use of any type of silicone-containing material is avoided, since significant heavy interference has been reported from this source (68).

3.3. NMR spectroscopy

All atoms possess positively charged nuclei which are surrounded by negatively charged electrons. Each nucleus rotates around its own axis and thus has a property called nuclear spin *I*. Only V_a nucleus with *I* different from zero can be used in NMRs. Because any moving charged particle has a magnetic field associated with it, a nucleus can be considered as a small magnet. In the absence of an external magnetic field, all orientations of the individual spins are possible, resulting in zero net magnetization. However, in the presence of an external magnetic field, only certain orientations in relation to the applied field (B_o) are permitted. The total number of spin orientations and, thus, the total number of nuclear energy levels is given by $2I + 1$ (for example, this value is 2 for ^{31}P, such that only one transition between these two energy states can occur). Transitions between nuclear energy states are induced by application of short pulses of radiofrequency, V_o, according to the equation V_o = Gamma * B_o / 2 Pi. Gamma is different for all nuclei: different nuclei in an applied field B_o resonate at different frequencies. In addition, the same nucleus in different chemical environments within the same molecule (or in different molecules) generally resonates at different frequencies, a process referred to as a chemical shift. The sensitivity (signal-to-noise ratio) of an NMR experiment depends on the particular nucleus, the volume and concentration of the sample (high density suspensions are usually required) and the magnetic field strength of the spectrometer. In order to obtain good results, the total accumulation time may amount to several days, which obviously excludes analysis of nuclei at low concentrations involved in very fast dynamic processes (70). Measurements of $[Mg^{2+}]_i$ can be performed by NMR spectroscopy using the ^{25}Mg, ^{31}P or ^{19}F nuclei (71,72).

3.3.1. ^{25}Mg NMRs

The use of this method is fundamentally limited by the low abundance of the isotope (approximately 10%). Thus, isotopic enrichment must be done frequently (73), which, apart from increasing the cost, leads to $[Mg^{2+}]_i$ much higher than those physiologically relevant. An additional problem is the existence of broad RMN lines or partial signal visibility (72).

Unlike the ^{25}Mg nucleus, the ^{31}P and ^{19}F nuclei (see below) give fully visible spectra and provide an indirect method for $[Mg^{2+}]_i$ analysis with relatively high sensitivity. ATP, phosphocreatine, diphosphoglycerate and ADP are endogenous indicators for ^{31}P (74), whereas NOTPME is an exogenous one (75).

3.3.2. ^{31}P NMRs

ATP is the most common indicator for ^{31}P NMRs which is currently the only one to provide totally non-invasive *in vivo* measurement of $[Mg^{2+}]_i$ in tissues and organs. Cellular ATP is present in the form of various magnesium complexes. The resonance frequency (or chemical shift) of ATP alpha, beta and gamma phosphates varies depending of the degree of magnesium complexation to ATP. Thus, $[Mg^{2+}]_i$ is estimated on the basis of the distance separating the phosphorus peaks of ATP (72). Complexation with calcium is usually ignored due to much lower levels of this cation in relation to magnesium. There are some very important points to take into account using this method: i) Modern peak identification methods are accurate when the magnetic field is homogeneous and the sensitivity good, but these do not seem to be frequent in *in vivo* NMRs experiments (76). ii) The chemical shifts of the ^{31}P NMR resonances of ATP are also dependent upon pH, temperature and ionic strength (72). iii) Accurate assessment of the final $[Mg^{2+}]_i$ depends on the choice of a K_D for Mg-ATP, which has been the object of great controversy until recently (77-79). iv). It is worth noting that, since changes in the intracellular ATP concentrations and pHi affect the complexation of magnesium, it may be difficult to use this technology for $[Mg^{2+}]_i$ determination in certain pathological situations associated with drastic alterations in both parameters (ischemia, etc)(64). v) Similarly, in tissues such as skeletal muscle, where ATP is highly bound to magnesium, the distance between peaks is minimal, and thus small changes in $[Mg^{2+}]_i$ are not detectable (74).

3.3.3. ^{19}F NMRs

$[Mg^{2+}]_i$ can be determined by use of exogenous ^{19}F NMR indicators (74). These are structural analogs to EDTA and thus, in a way similar to fluorescent probes, this method is based on the affinity of the indicators for magnesium ions. The most frequently used indicators are 4F-APTRA, 5F-APTRA and the 4-methyl-5-fluoroderivative of APTRA (80). These indicators are very sensitive to changes in $[Mg^{2+}]_i$ but interference with Ca^{2+} may still exist, at least in those situations in which large increases of $[Ca^{2+}]_i$ are expected (80).

3.4. Metallochromic dyes

These are reagents that change their colour and, consequently, their optical spectrum, in the presence of different concentrations of free metal ions. Several dyes have been employed to determine $[Mg^{2+}]_i$. These include: eriochrome blue SE, arsenazo III and anti-pyrylazo III (81,82). The method requires loading the dye into the cytosol via injection and is restricted to large cells such as muscle fibers and squid axons. Baylor *et al*. (82) obtained very different estimates of $[Mg^{2+}]_i$ for the same tissue (varying by a factor of 30), depending on the particular dye and the pH. Interference with intracellular Ca^{2+} has been demonstrated, although it can be corrected by multi-wavelength spectrophotometry (81,83). Unless the spectral properties of the dye in a given intracellular medium are known, caution must be taken, because these properties are modified by binding of the dyes to cellular components (for review of the use of these dyes see 81,83,84).

3.5. Radiotracer methods

Magnesium has two radioisotopes, ^{27}Mg and ^{28}Mg, which have previously been employed (particularly ^{28}Mg) to investigate the mechanisms of cellular magnesium transport using scintillation analysis

method (85,86). Unfortunately, these isotopes are very difficult to manipulate, resulting in a limitation of their use. The reasons are two-fold: i) Very short half-lives: 9.46 minutes and 20.9 hours for ^{27}Mg and ^{28}Mg, respectively (87). ii) They are not commercially available. ^{27}Mg is usually obtained by thermal neutron irradiation of ^{26}Mg from magnesium of natural isotopic composition or magnesium enriched in ^{26}Mg (which involves considerable expense). ^{28}Mg can be obtained from reactor-neutron irradiated Li/Mg alloy (88). The determination of both isotopes can be performed by gamma-spectrometry amd liquid scintillation counting (87).

3.6. Null-point method for plasma membrane permeability

This method is based on the observation that the plasma membrane permeability for magnesium increases when the cells are incubated in a Ca^{2+}-free medium in the presence of the ionophore A23187, which mediates the electro-neutral exchange of 1 Mg^{2+} for 2 H^+. Using incubation media with varying concentrations of Mg^{2+}, $[Mg^{2+}]_i$ can be calculated from the pH gradient and from the extracellular Mg^{2+} concentration ($[Mg^{2+}]_o$) according to the following equation:

$$[Mg^{2+}]_i = [Mg^{2+}]_o * [H+]_i^2 / [H+]_o^2$$

Since A23187 permeates both plasma and organelle membranes (83), some authors (89) have employed digitonin, which is selective for the plasma membrane. The technique is mainly limited by its requirement for high-density cell suspensions. Details of the method have been published elsewhere (90).

