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Magnesium Homeostasis in Heart: A Critical Reappraisal

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Ionised magnesium concentration in heart cytosol ($[Mg^{2+}]_i$) at ~1 mmol/l is far from equilibrium, making an efflux mechanism essential and various possibilities are first considered. Then the methods available to study such mechanisms are described. New methods include 1) the study of net Mg^{2+} flux from cell suspensions using novel Mg^{2+}-macroelectrodes 2) the self referencing probe to measure net flux from single myocytes and 3) molecular methods in eukaryotes

The mechanism of influx is unclear. Efflux is via a Na^+/Mg^{2+} antiport, but the stoichiometry and characteristics await clarification. Little is known about other extrusion mechanism. The parameters of electromotive force and ion concentration, which predicate the most probable mechanisms are considered. In regulation of $[Mg^{2+}]_i$, limited diffusion in the submembrane "fuzzy" space, cytosolic buffering and uptake/release by mitochondria and SR play a role. Binding of Mg^{2+} to ATP has been determined but the other aspects of $[Mg^{2+}]_i$ regulation are unclear.

Amongst hormones, catecholamines cause a net loss, and carbachol and insulin a net gain, with little change in $[Mg^{2+}]_i$; but the physiological functions of these changes remain to be determined. Recovery from such changes awaits study.

This is a dismal picture, but two major advances make a reinvestigation of Mg^{2+} homeostasis in heart opportune. The first is conceptual in that both total and ionised concentrations must be measured and a clear distinction made between cytosol, mitochondria and SR. The second is developments in methodology. Combination of these two makes advances in our understanding of Mg^{2+} regulation in heart likely. *J Clin Basic Cardiol 2002; 5: 5–22.*

Key words: heart, magnesium, membrane transport, cytosolic buffering, magnesium regulation

The present issue of the Journal of Clinical and Basic Cardiology illustrates that there is debate regarding the role of magnesium in prevention of cardiovascular disease, as treatment after an infarct and in cardiac surgery. The problem when considering the use of magnesium in a clinical setting is the fundamental lack of understanding of Mg^{2+} transport and homeostasis in heart. Indeed, as this article will show, Mg^{2+} research in heart if not in crisis, is certainly in the doldrums. We begin with a brief overview of the "Fundamental thermodynamic properties of transport systems", before discussing "Possible Mg^{2+} extrusion mechanisms". In the study of any transport system it is necessary to perturb the system and study the recovery. In "Perturbation of the intracellular magnesium concentrations" we consider how this could be achieved and then go on to describe the "Methods used to study Mg^{2+} transport and its regulation". We then discuss "Mg^{2+} homeostasis in heart" and finally in the "General Conclusions" we reflect on the present state of the art, and on the strategies that could be employed to resolve the present crisis in our understanding of Mg^{2+} transport and homeostasis in cardiac tissue.

Fundamental Thermodynamic Properties of Transport Systems

Extrusion Mechanisms

The ionised magnesium concentration ($[Mg^{2+}]_i$) in heart is just under 1 mmol/l [1, 2], making an extrusion mechanism essential, since the equilibrium $[Mg^{2+}]_i$ at 25 °C and for a membrane potential (E_m) of –78 mV, would be around 250 mmol/l. There are in theory, four types of extrusion mechanisms, namely:

1) Passive (dependent on E_m) changes during the cardiac cycle.
2) Symport, extrusion of Mg^{2+} accompanied by some other ion(s).
3) Antiport, an exchange of Mg^{2+} for some extracellular ion(s).
4) Pump:
   a) directly coupled to the utilisation of ATP.
   b) antiport/symport coupled to utilisation of ATP.

In this section, the various possibilities for extrusion will be discussed. The calculations are based on the equations in Stein [3]. An Appendix, in which the equations are derived, is available on request (J. McGuigan).

Chemical Potential (μ)

To define the chemical potential, or the energy available for extrusion from the concentration gradients, it is essential to have accurate measurements of the inorganic ionised intracellular concentrations and such measurements are available for ferret heart at 25 °C. The concentrations are given in Table 1.

The Table also includes the intra/extracellular bicarbonate concentrations estimated from a P_{CO_2} of 40 mmHg and for Tyrode in equilibrium with air (0.03 % CO_2) and assuming an atmospheric pressure of 760 mmHg. In the second half of the Table the concentrations of organic anions which occur during the Krebs cycle are also shown, taken from measurements on rat heart. The mean resting E_m in ferret trabeculae was –78.0 mV [4].

To decide on possible antiport, symport or pump mechanisms in heart, it is essential to calculate not only the chemical potential available from the ionic gradients and the E_m, but also the energy necessary to extrude Mg^{2+}. These are given in Table 2 and illustrated in Figure 1.

The equilibrium potential (E_{ion}) of the various ions has been calculated from the measured concentrations of the ions using the Nernst equation; E_{ion} is that potential at which there would be no net flux of the respective ion. The normal range of potentials in heart varies from roughly –80 mV to 20 mV at the upstroke of the action potential [5], which means that the driving forces for both Na^+ and Ca^{2+} are always inward and that for K^+ always outward. For the other cations, the driving force on the ion reverses during the course of the normal action potential ie at potentials more

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negative than E_{ion} the driving force is inward, at potentials more positive, outward, and vice versa for the anions.

The energy necessary to extrude Mg^{2+} has also been calculated and Figure 1 shows that to extrude Mg^{2+} roughly 15 KJ/mol are required. This energy level is exceeded by Ca^{2+}, and the organic anions malate and citrate. That from Na^+ is just slightly less but for the others it is considerably less. From the available energy and the energy required to extrude Mg^{2+}, it is possible to estimate the minimum number of ions (n) that would be required for Mg^{2+} extrusion by each ionic gradient, in order to maintain the value of $[Mg^{2+}]_i$ in a non-beating heart. This minimum number is 1 for Ca^{2+}, malate and citrate, 2 for Na^+ and lactate, 3 for H^+ or HCO_3^- and pyruvate, 7 for Cl^- and 26 for K^+ ions; the latter two would appear to be unlikely transport mechanisms. Since the $[HCO_3^-]$ is calculated from the measured pH values, the energy, driving force and necessary stoichiometry are similar for H^+ and HCO_3^-.

Table 1. Mean ionic concentrations in cardiac muscle

Ion	$[Ion]_o$	$[Ion]_i$	Temp. (°C)	Reference
K^+	5 mmol/l	140.5 mmol/l	25	[51]
Na^+	155 mmol/l	14.1 mmol/l	25	[4, 127]
$H^{+\S}$	51.35 nmol/l	77.42 nmol/l	25	[195]
Ca^{2+}	2.7 mmol/l	200 nmol/l	25	[81]
Mg^{2+}	0.5 mmol/l	0.81 mmol/l	25	[1]
Cl^-	164.5 mmol/l	17 mmol/l	25	[196]
HCO_3^{-*}	36.9 mmol/l	24.4 mmol/l	25	P_{CO_2} = 40 mmHg
HCO_3^{-*}	0.210 mmol/l	0.140 mmol/l	25	P_{CO_2} = 0.228 mmHg
$Lactate^-$	0.71 mmol/l	1.12 mmol/l	30	[13]
$Pyruvate^-$	0.13 mmol/l	0.068 mmol/l	30	[13]
$Malate^{2-\#}$	0.037 mmol/l	0.10 mmol/l	37	[197]
$Citrate^{2-\#}$	0.125 mmol/l	0.24 mmol/l	37	[197]

*Calculated from pH and pK values taken from Burton [198]; §Calculated from the measured hydrogen activities, using the mean activity coefficient of 0.7789 taken from Hamer and Wu [199]; #Intracellular concentrations calculated from concentrations given per dry weight using measurements for intracellular water in Polimeni [200]; they are for hearts perfused with glucose. Extracellular organic anions concentrations are for plasma taken from Documenta Geigy [201]. Charge on organic anions is for a physiological pH.

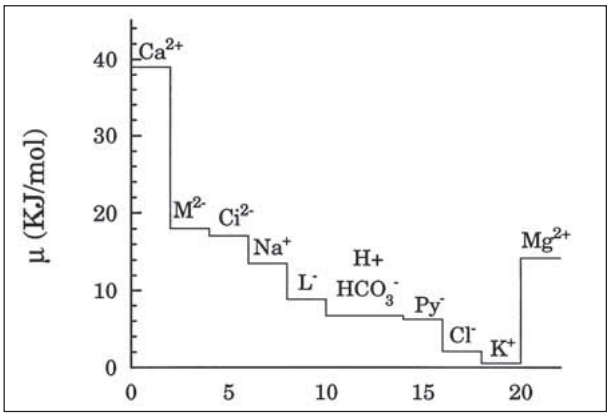

Figure 1. Chemical potential for the ionic gradients. The organic anions, malate, citrate, lactate and pyruvate are labelled M^{2-}, Ci^{2-}, L^- and Py^- respectively. This illustrates that due to the difference in the chemical potential between that required to extrude Mg^{2+} and that of Cl^- and K^+ these ions *a priori* would be unlikely to form a symport with Mg^{2+}

Equilibrium Concentration and Reversal Potential

An antiport/symport system will strive towards a $[Mg^{2+}]$ at which there is no net flux of Mg^{2+}; this can be considered the equilibrium concentration for the system or $[Mg^{2+}]_{Eq}$; $[Mg^{2+}]_{Eq}$ is that concentration that could be maintained by the transporter in a non-beating heart and with no background leak of Mg^{2+}. If the system is electrogenic, there is also a potential at which there is no net transport of Mg^{2+} (E_r; [6]). If net positive charge is being transported into the cell (equivalent to net negative charge being transported out) then, if E_m is more negative than E_r, the flux is outward; at potentials less negative, the flux is inward. On the other hand, if net positive charge has to be transported out of the cell (equivalent to net negative charge being transported into the cell) at an E_m more negative than E_r, the flux is inward; at potentials less negative, outward.

Possible Mg^{2+} Extrusion Mechanisms

The bases for Mg^{2+} extrusion mechanisms have been discussed above and it has to be emphasised that when considering such mechanisms they have to be thermodynamically possible. Moreover, before any antiport/symport can be seriously considered for Mg^{2+} extrusion not only has the energy available from the system to be calculated but also the $[Mg^{2+}]_{Eq}$ that the system would reach. Since heart cells are permeable to Mg^{2+} [7] the calculated $[Mg^{2+}]_{Eq}$ has to be below the measured value of $[Mg^{2+}]_i$ to allow for the background leak of Mg^{2+}.

Passive, Dependent on E_m, Changes During the Cardiac Cycle

The equilibrium potential for Mg^{2+} ions is –6.2 mV. During the cardiac cycle the passive flux is inward at E_m values more negative than this, and outward at potentials more positive. Depending on the characteristics of the passive flux, the net flux over the cardiac cycle could be zero (see [8]). In a beating heart it is possible to define a "mean membrane potential" similar to the "mean blood pressure" [9]; rough graphical analysis for a heart rate of 60 per minute gives a value of around –55 mV for this "mean potential". As the heart rate increases, the mean potential becomes less negative and the proportion of time spent at potentials more positive than –6.2 mV increases, relative to that at potentials more nega-

Table 2. Energy available for Mg^{2+} extrusion from ionic gradients and membrane potential

Ion	E_{ion} (mV)	TEMP (°C)	Direction of driving force during AP	μ KJ/mol	n(i)
K^+	–85.7	25	Out	0.55	25.90 (26)
Na^+	+61.4	25	In	13.65	1.04 (2)
H^+	–10.6	25	In/Out	6.70	2.13 (3)
Ca^{2+}	+122.2	25	In	39.01	0.36 (1)
Mg^{2+}	–6.2	25	In/Out	14.24	—
Cl^-	–58.3	25	In/Out	2.09	6.80 (7)
HCO_3^{-*}	–10.6	25	In/Out	6.69	2.13 (3)
$HCO_3^{-\#}$	–10.6	25	In/Out	6.69	2.13 (3)
$Lactate^-$	+11.9	30	In/Out	8.87	1.61 (2)
$Pyruvate^-$	–16.9	30	In/Out	6.09	2.34 (3)
$Malate^{2-}$	+13.3	37	In/Out	18.00	0.79 (1)
$Citrate^{2-}$	+8.7	37	In/Out	17.12	0.83 (1)

n(i) = the integer minimal number of ions necessary to extrude Mg^{2+}; *HCO_3^-, with a P_{CO_2} of 40 mmHg; #air.

tive. This means that $[Mg^{2+}]_i$ would be rate dependent, decreasing as the frequency increased. In a non-beating heart there would be a slow gain of Mg^{2+}, but depending on the size of the background leak, buffering and uptake by intracellular organelles, the change in $[Mg^{2+}]_i$ could be minimal over the time course of an experiment. However, as no great difference in the mag-fura-2 signal from non beating myocytes was found, on the second day after preparation [1], it suggests that active extrusion mechanisms are present.

Antiport, Symport and Combined Antiport/Symport Mechanisms

Examples of possible antiport, combined antiport and symport and symport extrusion systems for heart are listed in Table 3. The Table also includes whether the systems are electrogenic or neutral, the equilibrium $[Mg^{2+}]_{Eq}$ and the reversal potential, E_r for each system, if it is electrogenic. Since extrusion of HCO_3^- is equivalent to an uptake of H^+ [10], H^+/Mg^{2+} antiports are equivalent to HCO_3^-, Mg^{2+} symports.

1 Ca^{2+}, 2 Na^+ or 3 H^+ (or 3 HCO_3^- symport) to 1 Mg^{2+} antiporters would be capable of maintaining the level of $[Mg^{2+}]_i$ in a non-beating heart. 2 Na^+ and 1 Ca^{2+} to 1 Mg^{2+} antiporters would be electrically neutral, would not depend on changes of the membrane potential and would be effluxing Mg^{2+} during the course of the action potential. However, this is not the case, with either a 3 H^+ antiporter or 3 HCO_3^- symport which would change direction during the course of the cardiac cycle. A disadvantage of H^+/HCO_3^- coupled transport systems is that they are highly pH dependent.

From the values in Table 1, a 1 $Na^+/1$ Mg^{2+} antiport at a E_m of –78 mV would be incapable of maintaining the resting level of 0.81 mmol/l for the $[Mg^{2+}]_i$. However, it is potential-dependent and over the heart cycle it could maintain a lower $[Mg^{2+}]_i$ than the value calculated for –78 mV, since at potentials less negative than –74.0 mV the transporter is effluxing Mg^{2+}. If a 1 $Na^+/1$ Mg^{2+} transport system is present in heart, then an additional transport system has to be present to maintain the resting $[Mg^{2+}]_i$ at 0.81 mmol/l or there has to be an input of energy from ATP hydrolysis (cf. [11]). However, an advantage of a 1 $Na^+/1$ Mg^{2+} transport system is that the $[Mg^{2+}]_i$ would be a function of the heart rate. As the heart rate increases, the mean potential over the cardiac cycle becomes less negative which could cause a decrease in $[Mg^{2+}]_i$. However, such calculations are based on mean values, ie the values of $[Na^+]_i$, $[Mg^{2+}]_i$ and E_m have not been measured simultaneously in the same cell. This will be taken up again below (see "Stoichiometry of Na^+/Mg^{2+} Exchange").

There are other possibilities that can be considered. A 1 Na^+, 1 $H^+/1$ Mg^{2+} transporter (or 1 $Na^+/1$ Mg^{2+}, 1 HCO_3^-) would be electroneutral and give an equilibrium $[Mg^{2+}]_i$ of 0.07 mmol/l. A 1 Na^+, 2 $H^+/1$ Mg^{2+} transporter (or 1 $Na^+/1$ Mg^{2+}, 2 HCO_3^-) would be potential dependent and effluxing Mg^{2+} during the cardiac cycle. Again, such extrusion systems are pH dependent. Two other antiport/symport systems could, in theory, maintain the $[Mg^{2+}]_i$ namely, a 1 $Na^+/1$ Mg^{2+}, 1 Cl^- and a 1 $Na^+/1$ Mg^{2+}, 2 Cl^-. A 1 $Na^+/1$ Mg^{2+}, 1 Cl^- would be electrically neutral, and 1 $Na^+/1$ Mg^{2+}, 2 Cl^- would reverse at –43.8 mV.

Cl^- and HCO_3^- symports are possible extrusion mechanisms. A symport with Cl^- alone would require 7 Cl^- to 1 Mg^{2+} to maintain the resting $[Mg^{2+}]_i$ at an E_m of –80mV. In such a symport, the flux changes from efflux to influx at a potential of –79.2 mV and would be influxing Mg^{2+} for most of the cardiac cycle. It can be excluded on these grounds alone. A combined 1 Cl^-, 2 HCO_3^- and Mg^{2+} is another possibility. With a reversal at –67.2 mV it too is rather unlikely.

Symports with organic anions that are part of the Krebs cycle are also illustrated. In view of the recently described malate, Mg^{2+} symport in leech neurones [12], such mechanisms have to be seriously considered for heart. Since the concentrations change during activity in heart [13] such symports would allow a feedback between activity and the $[Mg^{2+}]_i$.

Pumps

In the previous sections the transport of Mg^{2+} depended on the ionic gradients between the cytosol and the surrounding milieu. These gradients were themselves maintained by active transport processes, but in neither the antiport nor the symport was there a direct energy input into the system through the hydrolysis of ATP. In a pump mechanism, Mg^{2+} would be extruded directly by a mechanism similar to the Ca^+ pump (cf. [3]). Under physiological conditions, the splitting of one mole ATP yields about 50–60 kJ/mol [14]. A Mg^{2+} ATPase would thus be able to transport up to 3 Mg^{2+} per ATP (3 × 14.24 kJ/mol; see Table 3) while maintaining physiological $[Mg^{2+}]_i$ levels. Such a transport would, however, be highly electrogenic.

Another possibility is the coupling of ATP hydrolysis to an antiport/symport system. As shown above, the energy available from a 1 $Na^+/1$ Mg^{2+} exchanger is insufficient to maintain the measured resting level of $[Mg^{2+}]_i$. It has been suggested that additional energy input from ATP would make such a mechanism possible [11]. However, such a mechanism would gain energy from dissipating the Na^+ gradient (13.65 kJ/mol) and from splitting ATP, a total of about 70 kJ/mol

Table 3. Characteristics of possible extrusion mechanisms

Group	Transport/system	Antiport/ symport	Neutral/ E_m dependent	$[Mg^{2+}]_{Eq}$ (mmol/l)	E_r (mV)
Ca^{2+}	Ca^{2+}/Mg^2	Antiport	Neutral	37 nmol/l	—
Na^+	1 $Na^+/1$ Mg^{2+}	Antiport	E_m dependent	1.02	–74.0
	2 $Na^+/1$ Mg^{2+}	Antiport	Neutral	0.0045	—
H^+/HCO_3^-	3$H^+/1$ Mg^{2+} or 3 HCO_3^- and 1 Mg^{2+}	Antiport Symport	E_m dependent	0.07	–19.3
	1 Na^+, 1 $H^+/1$ Mg^{2+} or 1 $Na^+/1$ Mg^{2+}, 1 HCO_3^-	Antiport Antiport and Symport	Neutral	0.07	—
Na^+, H^+/HCO_3^-	1 $Na+$, 2 $H^+/1$ Mg^{2+}, or 1 $Na^+/1$ Mg^{2+}, 2 HCO_3^-	Antiport Antiport and Symport	E_m dependent	0.006	+52.7
Na^+, Cl^-	1 $Na^+/1$ Mg^{2+}, 1 Cl^-	Antiport and Symport	Neutral	0.44	—
	1 $Na^+/1$ Mg^{2+}, 2 Cl^-	Antiport and Symport	E_m dependent	0.19	–42.3
Cl^-, HCO_3^-	7 Cl^- and 1 Mg^{2+}	Symport	E_m dependent	0.69	–79.2
	1 Cl^-, 2 HCO_3^- and 1 Mg^{2+}	Symport	E_m dependent	0.49	–67.2
	2 lactate$^-$ and 1 Mg^2	Symport	Neutral	0.20	—
Organic anions	3 pyruvate$^-$ and 1 Mg^2	Symport	E_m dependent	0.16	–38.4
	1 malate^{2-} and 1 Mg^2	Symport	Neutral	0.071	—
	1 citrate^{2-} and 1 Mg^2	Symport	Neutral	0.14	—

for transporting one Mg^{2+} requiring 14.24 kJ/mol. In other words, about 80 % of the energy spent in such a process would be wasted. Other combinations of ATP hydrolysis and antiport/symport systems are possible but purely hypothetical.

Perturbation of the Intracellular Magnesium Concentrations

In order to investigate any transport system it is essential to perturb the system and study the recovery from the perturbation. If the ionised and/or total magnesium concentrations in the cytosol can be increased or decreased or the net membrane flux changed, then it is possible to investigate how and under what conditions the concentrations and or flux return to normal. The methods used to perturb cellular Mg are described below.

Increase in Total/Ionised Magnesium Concentrations

Various strategies have been tried and these include the following.

Reversal of the Na^+/Mg^{2+} Exchanger

The Na^+/Ca^{2+} exchanger in heart can influx/efflux Ca^{2+} [4, 15] but attempts to increase $[Mg^{2+}]_i$ in heart by Na^+ removal ie by reversing a putative Na^+/Mg^{2+} transporter have been unsuccessful [1].

Decrease of the $[ATP]_i$

If the ATP concentration is decreased by the use of KCN and iodoacetic acid [16] then the intracellular buffering capacity is reduced, and the $[Mg^{2+}]_i$ increases. There are at least two disadvantages of this method. Firstly, the reduction in the buffering capacity per se. Secondly, the reduction of the ATP concentration may influence the Mg^{2+}-transport directly by inhibiting a Mg^{2+} pump or indirectly, by reducing the Na^+ gradient due to inhibition of the Na^+/K^+ pump.

Loading Solutions

In leech neurones increasing the $[Mg^{2+}]_o$ from 1 to 30 mmol/l caused on average, over a 5 minute period, an increase from 0.45 to 0.95 mmol/l [17]. In heart however, increasing the $[Mg^{2+}]_o$ in normal Tyrode from 0.5 to 20 mmol/l gave only an increases of 0.33 ± SD 0.19 mmol/l (n = 5) over 5 minutes [1] but larger concentrations of $[Mg^{2+}]_o$ have not been tried in heart. Empirically, Handy, et al. [18] discovered that myocytes could be Mg^{2+} loaded in a zero Na^+, Ca^{2+}, 5 mmol/l Mg^{2+} solution. The problem with this solution is that it is uncertain what is happening. The E_m of the cells was not measured, it is uncertain if the ATP concentrations are maintained and there could well be changes in the intracellular distribution of Mg^{2+}. Despite these disadvantages, it was possible to investigate the membrane transport system.