4. MAGNESIUM TRANSPORT

Prior to our specific discussion about Mg^{2+} transport, it is important to understand the intracellular distribution of magnesium. Improvement of the methods to determine both total and free magnesium concentrations indicates that only about 5-10% of intracellular magnesium is free, the remainder binds to highly charged anionic ligands such as ATP, ADP, RNA, polyphosphates, proteins and citrate (85,91-93). Studies with the radioactive isotope ^{28}Mg show that, depending on the cell type, free and bound magnesium are completely or partially exchangeable (85). The first approach to the morphological distribution of magnesium within the cell was done by homogenizing the tissue in isotonic medium and using differential centrifugation to separate the organelles and particles that are liberated. Using this method, investigations carried on in rat liver cells showed that the largest fraction of magnesium occurs within the microsomal and mitochondrial fractions of the cell (94-96). In cardiac cells, it was estimated that about 12% of magnesium is found in mitochondria (97). In pancreatic acinar cells, magnesium was believed to be distributed around rough microsomes (98) and also stored in zymogen granules. This latter suggestion was supported by the fact that magnesium is released with enzymes in both saliva (99) and pancreatic juice (100) upon stimulation with secretagogues. This has been confirmed more recently (101) and, moreover, studies performed in our laboratory, using digital imaging of Magfura-2-loaded single mouse pancreatic acinar cells, show that at rest, the intensity of the fluorescence is non-uniform. Intracellular magnesium is more localized towards the secretory pole, an area which, according to parallel differential interference contrast (DIC) microscopy experiments, contains the largest number of zymogen granules (102). This is also in close agreement with the earlier finding by Harper *et al.* (103) that the pancreatic zymogen membrane contains strong Mg^{2+}-requiring activity towards the hydrolysis of all nucleosides and di-triphosphates. Other authors have found a homogeneous distribution of magnesium in cultured mammalian cell lines including LLC-PK1 porcine kidney epithelial cells, as analyzed by laser scanning confocal microscopy and ion microscopy (104). Nevertheless, there is some conflicting evidence on the distribution of magnesium in different types of epithelial cells. This is logical if we consider the high degree of specialization that pancreatic acinar cells have with respect to their secretory function.

Returning to Mg^{2+} transport, it must be noted that the exocrine rat pancreas has been used extensively to study the molecular mechanisms of ion transport. Several transport systems for Na^+ (105), K^+ (106) and Ca^{2+} (107) have been well characterized. However, Mg^{2+} transport has remained unexplored for a long time because of the lack of suitable methods for the accurate measurement of this divalent cation. This section focuses on the results obtained in our laboratory, particularly in relation to the exocrine pancreas. The main findings are summarized in Figure 1 in this review. The most important evidence in other tissues are also included for comparison.

In investigating Mg^{2+} transport, there does not appear to be a completely adequate method for the measurement of net Mg^{2+} fluxes. The development of new methods for the continuous monitoring of cytosolic free magnesium concentrations has encouraged many authors to examine resting or stimulated Mg^{2+} fluxes with the aid of Mg^{2+}-sensitive electrodes (47,48,61,64,108) or Mg^{2+}-sensitive fluorescent dyes (28,36-39). However, although accepted for this purpose, caution has to be taken in the interpretation of the results, since $[Mg^{2+}]_i$ may be altered by interaction with other ions for the same binding sites and by changing the distribution of magnesium among organelles without affecting Mg^{2+} fluxes across the plasma membrane. On the other hand, as reported by Vormann and Günther (86), studies using $^{28}Mg^{2+}$ confirm that, in most instances, incubation of cells with $^{28}Mg^{2+}$ under physiological conditions results in the uptake of the isotope without change in total magnesium content, this representing a $^{28}Mg^{2+}$-$^{24}Mg^{2+}$ exchange, Thus, determination of $^{28}Mg^{2+}$ fluxes can be an indication of net Mg^{2+} transport only in certain situations such as $^{28}Mg^{2+}$ efflux from $^{28}Mg^{2+}$-loaded cells into Mg^{2+}-free medium.

These problems may be circumvented by artificially modifying Mg^{2+} concentrations. Thus, in certain cell types (109), it has been possible to detect

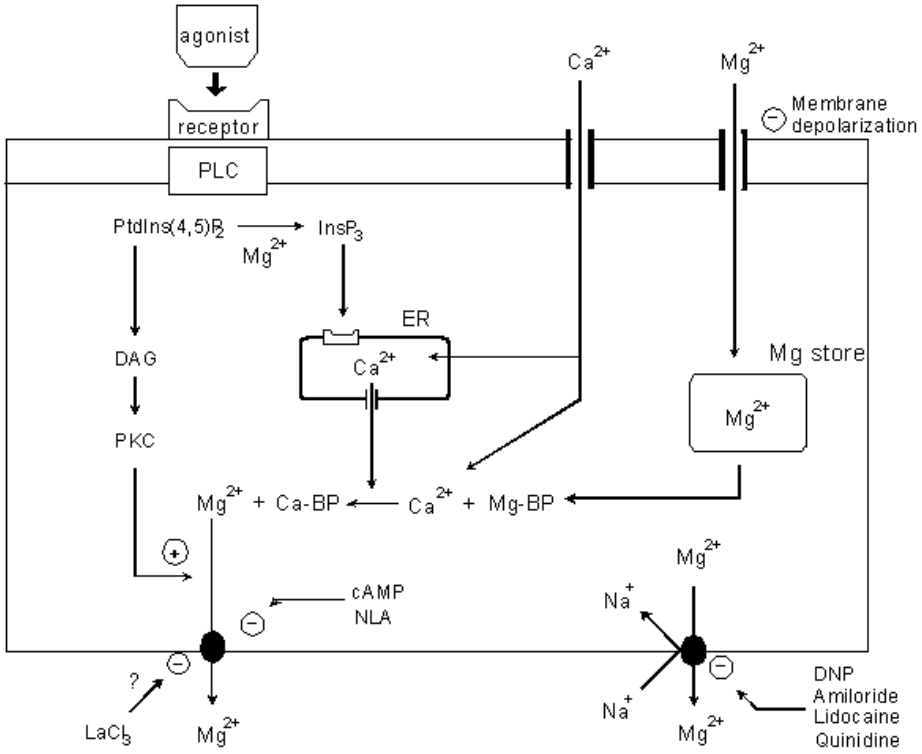

Figure 1: Schematic model of the events in the regulation of magnesium homeostasis in rat secretory epithelial cells. It is proposed that the influx of Mg^{2+} is associated with changes in the membrane potential since it can be inhibited by either monensin, ouabain or perfusion with K^+-rich solution. Once in the cell, Mg^{2+} is taken into (possibly by the mitochondria) stores and subsequently released, where it combines with cytoplasmic proteins. In relation to Mg^{2+} efflux, Ca^{2+} released from the stores (eg. endoplasmic reticulum) displaces magnesium from the binding proteins (BP). Agonists such as ACh and CCK-8 can now evoke Mg^{2+} efflux, which is sensitive to nitro-L-arginine (NLA), cAMP and perhaps $LaCl_3$. On the other hand, protein kinase C can stimulate Mg^{2+} efflux. Passive Mg^{2+} efflux is Na^+-dependent and it is sensitive to dinitrophenol (DNP), amiloride, quinidine and lidocaine.