Ionophores

In erythrocytes loading of cations has been successfully carried out by the use of the ionophores A23187 [19] and p-chloromercuribenzene-sulphonate (PCMBS, [20]) and the A23187 method has been used in other cell types. Cittadini and Scarpa [21] found that Ehrlich tumour cells accumulated Mg^{2+} with A23187 and 10 mmol/l Mg^{2+} and Günther and Vormann [22] loaded thymocytes with the ionophore and normal concentrations of Mg^{2+}. The position with myocytes is somewhat uncertain. PCMBS has not been tried and the reports of success with ionophores are varied. Raju et al. [23] were not successful with an intracellular calibration using ionomycin as were Fry and McGuigan (unpublished data) using 4-bromo-A23187. However, Silverman et al. [24] were successful with such calibrations using either ionomycin or 4 bromo-A23187. Recent experiments by Tashiro and Konishi [25] were also successful in loading rat ventricular cells with Mg^{2+} by the use of ionomycin, so it does appear ionophores could be used in future studies.

Direct Injection of Mg^{2+}

Direct pressure injection of Mg^{2+} has been successfully carried out in skeletal muscle fibres [26] and in leech neurones Mg^{2+} has been injected iontophoretically [27]. While such methods have not been tried in myocytes it would seem worthwhile to do so.

Decrease in the Total/Ionised Magnesium Concentrations

It would be advantageous if it was also possible to deplete cells of Mg^{2+} in order to study the recovery to normal levels. The procedures used include the following.

Low $[Mg^{2+}]_o$

Exposure of ferret trabeculae to zero Mg^{2+} for periods up to 30 minutes did not change the $[Mg^{2+}]_i$ [McGuigan, unpublished data]. However, Quamme and Rabin [28] found that chicken embryo heart cells cultured for 16 hours in 0.16 mmol/l Mg^{2+} had lower than normal $[Mg^{2+}]_i$ and in these cells recovery could be studied. More recently Griffiths [29] found it was possible to deplete rat ventricular myocytes of Mg^{2+} by incubation in 0.1 mmol/l Ca^{2+} and zero Mg^{2+} at 37 °C for 90 minutes.

Ionophores

Günther and Vormann [30] found that rat and chicken erythrocytes as well as rat thymocytes and Ehrlich tumour cells could be depleted using the ionophore A23187 and EDTA to chelate the extracellular Mg^{2+}. Such methods have still to be applied to myocytes.

Direct Injection of Mg^{2+} Buffer Substances

If injection of Mg^{2+} was feasible, then injection of Mg^{2+} buffer substances, such as EDTA to lower the $[Mg^{2+}]_i$ should also be possible. The binding of Mg^{2+} to EDTA has been characterised [31] so if the amount of EDTA injected was known, then the total Mg^{2+} bound to the ligand could be calculated.

Change in Net Membrane Flux

In both heart and myocytes, the net efflux is increased by catecholamines and net influx by carbachol [32]. Insulin also causes an increase in the net influx in myocytes [33]. In neurones, activation of ionotropic glutamate receptors (NMDA-receptor) [34]; non-NMDA-receptors [35, 36] have been demonstrated to cause an influx of Mg^{2+} through the receptor coupled ion channels. As such receptors have recently been shown to exist in heart tissue [37, 38], glutamate-induced changes in $[Mg^{2+}]_i$ might also be relevant in cardiology.

Conclusions Perturbation

In any study of Mg^{2+} transport it is essential to perturb the system and study the recovery. Because cytosolic muffling [see below] dampens changes in the cytosolic $[Mg^{2+}]$, it is difficult to perturb the system and because of this, rather extreme measures have to be employed. These include, marked alterations in extracellular $[Mg^{2+}]$ either alone or in combination with changes in $[Na^+]$ and $[Ca^{2+}]$, ionophores, direct injection, changes in the cytosolic buffering and application of hormones. Such methods could very well have actions other than simply changing the $[Mg^{2+}]$, actions that could influence the interpretation of the results. This has to be borne in mind when the methods are used.

Methods Used to Study Mg^{2+} Transport and its Regulation

If there is a perturbation of either the total or ionised magnesium concentrations in cytosol/organelles, investigating the recovery from such changes provides insight into the membrane transport systems as well as the regulatory mechanisms. Mg^{2+} research has in the past, been hampered by the lack of suitable techniques to investigate Mg^{2+} transport and its regulation, not only in heart but also in other tissues. The goals are to be able to accurately measure changes in the ionised and total Mg^{2+} concentrations, alterations in the intracellular distribution of the ion, including uptake/release from organelles, net fluxes and their regulation and the cytosolic buffer capacity. To study intracellular perturbations and their recovery, membrane fluxes can be measured with isotopes, voltage clamp procedures can be applied to single myocytes or isolated patches, ionised and total magnesium concentrations can be measured both intra/extracellularly and the changes in intracellular distribution between cytosol and intracellular organelles can be determined by electron probe X-ray microanalysis (EPXMA). More recent techniques include the application of molecular methods to the study of Mg^{2+} transport systems.

Isotopes

Isotopes of Mg^{2+} offer a direct method to study the membrane influx/efflux of Mg^{2+} with physiological concentrations of Mg^{2+} in the external solution, in cells, vesicles or isolated organelles. $^{28}Mg^{2+}$ with a half life of 21.3 hours [39] and β-emission is ideally suited for flux studies (see Schweigel et al. [40]). While the isotope can be obtained from Brookhaven National Laboratory (isotopes@bnl.gov) the problem is expense, as a minimum of 400 μCi have to be ordered at a cost of $4,186. Because the isotope has had, and could have important research applications in the future, serious considerations should be given to forming a research consortium from interested groups to share deliveries on designated dates per year at preplanned locations. $^{27}Mg^{2+}$, also a β-emitter, has been used to measure uptake in intestinal vesicles of the fish [41]. However, the half life is only 9.46 minutes, severely limiting its use.

The stable isotope $^{26}Mg^{2+}$ has been used to study Mg^{2+} transport in renal tubules of the fish [42]. This was in combination with ion microscopy imaging so again it is not suitable as a routine method. $^{25}Mg^{2+}$ has also been used [43]. The disadvantage of this method is that each method requires several hours on a mass spectrometer, making it unsuitable for routine use. However, it is the type of method that should be borne in mind for very specific experimental protocols (personal communication, Gary Quamme). Despite the potential for the use of stable Mg^{2+} tracers, the possibilities for their general application are at present limited.

Voltage Clamp

Standard electrophysiological techniques for measuring membrane potentials have routinely been used on small trabeculae (see [1]). Whole cell clamp to change the membrane potential has been applied in combination with magfura-2 to study the influence of the membrane potential on Mg^{2+} transport [8, 44]. Patch clamping to study putative Mg^{2+} channels has not yet been applied to cardiac cells, but has been used to study Mg^{2+} channels in paramecium [45] and divalent cation channels in chicken lymphoma cells [46].

Microelectrodes

Such electrodes can be used to measure either the intracellular or extracellular ionised ionic concentrations. Intracellularly the measurement is in the cytosol and extracellularly, microelectrodes are used to measure net membrane fluxes from single cells with the self referencing probe (SRP).

Intracellular Ionic Concentrations

Single barrelled microelectrodes have been used to measure the ionised concentrations of Na^+, K^+, Mg^{2+}, pH and Cl^- in heart muscle (see Table 1, for references). Ca^{2+} microelectrodes have been used in heart (see [47–49]). It is, however, very difficult to manufacture reliable Ca^{2+} microelectrodes suitable for use in heart cells, as the tips of functioning Ca^{2+} microelectrodes are normally too large to penetrate heart cells [McGuigan, unpublished data]. Microelectrodes can be exactly calibrated but there is also the necessity to independently measure the E_m. In trabeculae the two electrodes are not in the same cell, but since heart is a functional electrical syncytium the reasonable assumption is made that the E_m throughout the trabeculae is similar. If the cells become uncoupled this basic assumption is no longer valid [50]. The main problem with the method is the technical difficulty of maintaining impalements in beating tissue or during Na^+-free contractures, problems which have severely restricted the use of the method.

Double-barrelled ion selective microelectrodes have been used to measure the $[K^+]_i$ in ferret trabeculae [51], and pH_i [50]. Triple barrelled microelectrodes have been used in leech neurones to measure simultaneously the $[Na^+]_i$, $[Mg^{2+}]_i$ and E_m in order to directly estimate the stoichiometry of the Na^+/Mg^{2+} exchanger [17]. Triple barrelled microelectrodes have not been used in cardiac tissue and since heart cells are around 8 μm in radius, they would most probably damage the cell. However, as pointed out above, E_m does not have to be measured in the same cell, so doubled barrelled Na^+/Mg^{2+} microelectrodes coupled with an independent measurement of E_m would suffice. This technique has not yet been used in heart.

Microelectrodes have been used in single myocytes. Double barrelled pH microelectrodes [52] have been used. Single barrelled measurements of $[K^+]_i$, $[Na^+]_i$ and $[Cl^-]_i$ [53, 54], [Na+]$_i$ [55], $[K^+]_i$ and $[Ca^{2+}]_i$ [56] have also been carried out; although as the authors themselves point out the Ca^{2+} measurement were subject to several artifacts. Due however, to the difficulties involved with the technique, it has not found widespread application. Rodrigo and Chapman [57] modified the method by using suction type microelectrodes with tips of 1 μm. Because the tip is larger than conventional ion selective microelectrodes the time constant of response is correspondingly less, Na^+, pH around 50 ms, that of Ca^{2+} around 200 ms. They have been successfully used to measure $[Na^+]_i$, $[Ca^{2+}]_i$ and pH_i [57, 58] and $[K^+]_i$ [59]. The method can also be combined with whole cell clamping (see [58, 59]). The method has not as yet been used to measure $[Mg^{2+}]_i$ under voltage clamp conditions but it would appear to be worth trying.

Microelectrodes can be regarded as measuring the concentration of a point source in the cytosol; they cannot measure changes in intracellular organelles and would not respond to changes in concentration in the submembrane space. Their great strength is the accurate establishment of resting values of ionic concentrations in heart and in other tissues.

Self Referencing Probe (SRP)

In a single cell, a net Mg^{2+} efflux (or influx) across the membrane will increase (decrease) the Mg^{2+} concentration immediately surrounding the individual cell, and there will be decrease (increase) in Mg^{2+} concentration as a function of distance from the cell surface. If a Mg^{2+} selective microelectrode is oscillating at 0.3 Hz between a point at the cell

surface and a reference point in the bathing solution then a potential difference, corresponding to the Mg^{2+} concentration difference will be registered. This is the self referencing probe which has been very successfully applied to the measurements of Ca^{2+} fluxes [60, 61]. No measurements have yet been made of Mg^{2+} flux with this technique. However, in view of the fact that the new neutral Mg^{2+} carrier ETH 5504 has an improved selectivity against Ca^{2+} and microelectrodes with relatively large tip diameters (> 5 μm) have been successfully manufactured using this neutral carrier [62, 63], the time would appear ripe to apply the technique.

Macroelectrodes

It is now possible to manufacture and calibrate Mg^{2+}-macroelectrodes down to 1 μmol/l in intracellular like physiological solutions and 10 μmolar in extracellular like physiological solutions; Ca^{2+}-macroelectrodes for use in intracellular like solutions have also been manufactured [31, 64]. These macroelectrodes are stable, can be used for determining binding constants [65] and/or to record changes in $[Mg^{2+}]$ and/or $[Ca^{2+}]$ in cell suspensions. Fry and Langley [66] not only give details of their construction but also the compositions for the manufacture of other cation selective macroelectrodes. The use of such electrodes requires the use of accurate buffer solutions for Mg^{2+} and Ca^{2+}. Methods are available to manufacture such solutions [31, 67]. It is not recommended to try to calculate the ionised concentration in buffer solutions as the method is not accurate enough (see [31]).