Mg^{2+} efflux simply by incubating the cells in a Mg^{2+}-free medium and then measuring the magnesium concentration in the supernatant (magnesium released) or the total magnesium content in the cells at different times by an established method, for example, AAS. However, in many other instances (particularly in non-stimulated cells), net Mg^{2+} fluxes are, at physiological $[Mg^{2+}]_i$, too small to be measured and the only way to overcome this difficulty is to increase or decrease $[Mg^{2+}]_i$ and therefore measure its return to normal, since the transported amount of Mg^{2+} is, under these conditions, high enough to allow a precise measurement of net Mg^{2+} fluxes.

4.1. Magnesium transport in non-stimulated cells

The intra- and extracellular concentration gradient of Mg^{2+} in most cell types is small, as is the diffusion force for Mg^{2+} entry into cells. By contrast, a resting cell membrane potential of -70 mV together with an equilibrium potential for Mg^{2+} around 3 mV constitute a large inward driving force for magnesium ions. If these were allowed to distribute themselves at equilibrium with this membrane voltage, $[Mg^{2+}]_i$ would be approximately 214 mM. The fact that studies performed during the last years give a value lower than 1 mM for this parameter, well below the one predicted by the Nernst equation, is strongly suggestive of at least one active Mg^{2+} transport system which would control $[Mg^{2+}]_i$ by extruding Mg^{2+} out of the cell. In order to study Mg^{2+} efflux properties in resting pancreatic acinar cells, these have to be loaded with magnesium. For this purpose, we used the method described by Günther *et al.* (110,111) by incubating the cells in Krebs-Ringer-Hepes (KRH) solution at increased $[Mg^{2+}]_o$ (12 mM) in the presence of the divalent cation ionophore A23187. After 30 minutes at 37°C in a shaking water bath, a 2-fold increase in $[Mg^{2+}]_i$ became apparent as measured by Magfura-2 AM. Prior to the examination of net Mg^{2+} efflux from magnesium-loaded cells, a set of experiments was performed to ascertain the effect of loading upon intracellular magnesium redistribution due to the presence of the ionophore. When either magnesium-loaded or unloaded cells were treated with digitonin to increase the permeability of the plasma membrane, a significant increase in $[Mg^{2+}]_o$ was shown as determined by the impermeant form of Magfura-2, the tetrapotassium salt. Further addition of Triton-X 100 (which permeabilizes the cytosolic organelles) produced small non-significant increases in $[Mg^{2+}]_o$ in both cases, indicating that the magnesium-

loading procedure does not cause any significant redistribution of internal magnesium (45).

On removal of A23187, Mg^{2+}-loaded pancreatic acinar cells re-incubated in a Mg^{2+}-deficient KRH solution show a high rate of Mg^{2+} efflux (as measured by analysis of magnesium in the effluent samples by AAS) which is not detected in unloaded cells unless they are stimulated with secretagogues. Using this approach, the characteristics of the Mg^{2+} efflux system are:

a.- Release of Mg^{2+} is not constant but shows a maximum at around 50 minutes. Thereafter, Mg^{2+} efflux

is reduced, suggesting that the process stops when the physiological $[Mg^{2+}]_i$ is reached (45,112). The same has been observed by us in red blood cells (RBC) from rat, pig, cow and human (113) and by others in chicken RBC (110).

b.- Mg^{2+} efflux is insensitive to ouabain, bumetanide and SITS (4-acetamido-4´-isothiocyanatostilbene-2,2´-disulphonate), indicating that Mg^{2+} extrusion is not associated with either the Na^+/K^+ ATPase, the $Na^+/K^+/Cl^-$ co-transporter or the Cl^-/HCO^-_3 exchanger (112).

c.- In rat pancreatic acinar cells, Mg^{2+} efflux occurs mainly through a Na^+-dependent transport system, since it is markedly attenuated by different doses of either amiloride, quinidine or lidocaine (by 74, 73 and 80%, respectively)(112)(see Figure 1). This is in close agreement with the results obtained in a wide range of tissues: inhibition of Mg^{2+} efflux by amiloride from preloaded cells has been shown in rat, pig and cow RBC (113), chicken RBC (114), rat thymocytes (115) and rat sublingual acini (39). In addition, in ferret RBC, which do not need a loading procedure for Mg^{2+} efflux to be detected, significant inhibition of Mg^{2+} efflux by amiloride, quinidine and imipramine has been reported (116). The specificity of this transport system for extracellular Na^+ in pancreatic acinar cells is further supported by experiments with isotonic substitution of Na^+ with either choline chloride, Tris or the large impermeant cation NMDG (N-methyl-D-glucamine), since these operations almost abolish Mg^{2+} efflux (45,112)(see Figure 1). The same dependence has been observed using NMDG in rat, cow and pig RBC (113), and either choline, Li^+ or K^+ in chicken RBC (114), all suggesting that it is the removal of Na^+ and not the presence of the substitutes which inhibits Mg^{2+} efflux. The finding in some of the above studies that preventing Na^+ entry (in one way or another) does not totally block Mg^{2+} efflux (45,112,113), together with the results of other authors (117) indicate that some Na^+- independent Mg^{2+} efflux may exist, at least in pre-loaded chicken, rat, cow and pig erythrocytes, as well as in pancreatic acinar cells. By contrast, in human RBC, the major fraction of Mg^{2+} efflux does seem to occur in a Na^+-free medium (113,118), suggesting differences in the characteristics of Mg^{2+} transport between species.

d.- In rat pancreatic acinar cells, Mg^{2+} efflux is energy-dependent: addition of the metabolic inhibitor dinitrophenol (DNP) drastically reduced (80%) Mg^{2+} efflux when compared to control (45,112). A similar effect has been seen in chicken, rat and human RBC (110,111, 119) and in squid axons (120).