Macroelectrodes also have their drawbacks. As the initial $[Mg^{2+}]_o$ or the appropriate ion increases, the sensitivity of the method decreases. To illustrate this, the ratio of the final $[Mg^{2+}]_o$ to the initial $[Mg^{2+}]_o$ or fractional increase has been calculated after the application of adrenaline to a cell suspension. This was calculated, assuming a 5 % release of total Mg^{2+} from 10^6 heart cells/ml on adrenaline stimulation (see [68]). If the initial $[Mg^{2+}]_o$ is less than 50 μmol/l the $[Mg^{2+}]_o$ in the extracellular milieu, after the application of adrenaline would increase by more than one and a half times or greater. At $[Mg^{2+}]_o$ above 50 μmol/l the fractional increase rapidly decreases, at 100 μmol/l it has declined to 1.3 times and at 250 μmol/l the increase is only 1.1 times. While increasing the cell density by centrifugation makes the changes correspondingly great, it does not get round the problem of a decreasing sensitivity as the $[Mg^{2+}]_o$ increases. Other problems with macroelectrodes are that changing the composition of the extracellular fluid will alter the calibration curve for the electrode. These problems have been well characterised and do not represent an insurmountable barrier for their use [68].

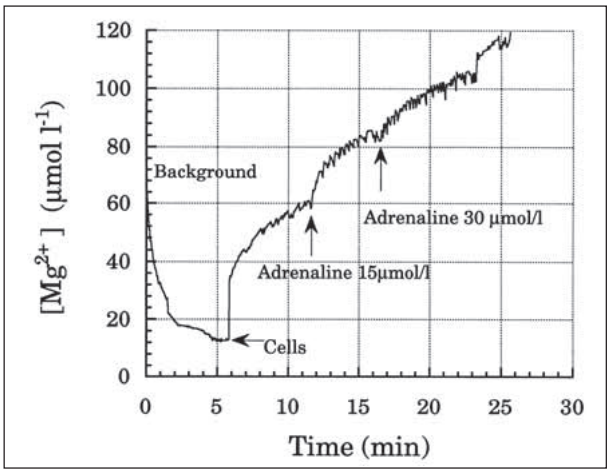

Figure 2. Preliminary measurements of Mg^{2+} efflux from cultured atrial cells. The background solution contained in mmol/l: Na^+, 155; K^+, 5; Ca^{2+}, 0.9; and no added Mg^{2+}, buffered to pH 7.4 with 5 mmol/l HEPES. After 6 minutes the cell pellet was added to the background solution. Because of contamination this increased the $[Mg^{2+}]$ from around 15 μmol/l to 60 μmol/l. Addition of adrenaline caused an efflux of Mg^{2+} from the cells. The electrode did not react to 30 μmol/l adrenaline

In cell suspensions which contain no Mg^{2+} buffer the ionised Mg^{2+} concentration is equal to the total concentration and macroelectrodes measure changes in the total concentration. The electrodes can be used in 0.5 to 1 ml of cell suspensions and, unlike atomic absorption spectrophotometry, the recording is continuous and on-line. The measured potentials can be directly converted into concentrations with use of the calibration curve. A preliminary experiment [McGuigan, Elder and Johnson, unpublished data] illustrating the increased efflux of Mg^{2+} from cultured atrial cells on addition of adrenaline is shown in Figure 2. This experiment demonstrates the possibilities for the use of Mg^{2+} macroelectrodes and on-line registration.

Fluorochromes

Intracellular dyes such as mag-fura-2 also known as furapatra [23, 69] or mag-indo (Molecular probes) possessing fluorescent characteristics which depend on the $[Mg^{2+}]_i$, would appear to be an ideal solution to the problem of measuring $[Mg^{2+}]_i$. The ratiometric method (see [70]) which measures the $[Mg^{2+}]_i$ independently of dye concentration and path length has allowed a wide application of the method in a variety of tissues. There are, however, two main problems associated with their use namely, calibration and their selectivity over Ca^{2+}. The fluorochromes can be distributed between cytosol and intracellular organelles; this can be a disadvantage if both are loaded and only cytosolic changes are to be measured. On the other hand, if preferential loading of organelles is possible, this would be an advantage as it would allow measurements of the changes in $[Mg^{2+}]$ in these organelles under physiological conditions.

Calibration

Loading mag-fura-2/mag-indo into cells is often carried out by using the acetoxy-methly ester form. This is membrane permeable and intracellularly it is then split by endogenous esterases in the cell into the active form [71]. However, though convenient, loading this way has a number of difficulties. The ester may not be fully split, making the calibration curve obtained *in vitro* differ from that *in vivo*. Silverman et al. [24] attempted both calibrations in that they permeabilised the cell membrane by using ionophores and found what can only be described as minor differences. Csernoch et al. [72] made a similar attempt but increased the membrane permeability of mouse skeletal muscle fibres using saponin. In contrast to Silverman et al. [24] they found that the apparent binding curve *in vivo* compared to *in vitro* was shifted by a factor of roughly 2 to higher $[Mg^{2+}]$. Westerblad and Allen [26] did both calibrations in mouse skeletal muscle, increasing the $[Mg^{2+}]_i$ by injecting suitable Mg^{2+} buffer mixtures. They found little difference between *in vitro* and *in vivo* calibrations. Even if there are differences between the *in vivo* and *in vitro* calibrations the direction of subsequent experimentally induced changes will be correct, although the exact magnitude might be in doubt.

Selectivity Over Ca^{2+}

This is a confusing problem, made worse by the fact that there are no standardised Ca^{2+} buffer solutions. K_d values quoted in the literature for the binding of Ca^{2+} to the fluo-

rescent probe depend in many cases not only on Ca^{2+} binding but also on the purity of EGTA, the Ca^{2+} contamination of the distilled water and the programme used to calculate the concentrations, making it difficult to compare K_d values (see [31, 67]). The original paper on mag-fura-2 [23] quoted a K_d for Ca^{2+} of 53 μmol/l suggesting that Ca^{2+} interference would not be a serious problem with an $[Mg^{2+}]_i$ around 0.8 mmol/l. However, Hurley et al. [73] in acini and Buri et al. [1] in heart concluded that Ca^{2+} interference was a major problem, severely limiting the use of the probe. Problems with Ca^{2+} interference with mag-fura-2 in skeletal muscle during stimulation but not at rest, were found by Konishi et al. [74] and by Westerblad and Allen [26] during repetitive stimulation in skeletal muscle. On the other hand, Li and Quamme [75] found that the mag-fura-2 signal decreased in myocytes as Ca^{2+} increased providing evidence that Ca^{2+} only marginally interfered with the mag-fura-2. Silverman et al. [24] also reported marginal Ca^{2+} interference with mag-indo. The most direct way to measure the effect of Ca^{2+} on mag-fura is to compare directly the microelectrode and mag-fura-2 measurements in the same cell. Kennedy [76] made this comparison in snail neurones. She found that impaling a Mg^{2+}-microelectrode into the cell caused an apparent increase in the $[Mg^{2+}]_i$ as measured with mag-fura-2, but not with the Mg^{2+}-microelectrode. This difference was attributed to an increase in $[Ca^{2+}]_i$ due to damage caused by impalement, registered by mag-fura, but not by the Mg^{2+}-microelectrode. Clamping the neurone for 10 s from –60 mV to 0 mV to activate Ca^{2+} entry caused no change in the Mg^{2+}-microelectrode signal but an increase in the mag-fura-2 ratio, again attributed to an increase in the $[Ca^{2+}]_i$. The measurements of $[Mg^{2+}]_i$ in these experiments were carried out at a E_m of –60 mV where the $[Ca^{2+}]_i$ has been measured to be 40 nm/l. Kennedy [76] concluded that this $[Ca^{2+}]_i$ did not interfere with the Mg^{2+} measurements. A similar comparison was carried out [77] in pancreatic acini. In these experiments the initial increase in $[Mg^{2+}]_i$ in the mag-fura-2 experiments was shown to be interference due to Ca^{2+}. The problem could be avoided by loading the cells with the ester form of BAPTA. Because of the uncertainties involved in these measurements, the only sensible method is to measure the time course of both the Mg^{2+} and the Ca^{2+} signal in the preparation being investigated. If both are similar then caution should be exercised in attributing the changes in the mag-fura or mag-indo signal to changes in Mg^{2+}. Whether the increase in the ratio of mag-fura-2 is due to an increase in either Ca^{2+} or Mg^{2+} can be settled by either a direct comparison with Mg^{2+}-microelectrode (if possible) or by loading the cells with BAPTA. Despite this proviso, mag-fura-2 is a useful tool to study changes in the $[Mg^{2+}]_i$.

The problems discussed above, especially the calcium sensitivity, apply to mag-fura-2 and mag-indo. In a recent paper, Otten et al. [78] describe a new fluorescent indicator for Mg^{2+} based on 4-oxo-4H-quinolizine-3-carboxylic acids. *In vitro* experiments demonstrated that the indicator is not sensitive to mmolar concentrations of Ca^{2+}. This indicator could be a vast improvement on mag-fura-2, but at the time of writing no measurements on cells have been published.

Intracellular Distribution

With ester loading the fluorochrome is distribution between cytosol, mitochondria and SR. If the dye is distributed throughout all intracellular compartments then it is uncertain if changes in the signal are from cytosol and/or intracellular organelles, making interpretation of the results difficult. A substantial fraction of ester loaded dye is in intracellular organelles. Hongo et al. [79] concluded that, from mag-fura-2, 80 % was in cytoplasm and 20 % in the SR. Since release of Ca^{2+} or blocking Ca^{2+} uptake from the SR did not change the signal it was concluded that dye in the SR did not interfere with the signal. However, uptake of the dye by the mitochondria was not considered. With mag-indo, Silverman et al. [24] found that 60 % was in the cytosol and the remainder in the mitochondria. In their experiments the signal is considered to be an average of both components. However, it is possible to differentiate between cytosol and organelles by using the appropriate methods e.g. preferential loading of either cytosol or mitochondria or by using confocal microscopy.

Preferential loading of the cytosol: If the acid form of the probe is directly injected into single cells it is distributed only throughout the cytosol. This has been done by pressure injection of mag-fura-2 into single mouse skeletal muscle fibres [26], single frog skeletal muscle [74] and hippocampal CA1 neurones (Schulte, Günzel and Schlue, unpublished data; [80]) and for mag-indo into mouse skeletal muscle fibres [72]. The method has not yet been applied to myocytes, but aequorin has been successfully injected into cells in small trabeculae [81] and single myocytes [82]. There seems no reason to doubt that such a method would be successful in myocytes and it would get round the very serious problem of the distribution of mag-fura-2 between cytosol and mitochondria. To emphasise the seriousness of the problem, mitochondria in hepatocytes have been ester loaded with mag-fura-2 to measure *in situ* changes in the mitochondrial $[Ca^{2+}]$ [83]. Although technically more difficult than ester loading, direct injection of mag-fura/mag-indo into single cells is the method of choice.

Preferential loading of the mitochondria: In rat and rabbit heart, mitochondria make up some 35 % of the cell volume and the SR 3.5 % [84]. Two methods have been tried with fluorescent probes for Ca^{2+}. Miyata et al. [85] loaded cells with the ester form of indo-1 then used Mn^{2+} to quench the cytosolic signal. However, this method has the disadvantage that Mn^{2+} inhibits mitochondrial transport [86] so it is obviously not ideal. Griffiths et al. [87] loaded rat myocytes with the ester form of indo-1, a form that loads not only cytosol but mitochondria as well. Heat shock treatment at 25 °C for 2.5 hours followed by incubation at 37 °C for 1.5 hours removed indo-1 from the cytosol but not from the mitochondria. No attempts have been made so far to preferentially load heart cells with Mg^{2+} fluorochromes, but based on the results with the Ca^{2+} fluorochromes there is no reason to doubt that such attempts would be successful.