Thus far, our results with pancreatic acinar cells demonstrate the existence of a Na^+- and ATP-dependent transport mechanism for Mg^{2+} extrusion. Initially, this could be a Na^+-dependent Mg^{2+} pump, or a Na^+/Mg^{2+} exchanger (antiport). The available literature is rather confusing, supporting the proposition by Zhang and Melvin (121) that the Mg^{2+} efflux mechanism and the regulation of $[Mg^{2+}]_i$ is cell-type specific and may reflect differential expression of various transport systems. Thus, in loaded chicken (110,114) and rat RBC (111), Mg^{2+} efflux has been shown to be dependent not only on extracellular Na^+, but also coupled with the uptake of Na^+. The model proposed by Vormann and Günther (86) in rat and chicken RBC is based on a Mg^{2+} efflux system which, in non-loaded cells, exchanges extracellular Mg^{2+} for intracellular Mg^{2+}; however, after Mg^{2+} loading the system would change its properties thereby leading to Na^+/Mg^{2+} exchange. It is suggested by the authors that the effect may be due to an allosteric effect induced by the increased $[Mg^{2+}]_i$. They associate this allosteric effect with the irreversibility of the Mg^{2+} extrusion system in these cells: no significant Mg^{2+} reuptake is observed in Mg^{2+}-depleted RBC (122). The system depends on ATP, which might act by phosphorylating the exchanger resulting in gating of the transport system, or might increase the affinity of the exchanger at its internal site, as occurs with the Na^+/H^+ and Na^+/Ca^{2+} exchangers (123,124). Di Polo and Beauge (120) propose that a similar mechanism occurs in squid axons, ruling out the existence of an ATP-dependent Mg^{2+} pump and leaving the Mg^{2+}/Na^+ exchange as the only possibility for Mg^{2+} extrusion. In ferret RBC, there appears to be a reversible Na^+/Mg^{2+} exchanger, and the direction of the transport can be modified by sufficiently increasing $[Mg^{2+}]_o$ or lowering $[Na^+]_o$, both manoeuvres leading to net Mg^{2+} uptake (116). Nevertheless, these cells constitute a special case and behave differently, perhaps due to their physiologically high $[Na^+]_i$. Thus, two different transport systems for Mg^{2+} have been characterized in ferret RBC, one of which is little affected by amiloride. The other, which corresponds to the reversible Na^+/Mg^{2+} anti-porter, is amiloride-sensitive both in the efflux and influx components. In this system, a fall in ATP levels reduces Mg^{2+} uptake and favors Mg^{2+} efflux (116,125,126).

Using the method of microspectrofluorimetry to measure physiological intracellular magnesium content, we have shown that perifusion of magfura-loaded mouse pancreatic acinar cells with a Na^+-free solution results in only a small gradual rise in $[Mg^{2+}]_i$ (127). Similar results were obtained by Rotevatn *et al.* (36) in perfused chicken ventricular myocytes. From these data, however, it cannot be definitively concluded that a Na^+/Mg^{2+} antiport exists in these tissues.

Additional evidence was provided in the case of heart muscle cells when Fry (108), by means of both Mg^{2+}- and Na^+-sensitive electrodes, found that a reduction in $[Na^+]_o$ induced a rise in $[Mg^{2+}]_i$ and that increasing $[Mg^{2+}]_o$ caused a fall in $[Na^+]_i$. In spite of this, the mechanism of movement of Mg^{2+} across the myocardial cell membrane is still a matter of dispute. The results of a number of authors, again using electrodes, not only exclude the hypothesis of a Na^+/Mg^{2+} exchanger in heart tissue from sheep, ferret and guinea pig (47,48) but demonstrate an interference of some Na^+ substitutes with the Mg^{2+} electrodes used in earlier works (55,64). With a completely different preparation, i.e. paced hearts perfused with Krebs-Henseleit (KH) Mg^{2+}-free solution, we were able to show that, in these conditions, high $[Na^+]_o$ induces a large and significant increase in net Mg^{2+} efflux which is partially inhibited by amiloride. However, the ability of sucrose to produce the same effect in that preparation seems to indicate that the responses elicited by raised $[Na^+]_o$ may be related in part to an indirect consequence of its osmotic action (128,129). As far as pancreatic acinar cells is concerned, further research is needed to characterize more precisely this Na^+- and ATP-dependent process, given that many points remain still obscure. This is well illustrated by the fact that an increase in $[Mg^{2+}]_i$ can be measured in rat cells following a change in $[Mg^{2+}]_o$ from 1.1 mM to 10.0 mM (43,44,130). This could be due purely either to changes in Mg^{2+} gradient across the cell membrane acting upon passive Mg^{2+} flux, the consequence of a reversible Na^+/Mg^{2+} exchange as seen in ferret RBC (116), or even that there may be separate mechanisms for Mg^{2+} influx and efflux, the latter being a pump which uses Na^+ as an essential cofactor, as proposed by Lüdi and Schatzmann (131) in human RBC.

4.2. Regulation of magnesium transport and intracellular magnesium concentration

It is well known that magnesium ions can regulate innumerable processes *in vitro*. Since in several tissues $[Mg^{2+}]_i$ appears to be remarkably stable during several interventions, it has been questioned how intracellular magnesium can exert its modulatory actions. The role of Mg^{2+} should be considered from two viewpoints via its effect on membrane transporters, enzymes and other processes in the cell (22). First, it seems rather clear that the resting $[Mg^{2+}]_i$ will impart certain features to the cell physiology. Second, it is possible that, under certain conditions, $[Mg^{2+}]_i$ can change resulting in its biological actions, at least in part. However, very little is known about the physiological factors that influence $[Mg^{2+}]_i$ in most tissues, as well as the transport mechanisms involved (22).

4.2.1. Exocrine pancreas

Research done in our laboratory over the past few years has provided evidence that in the exocrine pancreas $[Mg^{2+}]_i$ is altered by mechanisms mediated by a number of receptors (see Figure 1). The changes are very rapid (a few minutes) and, as will be explained below, this may imply physiological importance.

Our experiments using either a suspension of rat pancreatic acinar cells or single mouse acinar cells loaded with Magfura-2 AM showed that acetylcholine (ACh), carbamylcholine cholecystokinin-octapeptide (CCK-8), thapsigargin and ionomycin, all well known Ca^{2+}-mobilising agents, induce marked changes in $[Mg^{2+}]_i$, consisting of a sharp and transient rise, immediately followed by a gradual decline to reach a steady-state significantly lower than the resting value, which is maintained throughout the experiment (40-43,45,46, 102,127,130). The decrease revealed a dose-dependency with secretagogues, and atropine blocked the ACh-evoked response. Control experiments indicated that the secretagogues-induced decrease can not be attributed to a bleaching effect caused by the excitation beam, which together with the dose-dependency and the effect of atropine confirms that the observed response is not artefactual (40). Different doses of adrenaline, noradrenaline and histamine were also examined, but no changes in $[Mg^{2+}]_i$ were seen (40,42). These latter experiments indicate that only Ca^{2+}-mobilising secretagogues (ACh or CCK-8) can elicit changes in $[Mg^{2+}]_i$, at least in pancreatic acinar cells. Moreover, once one secretagogue (e.g. ACh) has mobilized cellular magnesium, the other (e.g. CCK-8) has no detectable effect on $[Mg^{2+}]_i$, indicating that the two secretagogues stimulate Mg^{2+} release from a common intracellular pool (42).