Confocal microscopy: A rapidly growing family of techniques developed from conventional light microscopy permit optical sectioning of individual cells or thin tissue samples, thus allowing rejection of the large percentage of out-of-focus information, particularly from objective lenses of high numerical aperture, which degrades the image by conventional flood-beam light microscopy [88]. The laser scanning confocal form of microscopy in particular, with appropriate laser wavelength selection, is especially suitable for fluorescence applications such as the Fura fluorochromes, yielding on-line images with resolution at around the limit of optical resolution (see [89]). The problems of uneven partitioning of fluorochrome, following the acetoxy-methyl ester form of loading, amongst cytoplasm and organelles have been discussed above. Especially in heart cells in which the mitochondria are numerous and large (~1 mm in length) and lie in rows between the fibrils, confocal microscopy offers an alternative means of visualising differences in fluorescence particularly between sarcoplasm and mitochondria.

Comparison Between Mg^{2+}-Macroelectrodes and Fluorochromes

Normally macroelectrodes are used to measure $[Mg^{2+}]$ in the extracellular solution and fluorochromes to measure $[Mg^{2+}]$ in the cytosol or intracellular organelles. However, in permeabilised myocytes, bathed in an intracellular-like solution, both can be used to measure the $\Delta[Mg^{2+}]$ in the bathing solution. Typical calibration curves for both Mg^{2+}-macroelectrodes and mag-fura for measurements in a cuvette, at one wavelength are shown in Figure 3A.

In this type of experiment the macroelectrodes have several advantages. As illustrated in Figure 3A, they can be accurately calibrated down to 0.1 μmol/l (pMg 7) using Mg^{2+} buffer solutions [31]. In contrast, fluorochromes can only be calibrated without using Mg^{2+} buffer solutions from 0.25 mmol/l upwards. At concentrations less than this, Mg^{2+} buffers are essential and these can only be prepared using macroelectrodes (see [31]). The fluorochromes, unlike the macroelectrodes are not constrained to the extracellular solution but are distributed between extracellular and intracellular volumes. An intracellular effect on the signal can be disregarded, as the intracellular volume is less than 1 % of total volume at a cell density of 10^6/ml [68]. Another advantage of Mg^{2+}-macroelectrodes is that they can be used to measure changes in the μmolar range which, as illustrated in Figure 3B, would be difficult with mag-fura-2.

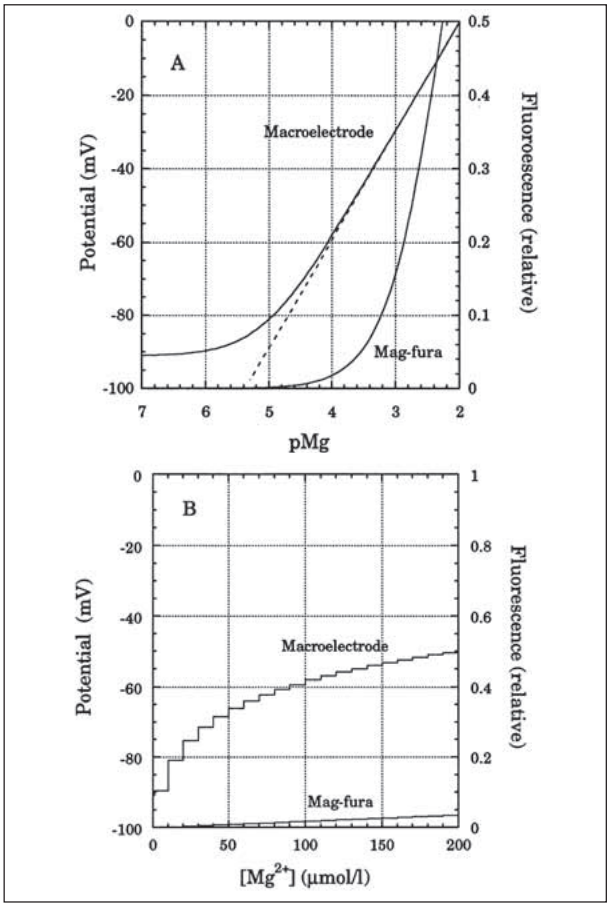

Figure 3. A) Calibration curves for Mg^{2+}-macroelectrode (ETH 7025) and mag-fura-2. The curve for the macroelectrode is based on Figure 2A of Lüthi et al. [31]. The dashed line represents the Nernstian response. The calibration curve for mag-fura is drawn for one wavelength using a K_d of 5.3 mmol/l at room temperature [1] **B)** Response of the Mg^{2+}-macroelectrode and mag-fura-2 for 10 μmol/l increases in $[Mg^{2+}]$ calculated from the respective calibration curves

^{31}P-NMR

This method depends on the shift of the β-phosphate peak of ATP relative to the α peak (see [90, 91]). The advantage of this method is that it is non invasive and can be applied to humans to estimate $[Mg^{2+}]_i$ (brain [92]; muscle [93]). The method can only be carried out in specialised units, is not continuous and requires the estimation of not only the α/β-shift but also pH_i, in order to estimate the Mg-ATP equilibrium constant. The estimated $[Mg^{2+}]_i$ depends directly on the Mg-ATP constant used for the calculation. Recent measurements of this equilibrium constant [65] under normal physiological conditions suggests that the most commonly used values in the literature are too low. Moreover, calculation of the effect of pH on the constant, assuming no binding of Mg^{2+} to $H\text{-}ATP^{3-}$, can introduce substantial errors into the estimation of the equilibrium constant. If the equilibrium constants in Lüthi et al. [65] are used, recalculation of the $[Mg^{2+}]_i$ from ^{31}P-NMR measurements brings them into line with more direct measurements using other methods [2]. A corollary to this is, that if the ionic conditions under which the ^{31}P-NMR measurements are carried out are vastly different from those in Lüthi et al. [65] then the Mg-ATP equilibrium constant has to be measured.

Atomic Absorption Spectrophotometry (AAS)

The normal method to measure μmolar concentrations of total Mg^{2+} or Ca^{2+} in cells in suspensions or in the extracellular solution is atomic absorption spectrophotometry (AAS [94]). Measurement of cellular uptake/loss over a given time period in cell suspensions or perfused tissues, requires repeated sampling of the extracellular milieu and/or repeated experiments to determine the total ionic content of the cells/tissues. Moreover, like measurements with macroelectrodes, the sensitivity of the method decreases as the $[Mg^{2+}]_o$ increases.

AAS has been the method of choice, used to measure net membrane fluxes in a variety of cells and tissues (see [95]). When measuring cellular changes in cell suspensions, allowances have to be made for A) the fluid trapped between the cells in the centrifuged cell pellet and B) the Mg^{2+} in this extracellular fluid. The volume of the trapped fluid can be estimated by using an extracellular marker such as ^{51}Cr-EDTA [19]. If the volume of the trapped fluid is measured, the extracellular Mg^{2+} content can be allowed for in the calculation of the total intracellular concentration: total cellular water being estimated from the wet/dry ratio of the centrifuged pellet [19].

Washing the cells with 150 mmol/l KCl [96] or centrifuging them through oil [97] will reduce the $[Mg^{2+}]_o$ to negligible levels. When washing with KCl or centrifugation through oil, the assumption is made that the $[Mg^{2+}]_o$ is approximately zero and can be ignored. This is a reasonable assumption, but the volume of the trapped fluid cannot be ignored. Ignoring the percentage of fluid trapped extracellularly, decreases the estimated intracellular concentrations by the same percentage. The errors are not large but an estimation of the amount of trapped fluid should at least be attempted. When studying total changes in whole heart preparations, the volume of the extracellular space has to be measured in order to calculate the total intracellular concentrations of the ions. Such experiments were carried out for Ca^{2+} by Bridge and Bassingthwaighte [98] who used ^{60}CoEDTA to estimate the volume of the extracellular space in rabbit heart.

Electron Probe X-Ray Microanalysis

Like AAS, EPXMA is used to measure the total elemental composition of the tissues and ionised and bound forms of the elements cannot be distinguished. Measurements are not

"on line" as elements present at the time of sampling are measured by these techniques. Elemental detection limits of EPXMA are several orders of magnitude poorer than AAS, being limited to mmol/l concentrations, but the important advantage is that EPXMA can yield spatial localisation of the elements to within small fractions of a micrometre [99] and thus cytosolic and individual organelle measurements can be distinguished. Preparation is by the technically demanding processes of ultra-fast freezing [100], usually followed by cryoultrathin sectioning and dry transfer for microanalysis when all of the main electrolyte elements can be measured simultaneously [101]. Amongst recent applications the technique has been used to measure changes in cells from ventricular trabeculae [102], cultured cell suspensions [103] as well as isolated heart cells [104].

Molecular Methods

While during the past decade attempts for a molecular identification of Mg^{2+} had only been successful in prokaryotes (for review see Smith and Maguire [105]) there is now some recent progress in eukaryotes. Shaul et al. [106] were able to identify a Mg^{2+}/H^+ exchanger in the vacuoles of the plant Arabidopsis thaliana (AtMHX), and homologues of the bacterial Mg^{2+} channel CorA have now been found in the yeast cell membrane (Alr1 [107]; [108]), as well as in yeast mitochondria (yMrs2, Lpe10 [109]; Gregan et al., unpublished data) and human mitochondria (hsaMrs2 [110]). Furthermore, a Ca^{2+}- and Mg^{2+}-permeable divalent cation channel was recently cloned from haematopoietic cells [46]. The proteins characterised so far either allow Mg^{2+} influx into the cytoplasm, or influx into cell organelles. As yet, no protein responsible for the extrusion of Mg^{2+} from the cell has been identified, however, this appears to be only a matter of time.

Mg^{2+} Homeostasis in Heart

There are several excellent reviews covering various aspects of Mg^{2+} transport and homeostasis ([8, 90, 105, 111–117]; Mineral and Electrolyte Metabolism, July/October issue 1993 contains a series articles about Mg^{2+}; the on-line journal, Frontiers in Bioscience, 5, 2000 also contains a series of review articles on magnesium).

An overview of magnesium homeostasis in heart is shown in Figure 4. There is a background influx of Mg^{2+} of 0.3 pmol/cm^2.s [7], which necessitates an active efflux mechanism or mechanisms [111]. Influx/efflux are under hormonal control [32] and intracellularly Mg^{2+} ions are not only buffered by ATP and other buffer substances but can also be taken up by intracellular organelles such as mitochondria and the sarcoplasmic reticulum (see [65]). Intracellular buffering can be modified and in the example illustrated this can occur through the binding of polyamines (eg spermine) to ATP [118]. A further possible complication when considering Mg^{2+} homeostasis in heart is the presence of a subcellular or "fuzzy" space where the concentrations of ions (eg Na^+, K^+, Ca^{2+}) can be temporarily higher than in the bulk cytosol [119, 120]. All these systems interact to regulate the $[Mg^{2+}]_i$. Each of these mechanisms will now be considered in detail.

Influx

It is uncertain how Mg^{2+} ions penetrate into heart cells. There are two possibilities, namely channels and/or a carrier mechanism. Channels have been found in Paramecium [45], and putative Mg^{2+} channels have been described in vesicles from the proximal tubule of the kidney and from the brush border of proximal intestinal epithelium of fish [121, 122]. Recently, Mg^{2+} permeable channels were cloned from yeast [107, 108] and from human haemopoietic cells [46]. In neurones it was found, that Mg^{2+} can pass through glutamate receptor-coupled cation channels, and NMDA-receptors [123], and AMPA-receptors [35, 36]. However, at present the relevance of these findings to heart is not known. Thirty years ago Page and Polimeni [7] interpreted their experimental findings as a carrier mechanism in rat heart and little has been added to our understanding of Mg^{2+} influx in heart since then.