In order to ascertain whether the secretagogue-induced change in $[Mg^{2+}]_i$ represents a Mg^{2+} efflux or a transport into intracellular stores, rat pancreatic segments were perfused with Krebs-Henseleit (KH) solution, and the effluent samples analyzed for magnesium by AAS. Under these conditions, ACh, CCK-8 as well as activation of intrinsic secretomotor nerve endings by electrical field stimulation (EFS), evoked a significant net Mg^{2+} efflux which declined rapidly to resting levels on cessation of stimulation (40,41,43-45,132). In addition, Mg^{2+} release into the extracellular medium was measured by incubating pancreatic acinar cells in a Ca^{2+}-free KRH solution containing 1 mM EGTA and 2.5 μM Magfura-2 tetrapotassium salt (the impermeant form of the probe): indeed ACh and CCK-8 produced a sustained increase in $[Mg^{2+}]_o$ (42). In more recent microspectrofluorimetric investigations performed using Magfura-2 AM-loaded single mouse pancreatic acinar cells with a system that permits perfusion of the cell, CCK-8 again induced an initial short rise in $[Mg^{2+}]_i$ followed by a progressive and significant decrease. Upon removal of the agonist, $[Mg^{2+}]_i$ returned to pre-stimulation resting values (46,102) indicating that the maintenance of the secretagogue-induced reduction in the cell suspension experiments was due to continued stimulation because of the static volume cuvette system. Taken together, the above observations confirm that in pancreatic acinar cells, ACh and CCK-8 cause a Mg^{2+} extrusion (in association with the corresponding fall in $[Mg^{2+}]_i$) followed by an uptake of the ion during the post-stimulation period until resting $[Mg^{2+}]_i$ is reached. Of course, it cannot be completely excluded that a small percentage of Mg^{2+} can be taken up and then released

from intracellular stores. Further experiments are required to measure intracellular organelle Mg^{2+} homeostasis in secretory epithelial cells both at rest and during stimulation with secretagogues.

Studies by other authors (54) show an interference of Magfura-2 by Ca^{2+}, which led us to attempt to find out whether this could be causing the short initial rise observed by us in $[Mg^{2+}]_i$ upon agonist stimulation. The idea was rejected initially due to several reasons, including the low affinity of Ca^{2+} for Magfura-2 (29), the fact that the time-course changes in $[Ca^{2+}]_i$ during stimulation are much larger compared to the increase in $[Mg^{2+}]_i$, and the temperature-dependence of the $[Mg^{2+}]_i$ increase which is not shared by $[Ca^{2+}]_i$ (41). Nevertheless, given that in our case dramatic increases in $[Ca^{2+}]_i$ were prolonged (40,41,102,130), subsequent to the employ of potent Ca^{2+}-mobilising agents such as ACh and CCK-8, it was decided to make this point clearer by co-loading either Fura-2 or Magfura-2 loaded cells with BAPTA-AM, a Ca^{2+} chelator (46). At a BAPTA concentration equal to 10 μM, which enabled a small rise in $[Ca^{2+}]_i$, not enough to interfere with Magfura, the typical decrease in $[Mg^{2+}]_i$ was found after the addition of CCK-8 but the initial rise was no longer present, indicating that it was an artifact. Concerning the secondary decrease, it might be suggested that it is a consequence of depletion of calcium stores since it has been reported that Magfura is a useful physiological tool to measure free calcium concentration within the stores (133), where the calcium concentration is quite high. However, both the conditions of our loading protocol (which has proved to cause no compartmentalization) and the efflux experiments indicate that we are observing a real decrease in $[Mg^{2+}]_i$.

The inverse direction of the $[Mg^{2+}]_i$ and $[Ca^{2+}]_i$ changes (decrease and increase, respectively) upon ACh and CCK-8 stimulation is by itself suggestive of some kind of reciprocal relationship between both ions during this process, this was confirmed in our work on mouse pancreatic acinar cells. The decrease of $[Mg^{2+}]_i$ induced by the secretagogues was abolished when intracellular Ca^{2+} was chelated with BAPTA at a concentration (50 μM) that totally prevents Ca^{2+} mobilization (127). In addition, application of thapsigargin (an inhibitor of the ATP-dependent Ca^{2+} pump of endoplasmic reticulum, SERCA) produced a large increase in $[Ca^{2+}]_i$ and a concomitant decrease in $[Mg^{2+}]_i$. After withdrawal of thapsigargin (which acts irreversibly) $[Mg^{2+}]_i$ did not return to resting values, and further addition of CCK-8 had no effect on either $[Ca^{2+}]_i$ or $[Mg^{2+}]_i$ (46). Moreover, in cells perfused with a Ca^{2+}-free solution (1 mM EGTA), the Ca^{2+} ionophore ionomycin produced a transient increase in $[Ca^{2+}]_i$ and a sustained decrease in $[Mg^{2+}]_i$. In this case, removal of the ionophore was associated with the usual increase in $[Mg^{2+}]_i$ towards pre-stimulation values (46). These results indicate the existence of a strong coupling between $[Ca^{2+}]_i$ increases and $[Mg^{2+}]_i$ decreases, and suggest that the release of Ca^{2+} from intracellular stores is necessary for the changes in $[Mg^{2+}]_i$ in response to CCK-8. The mechanism linking both processes is, however, still unknown, but several hypotheses have been proposed, including: i) An indirect action involving the hormonally-induced increase in $[Ca^{2+}]_i$ which may displace intracellular magnesium from certain binding sites to be then extruded to the outside (see Figure 1 of this review). ii) It is well established that, after CCK-8 stimulation it is the depletion of intracellular calcium stores which induces Ca^{2+} entry from the extracellular medium (134,135). Since some authors (136) support the production of a calcium influx factor (CIF) as being responsible, we speculate that this CIF could also promote Mg^{2+} extrusion.