Efflux

The various possibilities for extrusion are listed in Table 3. Na^+/Mg^{2+} in one form or other is a very probable candidate for such a mechanism and has been described in various cells (see [111, 114]). In heart however, the results are confusing. The results of microelectrode investigations into Na^+/Mg^{2+} extrusion in ferret and guinea-pig ventricular muscle [1, 70, 124] showed no change in $[Mg^{2+}]_i$ on reducing the $[Na^+]_o$ from 155 to 5 mmol/l and no change in $[Mg^{2+}]_i$ when the $[Na^+]_i$ was increased with cardiac glycosides. Increasing the $[Mg^{2+}]_o$ from 0.5 mmol/l to 10 or 20 mmol/l increased the $[Mg^{2+}]_i$ by 0.11 and 0.33 mmol/l respectively [1], but this was not associated with a decrease in the $[Na^+]_i$. These negative results, in view of the findings on the Na^+/Ca^{2+} exchange in heart [4, 15] were interpreted as not furnishing direct evidence for the presence of a Na^+/Mg^{2+} exchanger in ventricular muscle. In contrast to these experiments there is evidence to support a Na^+/Mg^{2+} antiporter in heart. Handy et al. [18] were able to load single ventricular cells with Mg^{2+} by using a solution containing no Na^+ or Ca^{2+} and 5 mmol/l Mg^{2+}. The recovery from this load was Na^+-dependent and could be inhibited by imipramine, suggesting the presence of a Na^+/Mg^{2+} antiporter. However, full recovery only occurred if both Ca^{2+} and Na^+ were present but the reason for this is not known. Romani et al. [32] found, in myocyte suspensions, that the net efflux with adrenaline was Na^+ dependent. Günther and Vormann [16] using perfused rat heart in which the $[Mg^{2+}]_i$ had been increased by reducing the ATP concentration with iodoacetic acid or potassium cyanide, found that recovery to normal $[Mg^{2+}]_i$ levels was Na^+-dependent, and that this efflux could be blocked by amiloride. Experiments using electron probe analysis on ventricular muscle showed that during perfusion with 5 mmol/l extracellular Na^+ there was an increase of total Mg in myofibrils and in the

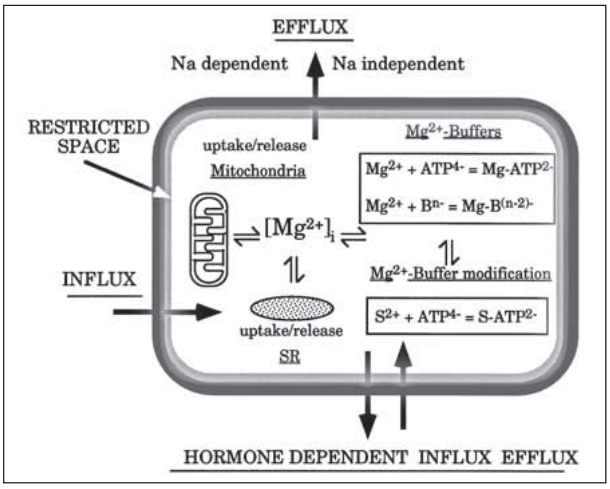

Figure 4. Diagram of a heart cell, illustrating influx, possible efflux pathways, subcellular or "fuzzy space" as well as various possibilities for maintaining $[Mg^{2+}]_i$ constant through uptake/release from organelles and buffering. Possible hormonal regulation is also included. Modified from Lüthi et al. [65]

mitochondria [102]. Fry [125] using the neutral carrier ETH 1117 did find evidence for a Na^+/Mg^{2+} in guinea-pig ventricle. However, ETH 1117 reacts to both Na^+ and K^+ as well as Mg^{2+}, making interpretation of these results difficult.

In view of the seeming incompatibility between the Mg^{2+}-microelectrode experiments and the fluorescent and total flux studies the question arises, whether the microelectrode investigations were carried out under the appropriate experimental conditions to demonstrate a Na^+/Mg^{2+} exchanger. We have examined in more detail the microelectrode experiments with special regard to a putative Na^+/Mg^{2+} exchanger and to the interaction between the Na^+/Ca^{2+} and Na^+/Mg^{2+} exchangers [126].

Interaction Between Na^+/Ca^{2+} and Na^+/Mg^{2+} Exchangers

At first glance it might be thought that reducing $[Na^+]_o$ from 155 mmol/l to 5 mmol/l should block a Na^+/Mg^{2+} exchanger and the $[Mg^{2+}]_i$ should increase due to the Na^+/Mg^{2+} reversing direction, effluxing Na^+, and influxing Mg^{2+}. This would cause a simultaneous decrease in $[Na^+]_i$ and an increase in $[Mg^{2+}]_i$. In 5 mmol/l $[Na^+]_o$ the background Mg^{2+} influx will continue, but with an estimated background leak of 0.3 pmol/cm^2.s [7] the gain of Mg^{2+} over one minute would result in a rise of 0.06 mmol/l. In contrast, the expected gain due to the reversal of the Na^+/Mg^{2+} exchanger would result in a rise of several mmol/l over the same period of time (cf. [4, 15]).

In heart cells the Na^+/Ca^{2+} exchanger, exchanges 3 Na^+ for Ca^{2+}. Because of this exchanger, $[Na^+]_i$ halves within 30 seconds when $[Na^+]_o$ is reduced from 155 to 5 mmol/l and is below 2 mmol/l after 6 minutes; most of this loss of intracellular Na^+, is in exchange with Ca^{2+} (see [4, 15, 98]). For a 2 Na^+/1 Mg^{2+} exchanger after about 1 minute, due to the decrease of the $[Na^+]_i$, the $[Mg^{2+}]_{Eq}$ would be under 0.81 mmol/l and the exchanger would be effluxing Mg^{2+}. Since most of the intracellular Na^+ loss is due to exchange with Ca^{2+} the actual uptake of Mg^{2+} can only be a small (unknown) fraction of the total cellular Na^+ loss. Cellular Mg^{2+} is well muffled (see below) so a marked increase in $[Mg^{2+}]_i$ on Na^+ removal in Ca^{2+} containing solutions is unlikely. An increase in $[Na^+]_i$ will only increase the $[Mg^{2+}]_i$ provided the shift in $[Mg^{2+}]_{Eq}$ is large enough to be measured. With a neutral 2 Na^+/1 Mg^{2+} exchanger, a doubling of the $[Na^+]_i$ would move the $[Mg^{2+}]_{Eq}$ by around 0.016 mmol/l; this difference in the $[Mg^{2+}]_i$ would not be measurable. Increasing $[Mg^{2+}]_o$ to 20 mmol/l, in the presence of 155 mmol/l Na^+ would not reverse a 2 Na^+/1 Mg^{2+} transporter and a decrease of $[Na^+]_i$ would not be expected under these circumstances.

If the stoichiometry of a Na^+/1 Mg^{2+} exchanger in heart is 1 to 1 then based on the mean concentration values, either it has to have an additional energy input or a second efflux system has to be present (see above). If a second Mg^{2+} efflux system was present it could modify the response to ionic changes. On Na^+ removal this second efflux system could maintain the $[Mg^{2+}]_i$ constant especially since Na^+ is exchanging for Ca^{2+}. However, the effects of increasing the $[Mg^{2+}]_o$ from 0.5 to 20 mmol/l in 155 mmol/l $[Na^+]_i$ would change $[Mg^{2+}]_{Eq}$ from 1.0 to 40 mmol/l and if the system, like the Na^+/Ca^{2+} exchanger could equally well influx as well as efflux Mg^{2+}, a decrease in $[Na^+]_i$ would be expected. This was never seen [1], so it can be concluded that if a 1 Na^+/1 Mg^{2+} exchange system is present in heart then it rectifies in an inward direction and in this sense is not reversible. If the system cannot reverse, then increasing the $[Na^+]_i$ by cardiac glycosides [4] would not be expected to change $[Mg^{2+}]_i$ due to a reversal of the exchanger.

In conclusion, when due consideration is given to the stoichiometry of a putative Na^+/Mg^{2+} exchanger, coupled with the possibility that it could be irreversible and the presence of a reversible Na^+/Ca^{2+} in this tissue, the microelectrode experiments do not negate the presence of a Na^+/Mg^{2+} exchanger in heart. There is now no contradiction in the literature and we regard the debate on the presence of a Na^+/Mg^{2+} exchanger in heart as settled in its favour.

Stoichiometry of Na^+/Mg^{2+} Exchange

Calculations of $[Mg^{2+}]_{Eq}$ using the mean values of $[Na^+]_i$ and $[Mg^{2+}]_i$ for stoichiometries of 1, 2 and 3 give values of 1.0 mmol/l, 0.0045 mmol/l and 0.02 μmol/l respectively at an E_m of –78 mV. These are mean values and it could be, that in those cells with a low $[Mg^{2+}]_i$, there is also a low $[Na^+]_i$ and a correspondingly less negative membrane potential, so that a 1 Na^+/1 Mg^{2+} exchanger would be sufficient to maintain the $[Mg^{2+}]_i$ in these cells. This was investigated by making use of the extensive data on E_m, $[Na^+]_i$ and $[Mg^{2+}]_i$ in ferret and guinea-pig heart [1, 4] and also the recalculations in Fry et al. [127]. The $[Na^+]_o$ and $[Mg^{2+}]_o$ were 155 and 0.5 mmol/l respectively. In microelectrode measurements it is log (concentration) that is normally distributed [127], but for simplicity the distribution of $[Mg^{2+}]_i$ has been linearised in Figure 5A. If a transporter is going to efflux Mg^{2+} then $[Mg^{2+}]_{Eq}$ has to be less than the measured $[Mg^{2+}]_i$ ie the distribution of $[Mg^{2+}]_{Eq}$ has to lie to the left of the $[Mg^{2+}]_i$ corresponding to curve "a". If the $[Mg^{2+}]_{Eq}$ is to the right of the $[Mg^{2+}]_i$ distribution curve such as in "b" then the exchanger would cause a Mg^{2+} influx and a concomitant decrease in the $[Na^+]_i$. The calculated $[Mg^{2+}]_{Eq}$ for a 1 Na^+/1 Mg^{2+} exchanger as well as the frequency distribution for $[Mg^{2+}]_i$

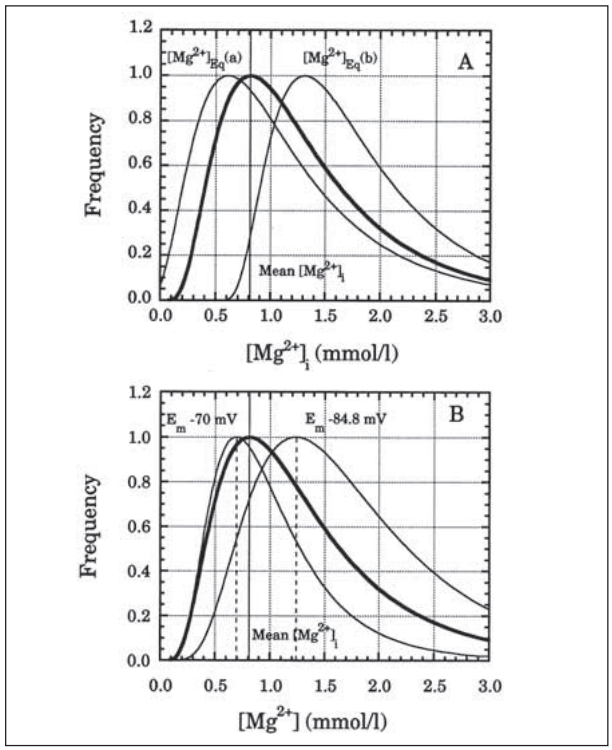

Figure 5. A) Frequency distribution of measured $[Mg^{2+}]_i$ from [1] is shown as thick line. The curve labelled "a" is the distribution that would allow a Na^+/Mg^{2+} exchanger to efflux Mg^{2+} at every measured $[Mg^{2+}]_i$, the curve labelled "b" and to the right of the measured frequency distribution would be influxing Mg^{2+} **B)** As in A the thick line is the frequency distribution of the measured $[Mg^{2+}]_i$. To illustrate the effect of membrane potential (E_m) on a 1 Na^+/1 Mg^{2+} exchanger the distribution of $[Mg^{2+}]_{Eq}$ for two E_m values, namely –70 mV, the lowest acceptable E_m value and –84.8 mV, a value 1 SD more negative than the mean E_m value from these experiments

are illustrated in Figure 5B, based on the measured $[Na^+]_i$ of Chapman et al. [4] as recalculated in Fry et al. [127]. In these experiments, the criterion for acceptance was an E_m more negative than –70 mV. This is the curve immediately to the left of the $[Mg^{2+}]_i$ measurements. At $[Mg^{2+}]_i$ greater than 0.81 mmol/l a 1 Na^+/1 Mg^{2+} exchanger could efflux Mg^{2+} at this membrane potential. However, at $[Mg^{2+}]_i$ less than this, $[Mg^{2+}]_{Eq}$ is very similar to $[Mg^{2+}]_i$ and with a background leak it is extremely doubtful if a 1 to 1 exchanger could maintain the $[Mg^{2+}]_i$ at this concentration.

The situation is even worse at more negative membrane potentials. The mean ± SD E_m in these experiments was –78.0 ± 6.9 mV and the curve calculated for an E_m of –84.9 mV (mean – 1 SD from mean) is also illustrated. This is shifted to the right and at that membrane potential a 1 Na^+/ 1 Mg^{2+} exchanger would not be effluxing Mg^{2+}. Similar calculations for a 2 Na^+/ 1 Mg^{2+} exchanger showed that even at the largest $[Na^+]_i$, $[Mg^{2+}]_{Eq}$ was still in the μmol/l range.

However, recent experiments on Mg^{2+} extrusion in rat myocytes have shown that the efflux is potential-dependent, when E_m was changed either by increasing the $[K^+]_o$ or by patch clamping. The efflux increased on depolarisation and decreased on hyperpolarisation; this is what would be expected of a one to one exchanger [8, 44]. The Na^+ dependence of the Mg^{2+} efflux was also consistent with a one to one exchanger [128]. Despite the reservation expressed above, this evidence points to the exchanger being 1 Na^+/1 Mg^{2+} and thus electrogenic. If this were so, then the $[Mg^{2+}]_{Eq}$ would depend on heart rate (see above) and there would be a feedback between heart rate and the $[Mg^{2+}]_i$.

The calculations shown in Figure 5, suggest that if a 1 Na^+/1 Mg^{2+} exchanger is present in heart, as can be concluded form the experiments of Flatman and colleagues, then a second extrusion mechanism is necessary. However, this conclusion is based on measurements of the intracellular concentrations and E_m measurements, obtained in different experiments using the same preparation. While such measurements are suggestive they are not conclusive. The only way to accurately determine the stoichiometry of the Na^+/Mg^{2+} exchanger is to measure $[Na^+]_i$, $[Mg^{2+}]_i$ and E_m simultaneously in the same preparation, as has been done in leech neurones using triple barrelled microelectrodes [17]. As discussed earlier, application of triple barrelled electrodes in heart is probably not possible, but doubled barrelled Na^+/Mg^{2+} microelectrodes coupled with an independent measurement of E_m would seem a possibility to decide the issue.

Other Efflux Mechanisms

As illustrated in Table 3, a Na^+/Mg^{2+} exchanger is only one of many possible extrusion systems. In the Table, eleven potential transport systems are anion-dependent. The experiments of Ödblom and Handy [129] in rat myocytes showed that if DIDS was added to the loading solution (zero Na^+ and Ca^{2+}, 5 mmol/l Mg^{2+}) of Handy et al. [18] there was a larger rise in $[Mg^{2+}]_i$ than in the absence of DIDS. This was attributed by these authors to a Mg^{2+} anion symport, most probably together with HCO_3^-. The function of Cl^- ions in these experiments remains unclear, as a 1 Cl^-, 2 HCO_3^-, Mg^{2+} symport would be unlikely (see Table 3). In view of the recent finding of Günzel et al. [12] of a malate Mg^{2+} symport in leech neurones it would seem worthwhile to consider the possibility of the presence of such an exchanger in heart. Since the concentrations of organic anions are metabolically controlled, this could be another example of feedback between activity and the $[Mg^{2+}]_i$.

No serious attempts have been made to investigate the presence or absence of other exchangers in heart. A Ca^{2+}/Mg^{2+} exchanger has been described in plasma membrane vesicles from rat liver [130] and a 2 H^+/1 Mg^{2+} exchanger has been proposed for Mg^{2+} extrusion in the epithelia cells from sheep rumen [40]. The relevance of these exchangers to heart is unknown.

Intracellular Muffling

It has become clear that the $[Mg^{2+}]_i$ in heart is maintained remarkably constant and the only known physiological method to change $[Mg^{2+}]_i$ over a 30 minute period is to reduce the buffering capacity by increasing the polyamine concentration [65]; other methods such $[ATP]_i$ reduction and loading solutions [18] cannot be considered physiological. Heart cells possess very effective mechanisms capable of damping the swings in the cytosolic $[Mg^{2+}]$.

There are several lines of defence to prevent changes in the $[Mg^{2+}]_i$ and as illustrated in Figure 4 these include, membrane transport systems, slow diffusion in the subcellular space, Mg^{2+} buffers and uptake/release from organelles. All these processes act together to maintain the $[Mg^{2+}]_i$ constant in the bulk cytosol. This has been referred to as "intracellular buffering" but buffering refers to a chemical process eg binding of Mg^{2+} to ATP and does not imply membrane transport or uptake/loss from organelles. To make a distinction between physical chemical buffering and transport processes, the term "muffling" was introduced to cover both [131] and will be used in this article to make the distinction between the two processes. This problem with terminology is not new for the difference between physical chemical buffering, and physiological regulation of pH was discussed at a symposium in 1971 [132]. The various aspects of muffling are discussed below.

Fuzzy Space

It has been demonstrated that in a nm region immediately adjacent to the cell membrane, there is limited diffusion and in this space the ionic concentrations can be different from those in the bulk cytosol. Because of this, this region next to the cell membrane has been designated the "fuzzy space" [99, 119, 120]. This space with restricted diffusion will contribute to muffling, in the sense that slow diffusion in this space will prevent marked changes in the $[Mg^{2+}]$ spilling over into the body of the cytosol and allow time for the membrane transport system to normalise the changes in the $[Mg^{2+}]_i$.

Cytosolic Buffers

The second line of defence is the physical buffer systems in the cytoplasm. While the characteristics of ATP Mg^{2+} binding, in solutions mimicking the intracellular cation concentration of heart cells have been determined [65], little or nothing is known about the characteristics of other buffer systems in heart. That such systems exist was demonstrated by Grubbs and colleagues in chick embryonic ventricular cells [133, 134] and it is of some interest to speculate what might be expected from such additional buffer system(s). The necessary equations for the calculations are from the Appendix to Lüthi et al. [65]. The ATP concentration was taken as 10 mmol/l [135] and the binding constants for Mg-ATP from Lüthi et al. [65].

Modelling a second system has to be, at present, arbitrary. The concentration of the second buffer was taken as 20 mmol/l, it was assumed that the efflux was completely blocked, there was no uptake into intracellular organelles and the influx was over a 10 minute period. The change in $[Mg^{2+}]_i$ ($\Delta[Mg^{2+}]_i$) under these circumstances, for fluxes of 0.3 pmol/cm^2.s (background, [7]) as well as for fluxes of 1 and 3 pmol/cm^2.s were calculated using the volume/area ratio for heart cells from Page [84]. The first question addressed was

"What would be the most advantageous K_{app} for a second system to minimise the changes in $[Mg^{2+}]_i$?" This is illustrated in Figure 6A where $\Delta[Mg^{2+}]_i$ for the three fluxes is plotted against K_{app}, the values varying from 0.1 to 8 mmol/l.

The Figure illustrates that not only is there an optimal K_{app} for a second buffer system but also, at a background influx of 0.3 pmol/cm^2.s there is under these conditions, little or no change in the $[Mg^{2+}]_i$. The optimal K_{app} increases as the load increases, but this is to be expected from the characteristics of a buffer. If the change in $[Mg^{2+}]_t$ is minimal, then buffering is most efficient at the midpoint of the curve; if on the other hand the change in $[Mg^{2+}]_t$ is large then buffering a load is most efficient if the initial $[Mg^{2+}]_i$ lies at the lower end of the buffer curve. This can be achieved by increasing K_{app}, which will move the buffer curve to the right. These calculations are for an increase in the total Mg^{2+} and similar calculations for a decrease would show the opposite effect. In heart the $[Mg^{2+}]_i$ of 0.81 mmol/l is at the upper end of the ATP buffer curve and is thus maximally buffered against a decrease in concentration (see Figure 4 in Lüthi et al. [65]).

At a resting flux of 0.3 pmol/cm^2.s the optimal K_{app} is 0.8 mmol/l, a value similar to that estimated for the second buffer system in Retzius neurones of the leech [27]. Using this K_{app} value, the increase in $[Mg^{2+}]_i$ can be calculated as illustrated in Figure 6B for a total buffer capacity of 10 and 20 mmol/l; also shown is the total increase in $[Mg]_t$ over the time period of 30 minutes. The Figure illustrates that, with a background leak of 0.3 pmol/cm^2.s and efflux completely blocked, some 80 % of the total Mg^{2+} uptake could be bound. Since mitochondria also take up Mg^{2+} [102, 136] the changes in $[Mg^{2+}]_i$ would be even less. Cytosolic Mg^{2+} buffer systems other than ATP have not been characterised in heart, but

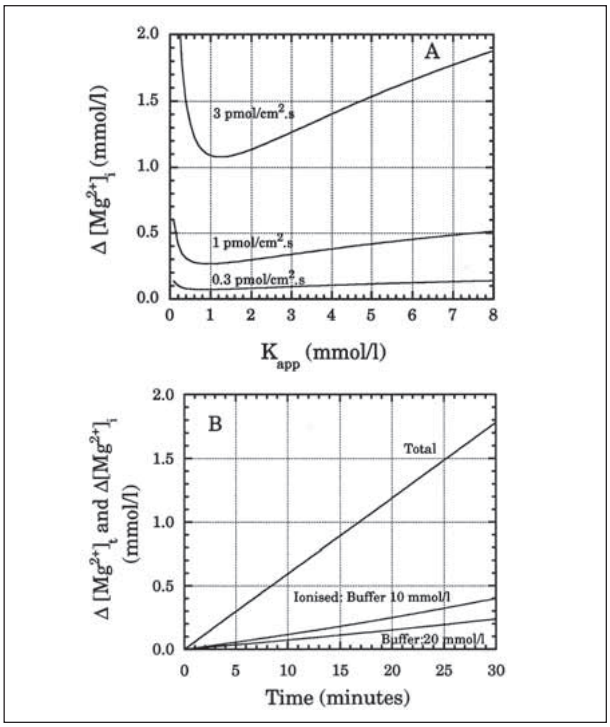

Figure 6. A) Calculation of the change ($\Delta[Mg^{2+}]$) in the $[Mg^{2+}]_i$ from the resting concentration of 0.81 mmol/l as the K_{app} of a second buffer system is varied from 0.1 to 8 mmol/l for the three fluxes; change calculated after a 10 minute time interval **B)** Comparison between the change in total Mg^{2+} ($\Delta[Mg]_t$) and the ionized ($\Delta[Mg^{2+}]_i$). Changes in ionized, calculated for two buffer concentrations and with a K_{app} of 0.8 mmol/l for second buffer system

Günzel et al. [27] have postulated that actin could be play an important role in cytosolic buffering in Retzius neurones of the leech. In view of the finding of an increase in the total Mg bound to myofibrils when the $[Na^+]_o$ was reduced from 155 to 5 mmol/l [102] it is possible that in heart actin could also be an important cytosolic buffer.