In close agreement with the concept of a Ca^{2+}-dependent Mg^{2+} transport, there is our observation that alteration of the metabolism of a number of intracellular mediators has a marked effect on CCK-8-induced Mg^{2+} extrusion (46). Thus, addition of the phorbol ester TPA (an activator of protein kinase C) enhances CCK-8-evoked $[Mg^{2+}]_i$ decrease, whereas either staurosporine (a protein kinase C inhibitor) or NLA (a nitric oxide synthase inhibitor) has the opposite effect. The precise mechanism whereby these mediators act to modulate changes in $[Mg^{2+}]_i$ is still unclear, mainly because their role in the stimulus-secretion-coupling process has not yet been fully defined. However, since all the above indicated pathways have been associated in different ways with the Ca^{2+} signal (137,138), it is tempting to suggest that the mediators involved may regulate Mg^{2+} transport in association with Ca^{2+} homeostasis. Furthermore, other intracellular pathways may also be involved in the process. Thus, secretin (alone) has been shown to increase $[Mg^{2+}]_i$ and evoke a net Mg^{2+} uptake in rat pancreas without any effect on Ca^{2+} mobilization.When combined with either ACh or CCK-8, it inhibits not only the secretagogue-evoked Mg^{2+} efflux and the decrease in $[Mg^{2+}]_i$ but also the parallel $^{45}Ca^{2+}$ influx and $[Ca^{2+}]_i$ increase (41,132), which could have important physiological consequences from the point of view of interaction between secretagogues.

Concerning Na^+-dependency of Mg^{2+} transport, studies in rat pancreatic acinar cells showed that when extracellular Na^+ is replaced with either choline or NMDG, both ACh and CCK-8 fail to produce a fall in $[Mg^{2+}]_i$. Thus, CCK-8 induces no net efflux from rat pancreatic segments in the absence of extracellular Na^+ or presence of amiloride (45, 112). A similar Na^+-dependency on Mg^{2+} transport has been observed in single mouse pancreatic acinar cells (102). The existence of a Na^+/Ca^{2+} exchanger has been demonstrated in rat and mouse pancreatic acinar cells (139) and, in fact, we were able to show that the application of a Na^+-free solution results in a marked increase in $[Ca^{2+}]_i$, this preventing a subsequent $[Ca^{2+}]_i$ change after CCK-8 (127). In contrast, when stimulation was initiated in a Ca^{2+}- and Na^+-free medium, there was the usual decrease in $[Mg^{2+}]_i$ with corresponding Ca^{2+} mobilization (stored calcium), which reveals that there is no real Na^+-dependency on Mg^{2+} transport. In addition, the continuous presence of quinidine or amiloride did no

affect the decrease in $[Mg^{2+}]_i$. No experiments employing both Ca^{2+}-Na^+-free solutions have been performed in rat pancreatic acinar cells, however, it is possible that the observed differences are due to a species variation in Mg^{2+} transport systems. Furthermore, this difference can also be explained in relation to the experimental protocol. In the rat pancreas we employed either segments or suspensions of acini and acinar cells whereas in the mouse pancreas we employed single magfura-loaded acinar cells. It is alos possible that different parts of the pancreas may have a different regulatory process for Mg^{2+} transport. However, further experiments are required to test these interesting observations. Similarly, blocking plasma membrane Ca^{2+} channels with lanthanum chloride ($LaCl_3$) did not modify secretagogue-evoked decrease in $[Mg^{2+}]_i$ in rat cells (45), whereas this was totally quenched in mouse cells (127).

Depolarization with either high $[K^+]_o$, monensin (a Na^+ ionophore) or ouabain did not altered CCK-8-induced decrease in $[Mg^{2+}]_i$ but abolished the rise in $[Mg^{2+}]_i$ subsequent to the removal of the secretagogue (127), showing that this process may occur via a conducting pathway along the electrochemical gradient. An inward-carrying system for magnesium ions has not yet been identified for pancreatic acinar cells. Interestingly, Mg^{2+} conductive pathways have been said to exist in other species and cell types (140,141). However, more studies will have to be done before reaching any conclusions regarding this important point.

Turning to Mg^{2+} extrusion during secretagogue-stimulation, the above results do not rule out the possibility of an active mechanism, since a decrease in $[Mg^{2+}]_i$ occurs in the presence of high $[Mg^{2+}]_o$ (43,44,127). Of course, given that resting $[Mg^{2+}]_i$ rises in a high (10 mM) $[Mg^{2+}]_o$ medium (43,44,130), the fall in $[Mg^{2+}]_i$ after CCK-8 does not reach the level observed under normal $[Mg^{2+}]_o$ (40-43,45).

4.2.2. Other tissues

There is strong evidence supporting a role for the cAMP pathway in the regulation of Mg^{2+} transport in different tissues. Many studies report that perfusion of the heart with catecholamines promotes a large Mg^{2+} efflux (142-145). We have also shown that this effect is evoked not only by catecholamines but also by an activator of adenylate cyclase, forskolin (128,146,147) and, in agreement with the findings of Vormann and Günther (142), this efflux depends in part on extracellular Na^+. Use of different preparations provide corroboration for the above observations. In previous studies we have demonstrated that perfusion of rat ventricular segments with either isoproterenol, epinephrine or norepinephrine in a Mg^{2+}-free solution results in a large significant increase in Mg^{2+} efflux which is inhibited by propranolol. This efflux was also associated with a concurrent increase in contractile force (129). In isolated cardiomyocytes, cAMP has been shown to induce Mg^{2+} efflux as well (143). In contrast, carbachol and phorbol myristate (TPA, an activator of protein kinase C), cause Mg^{2+} accumulation (148). Results in liver are very similar. Thus, Mg^{2+} efflux from isolated hepatocytes and perfused liver has been found to be evoked by, respectively, cAMP (149) and norepinephrine (150). In the latter study, the effect was dependent on extracellular Na^+ and inhibited by amiloride. Mg^{2+} efflux from heart and liver tissue induced by activation of the cAMP pathway has been shown to occur without changes in $[Mg^{2+}]_i$, this leading those authors to suggest that the released Mg^{2+} might come from an internal store (148,150). Other cell types seem to share this cyclic AMP-mediated modulatory system (143,150). As an example, the cAMP analogue dibutyryl-cAMP increases the rate of Mg^{2+} efflux from Mg^{2+}-loaded thymocytes (151). Concerning the precise mechanism for this action of catecholamines on Mg^{2+} movements, an increase in the affinity of the Mg^{2+} transporter for intracellular Mg^{2+} through cAMP-activated phosphorylation has been postulated (86).

5. RELATIONSHIP BETWEEN CALCIUM AND MAGNESIUM SIGNALING IN THE EXOCRINE PANCREAS

Our results thus far have shown that the classical secretagogues, ACh and CCK-8, can elicit marked and drastic changes in $[Mg^{2+}]_i$, and this, in turn, is associated with exocrine pancreatic secretion. It is apparent that the changes in $[Mg^{2+}]_i$ are closely related to the Ca^{2+} signaling process (130). In rat and mouse pancreatic segments, raising $[Mg^{2+}]_o$ in the perfusate to 10 mM results in a marked inhibition of ACh-, CCK-8- and EFS-induced protein, trypsinogen and amylase secretion as compared to the responses obtained in normal (1.1 mM and zero) $[Mg^{2+}]_o$ (43,44,102,152,153). In these experiments, the individual enzyme and total protein secretion were determined by on-line automated methods (152,154-156) based on either fluorimetric or colorimetric measurements. The same effect of high $[Mg^{2+}]_o$ is observed in isolated intact pancreas with respect to the pancreatic flow rate and total protein output (44).

The action of ACh and CCK-8 on pancreatic acinar cells involves the activation of phospholipase C, resulting in the hydrolysis of membrane-bound phosphatidylinositol 1,4-bisphosphate (PIP_2), which leads to the formation of diacylglycerol (DG) and inositol trisphosphate (IP_3). IP_3, in turn, mobilizes Ca^{2+} from endoplasmic reticulum (ER) stores (157), the Ca^{2+} -signaling events finally leading to exocytosis and enzyme secretion. Maximal agonist concentrations induce a large and dual Ca^{2+} response (40,41,44,152) in which the initial increase has been attributed to Ca^{2+} released from intracellular stores and the later sustained phase due to influx of Ca^{2+} across the basolateral membrane (158). Submaximal agonist concentrations evoke oscillating cytoplasmic Ca^{2+} spikes (46,102). According to the "two pool" calcium model (159,160), the initial IP_3-evoked Ca^{2+} release produces an emptying of separate intracellular calcium pools via the so called Ca^{2+}-induced Ca^{2+} release (CICR) through activation of

ryanodine receptors (161). This acts to bring about additional release phases of Ca^{2+} (160). Repetitive oscillations occur because of mechanisms capable of terminating and reinitiating the Ca^{2+} signal (162). Among the systems involved in the deactivation of the Ca^{2+} signal we have to consider the Ca^{2+}-activated plasma membrane Ca^{2+}-ATPase (PMCA), which actively extrudes Ca^{2+} (107,163,164) as well as active transport mechanisms (e.g. SERCA) that cause Ca^{2+} to be taken up again into both IP_3-sensitive and insensitive intracellular stores (165,166). On the other hand, there is activation of Ca^{2+} entry from the extracellular medium. Ca^{2+} influx is not fully characterized but one of the main events leading to the activation of Ca^{2+} entry into acinar cells is the release of Ca^{2+} from intracellular pools (134,135). Nevertheless, it has now been clearly demonstrated that blocking Ca^{2+} influx can result in a decreased frequency of Ca^{2+} spikes (167) or a failure to maintain the plateau phase of Ca^{2+} response in the dual-phase model (168).

Our results indicate that the inhibitory effects of high $[Mg^{2+}]_o$ on the secretory activity of the exocrine pancreas are mediated through a disruption of the Ca^{2+} signaling events, possibly by Mg^{2+} acting at different stages of Ca^{2+} mobilization process. In the presence of elevated $[Mg^{2+}]_o$, ACh- and CCK-8-induced increases in $[Ca^{2+}]_i$ are significantly reduced (both the peak and the plateau phases), together with an inhibition of stimulated $^{45}Ca^{2+}$ influx (152, 43, 44). Accordingly, a significant attenuation in both oscillatory spike amplitude and frequency is found after submaximal agonist stimulation (102). This action of extracellular Mg^{2+} is not exerted at receptor level due to either ACh or CCK-8 stimulation, since it is still observed when the receptor-binding step is bypassed by activation of G proteins with fluoroaluminate (130). Both the reduction in the plateau phase and the inability of the secretagogues to sustain the oscillations indicates that Mg^{2+} is acting, at least in part, by blocking Ca^{2+} uptake from the extracellular medium. This is confirmed by the attenuation of the secretagogue-evoked $^{45}Ca^{2+}$ influx by elevated $[Mg^{2+}]_o$ and by the results of more recent studies (127), in which Ca^{2+} entry was indirectly determined using Mn^{2+}. This cation has been shown to be a suitable substitute for Ca^{2+} for plasma membrane Ca^{2+} channels activated by depletion of intracellular calcium stores (169). It is, then, possible that Mg^{2+} behaves like a Ca^{2+} antagonist by blocking plasma membrane Ca^{2+} channels. The presence of high $[Mg^{2+}]_o$ also reduced the initial rise in $[Ca^{2+}]_i$, thus it seems that the release of Ca^{2+} from intracellular stores is also affected. For this reason, and based on the results of others and our own findings, we propose a number of mechanisms for the interaction between Mg^{2+} and Ca^{2+} signaling (see Figure 2), all of them requiring an increase in $[Mg^{2+}]_i$ subsequent to the rise in $[Mg^{2+}]_o$, an effect that, as mentioned in previous sections, has been amply demonstrated (43,44,130):

i) IP_3, the intracellular mediator that initiates Ca^{2+} release from the ER, is inactivated via dephosphorylation by the Mg^{2+}-dependent enzyme 5-phosphatase (170). It is possible that elevated $[Mg^{2+}]_i$ can increase the activity of the enzyme resulting in reduced concentrations of IP_3. It has been postulated, however, that once initiated by IP_3, CICR (calcium induced calcium release) is not further affected by changes in IP_3 (171), thereby leading to the concept that Mg^{2+} is acting, additionally, at other levels.

ii) Experiments with thapsigargin (which depletes IP_3-sensitive calcium stores) or ionomycin (which depletes all intracellular calcium stores) under high-Mg^{2+} Ca^{2+}-free conditions (127) show that the effects of Mg^{2+} are not due to the establishment, prior to ACh or CCK-8 stimulation, of different filling states of the stores. It is well established that CICR in the pancreas occurs via caffeine-sensitive channels (172). In sarcoplasmic reticulum of skeletal muscle these channels are inhibited by Mg^{2+} (173) and recent work indicates that this may also be the case in the pancreas (174). It is then suggested that a high $[Mg^{2+}]_i$ decreases the open-state probability of the caffeine-sensitive channels, this interrupting communication between different compartments of the calcium store. It is worth suggesting that Mg^{2+} may also affect the release of Ca^{2+} from IP_3-sensitive stores, as shown in studies with reconstituted membrane bi-layers (175).

iii) The third possibility is that a rise in $[Mg^{2+}]_i$ favors the action of the Ca^{2+}-ATPases in the plasma membrane (PMCA) and endoplasmic reticulum (SERCA). These enzymes responsible for active extrusion of Ca^{2+} into the external medium and active reuptake into the ER stores, respectively, and are Mg^{2+}-dependent enzymes (107,163-166) . At this point the evidence is less conclusive. In resting cells, $[Ca^{2+}]_i$ is the result of Ca^{2+} entry down its electrochemical gradient and the extrusion of cytosolic Ca^{2+} either outside or into the stores by the ATP-dependent systems indicated above. Elevation of $[Mg^{2+}]_i$ has been demonstrated to have no effect either on basal $[Ca^{2+}]_i$ or enzyme secretion. We also failed to observe any effect on the filling state of cytoplasmic stores of non-stimulated cells. However, we cannot completely exclude a possible effect under stimulated conditions.

Thus, it can be inferred that for optimal generation and maintenance of the Ca^{2+} signal and secretion of enzymes, it is necessary that $[Mg^{2+}]_i$ reaches a certain critical level. Indeed, we have previously described that pancreatic secretagogues induce a significant reduction in $[Mg^{2+}]_i$ by stimulating Mg^{2+} -efflux systems. In the presence of 10 mM $[Mg^{2+}]_o$, cells take up Mg^{2+} resulting in an increase of $[Mg^{2+}]_i$. In this situation, either ACh or CCK-8 do elicit a decrease in $[Mg^{2+}]_i$ as well, but without achieving the level seen in a normal medium, this is probably the reason for the observed inhibitory effects. This undoubtedly important role for intracellular Mg^{2+} in the stimulus-secretion coupling process is not restricted to the exocrine pancreas but may be shared by other secretory cells. This is suggested by the results of Kasahara *et al.* (19), who show a direct inhibitory effect of increased $[Mg^{2+}]_i$ on

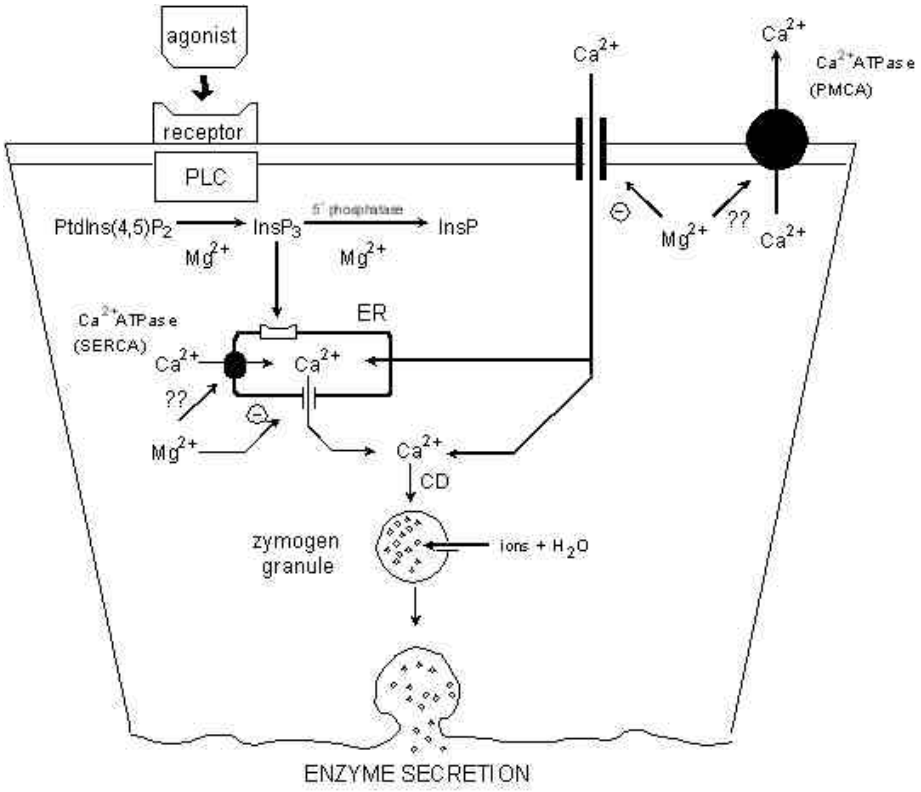

Figure 2. Schematic model illustrating the relationship between Mg^{2+} and Ca^{2+} signaling during enzyme secretion in pancreatic acinar cells in response to such agonists as ACh and CCK-8. Secretagogues-evoked an increase in $[Ca^{2+}]_i$ which in turn stimulates calmodulin (CD), Ca^{2+}-CD activates the phosphorylation of regulatory proteins on the zymogen granules resulting in the influx of ions and water and subsequent swelling of the granules. The granules then migrate towards the luminal pole where they dock and fuse with the membrane to bring about exocytosis and secretion. It is proposed that Mg^{2+} can regulate the metabolism of IP_3, Ca^{2+}-ATPase pumps (SERCA and PMCA) in the endoplasmic reticulum (ER) and plasmic membrane respectively, Ca^{2+} release from the ER, and Ca^{2+} influx from the extracellular medium. High $[Mg^{2+}]_o$, and subsequently high $[Mg^{2+}]_i$, seems to attenuate Ca^{2+} release from the ER and its entry into the cell whereas low $[Mg^{2+}]_o$ and subsequently $[Mg^{2+}]_i$ has the opposite effect. PLC=phospholipase C; IP_3=inositol trisphosphate.

TRH-induced Ca^{2+} mobilization and prolactin secretion in rat pituitary lactotropes. Similar results have been demonstrated in rat gastric parietal cells (51) where an increase in $[Mg^{2+}]_o$ (and subsequently $[Mg^{2+}]_i$) evoked an attenuated Ca^{2+} response to carbachol stimulation together with abolition of the corresponding acid production. In this case, apart from the mechanisms already proposed for the pancreas, additional ones may be operative, since on the one hand, elevated $[Mg^{2+}]_i$ did reduce both Ca^{2+} mobilization and acid secretion in non-stimulated cells, in addition, significant inhibition of histamine-stimulated cAMP production was observed, which is indicative of an involvement of Mg^{2+} in intracellular pathways other than phospholipid-calcium signaling.

6. PERSPECTIVES

The development of new analytic methods has enabled us to investigate the mechanisms for Mg^{2+} transport and the regulation of intracellular Mg^{2+} concentration in epithelial secretory cells. Interestingly, an important modulatory role for Mg^{2+} in the control of agonist-induced secretory responses has emerged from these studies. At this point, the natural Ca^{2+} antagonism that Mg^{2+} seems to exert may be crucial, not only in health states but also during the development of such pathologies as pancreatitis and gastric ulcers. Experiments in our laboratories are in progress to further characterize Mg^{2+} transport and homeostasis both its influx and its mobilization with intracellular stores and organelles in both normal and diseased conditions.

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Abbreviations: Ach;Acetylcholine, AAS:Atomic absorption spectrometry, CICR:Calcium-induced calcium release, CCK-8:Cholecystokinin-octapeptide, EFS:Electrical field stimulation, ER:Endoplasmic reticulum, $[Mg^{2+}]_o$:Extracellular magnesium concentration, IP_3:Inositol trisphosphate, $[Ca^{2+}]_i$:Intracellular free calcium concentration, $[Mg^{2+}]_i$:Intracellular free magnesium concentration, KRH:Krebs-Ringer-Hepes, NLA:N^w Nitro-L arginine, NMDG:N-methyl-D-glucamine,NMRs:Nuclear magnetic resonance spectrometry, PMCA:Plasma membrane calcium pump, RBC:Red blood cells, SERCA:Sarco-endoplasmic reticulum calcium pump

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