Modification of cytosolic buffers: At a given $[Mg]_t$, if the buffer capacity is reduced, the $[Mg^{2+}]_i$ will increase. However, the new increased $[Mg^{2+}]_i$ will only be maintained if there are concomitant changes in the efflux mechanism(s) to prevent Mg^{2+} being pumped out of the cell, reducing $[Mg]_t$ and returning the $[Mg^{2+}]_i$ to normal, at the reduced buffer capacity. Modification of the cytosolic buffer capacity will only produce a transient change in the cytosolic $[Mg^{2+}]_i$. With this proviso in mind, the cytosolic buffer capacities can be altered by polyamines, pH and possibly by intracellular Ca^{2+}. It is not known if other substances could modify intracellular cytosolic buffering. A decrease in the ATP concentration in heart, as does occur in ischaemia, will increase the $[Mg^{2+}]_i$ [137, 138]. However such a decrease is not physiological and will not be considered further.

The binding of Mg^{2+} to ATP is pH dependent [65] but there is little change in the binding until the pH is less than 6.5; thus under normal physiological conditions there will be little change in the ATP binding. The pH dependence of the other buffer systems has not been characterised in heart. Polyamines have a greater affinity for ATP than Mg^{2+} ions so any change in the intracellular concentrations of the polyamines will change the amount of Mg^{2+} bound to ATP. Polyamines are known to increase in hypertrophy of the heart and, in these hearts, less Mg^{2+} will be bound to ATP (see [65]), but whether this will cause an increase in $[Mg^{2+}]_i$ depends on the membrane transport systems. The relevance of polyamines to normal Mg^{2+} regulation in heart is unknown.

$[Ca^{2+}]_i$ increases from around 200 nmol/l to 10 μmol/l in ischaemia [139] but such changes would not affect the Mg^{2+} binding to ATP [65]. In cultured chicken heart cells, Na^+ removal caused an increase in $[Mg^{2+}]_i$ when measured with mag-fura-2 [140]. Under these conditions there is an increase in $[Ca^{2+}]_i$ and the increase in $[Mg^{2+}]_i$ was attributed to competition between Mg^{2+} and Ca^{2+} for common binding sites. In these experiments the $[Ca^{2+}]_i$ increased to 1.5 μmol/l so no Ca^{2+} binding to ATP would be expected; binding would have to be to other Mg^{2+} buffers. However, as discussed in page 12, mag-fura-2 also reacts to Ca^{2+}. In similar experiments with Mg^{2+}-microelectrodes no changes in $[Mg^{2+}]_i$ were found in ferret trabeculae on Na^+ removal and the changes measured in $[Mg^{2+}]_i$ myocytes with mag-fura-2 on Na^+ removal were attributed to the fluorochrome reacting to Ca^{2+}. It is difficult to say at present if the changes measured by Murphy et al. [140] are wholly due to the reaction between mag-fura-2 and Ca^{2+} or represent competition between Mg^{2+} and Ca^{2+}.

Mitochondria

It has been shown by EPXMA that mitochondria take up Mg in liver on hormonal stimulation with glucagon or vasopressin [141]. In HL60 cancer cells differentiation induced by retinoic acid induces a decrease in the total Mg content of the mitochondria [103] and in heart, on perfusion with 5 mmol/l Na^+ solution [102] there is also an increase in the Mg content of the mitochondria. Although not as yet measured by EPXMA, this Mg^{2+} must be released on returning to Na^+ containing solution to maintain a steady state. Release of Mg^{2+} on alkalisation of the mitochondria has been demonstrated in Retzius neurones of the leech. Since the response

was 50 % blocked by cyclosporin A, an inhibitor of the mitochondrial permeability transition pore (MTP), and increased by phenylarsine oxide, an activator of the pore, it was concluded that Mg^{2+} was released by opening of the MTP [142]. Such experiments demonstrate that there is a net flux of Mg^{2+} across the mitochondrial membrane.

There have been numerous studies of Mg^{2+} transport in isolated mitochondria but the initial studies were carried out in sucrose solutions, buffered with organic buffers. Moreover, in many cases no attempt was made to buffer the $[Ca^{2+}]$ and since contamination can be as high as 20 μmol/l [67] the $[Ca^{2+}]$ in these experiments could have been outwith the normal physiological range. Both these factors make the relevance of such studies doubtful to mitochondrial transport under intracellular conditions (see [143]).

Despite these reservations it appears that Mg^{2+} ions enter the mitochondria due to the membrane potential, $\Delta\Psi$. This pathway is separate from that of Ca^{2+} and is not blocked by ruthenium red. Efflux is probably via a H^+/Mg^{2+} antiport. However the characteristics of both the influx and efflux mechanisms, and how they are regulated, remain obscure (reviewed [144; 145]). Two studies on isolated mitochondria in physiological-like solutions [146] in 120 mmol/l KCl or NaCl, 20 mmol/l Tris, pH 7.4; [143], 100 mmol/l KCl, 10 mmol/l TES, 30 mmol/l EGTA, pH 7.2 have attempted to determine the $[Mg^{2+}]_i$ at which influx and efflux were in equilibrium finding values of 2.5 and 5 mmol/l respectively. This does not make physiological sense as the $[Mg^{2+}]_i$ is 0.81 mmol/l [1]. There are other differences between isolated mitochondria and mitochondria *in situ*. Jung and Brierley [145] draw attention to the fact that K^+ inhibits the Mg^{2+} uptake by isolated mitochondria; the uptake in 200 mmol/l sucrose is reduced to just under 1 % in 100 mmol/l KCl medium. However, Jung and Brierley [145] and Altschuld et al. [147] found that the specific activity for $^{28}Mg^{2+}$ in the mitochondrial fraction was the same as that of the whole cell over an extended time period, suggesting that the uptake *in viv*o was as least as rapid as across the cell membrane. This emphasises again the necessity to carry out such experiments in, as near as possible, physiological conditions.

A major advance in the study of mitochondrial transporters was the discovery that the mitochondrial proteins Mrs2p/Lpe10p, distant relatives of the bacterial CorA Mg^{2+} transporters present in yeast mitochondria, are also coded for in the human genome [148]. In yeast these proteins transport Mg^{2+} into mitochondria. Over-expression results in a significant increase in Mg^{2+} uptake by yeast mitochondria and a reduction in the expression of the proteins causes a reduction in the transport [136]. These results can only be described as intriguing as, due to the mitochondrial membrane potential, it would seem unnecessary to transport Mg^{2+} into mitochondria and moreover, no analogous proteins capable of transporting Mg^{2+} out of mitochondria have yet been described.

In summary while it is accepted that mitochondria can take up and release Mg^{2+}, there is little information on how this occurs or is regulated under physiological conditions, specifically at physiological $[Ca^{2+}]_i$. Depending on the characteristics of uptake/release of Mg^{2+} the mitochondria, because of their size and distribution in cardiac muscle, could well be in the position to regulate the cytosolic $[Mg^{2+}]$ since changes in $[Mg^{2+}]_i$ in the vicinity or domain of a mitochondrion could be compensated for by uptake or release of Mg^{2+} [149].

Conclusions Muffling

It has been established that the cytosolic $[Mg^{2+}]$ is maintained constant despite numerous experimental manipulations [1, 70]. This arises (see Figure 4) due to membrane transport systems, "fuzzy space", cytosolic buffers, uptake/release from mitochondria and an initial concentration of around 1 mmol/l. All these processes combine to dampen changes in the cytosolic $[Mg^{2+}]_i$. An important corollary to this is that if putative changes in the $[Mg^{2+}]_i$ are measured, then the possible flux across the membrane has to be calculated with due consideration of the intracellular muffling. This muffling also makes it difficult but not impossible to perturb the system in order to investigate membrane transport. To transiently change the cytosolic $[Mg^{2+}]$ either influx has to be increased or efflux decreased, cytosolic buffering capacity changed and/or the mitochondrial uptake or release mechanisms altered.

Hormones

Using AAS it has been established that catecholamines cause a net loss of Mg^{2+} from perfused heart and from myocytes, and that carbachol causes an increased uptake of Mg^{2+}. On the bases of pharmacological studies it was proposed that the catecholamines release Mg^{2+} from the mitochondria and that the carbachol induced uptake is into the SR, although there is no direct evidence to support this claim [150]. Although doubt was cast on the original experimental findings by [147], the experiments have been successfully repeated by other groups ([151, 152]; Figure 2). More recent experiments have demonstrated that insulin in the presence of glucose can cause an uptake of Mg^{2+} and could also inhibit the loss caused by catecholamines [153]. Other hormones have not been studied, nor has the action of glutamate on the heart been studied (see above).

Changes in Cytosolic $[Mg^{2+}]$

Since catecholamines cause a loss of Mg^{2+} from myocytes, it might be surmised that there should be an increase in the $[Mg^{2+}]_i$, so that efflux mechanisms could be activated to transport Mg^{2+} out of the cell. This does not appear to be the case as long as the $[ATP]_i$ is not reduced. Buri et al. [1] with microelectrodes in ferret trabeculae, Gow et al. [154] and Hongo et al. [79] using mag-fura-2 in isolated rat ventricular cells, found no change in $[Mg^{2+}]_i$ on application of catecholamines. That the changes in cytosolic $[Mg^{2+}]$ were either absent or minimal has been shown in a recent study with mag-fura-2 on myocytes and ^{31}P-NMR in perfused hearts [33]. This study (mag-fura-2) showed, in myocytes, that mimicking catecholamine action with cAMP caused a maximal decrease of $[Mg^{2+}]_i$ of 0.149 ± 0.016 mmol/l, and SAG (1-stearoyl-2-arachidonoyl-sn-glycerol) to activate protein kinase C and mimic the action of carbachol, caused an increase of 0.09 ± 0.01 mmol/l. In perfused hearts however, β-adrenergic stimulation showed no change in $[Mg^{2+}]_i$ as measured with ^{31}P-NMR. The reason for the difference between myocytes and perfused hearts is unclear, but any measured changes in $[Mg^{2+}]_i$ were minimal.

If, during the application of catecholamines, the $[ATP]_i$ decreases then $[Mg^{2+}]_i$ would be expected to increase and this has been found to be the case [155, 156], In a ^{31}P-NMR study Nishimura et al. [157] applied isoprenaline for 100 minutes. During the first 25 minutes there was an initial increase in $[Mg^{2+}]_i$ followed by a decrease. On reperfusion with normal solution the $[Mg^{2+}]_i$ recovered to normal levels, although the $[ATP]_i$ had decreased to 46 % of the initial concentration. Assuming that ATP is the main buffer, these hearts would have had to lose around 50 % of their total Mg^{2+} during perfusion with isoproterenol in order for the $[Mg^{2+}]_i$ to have returned to resting levels. A more recent paper by the same group [158] found only a decrease on appli-

cation of isoprenaline. Again it has to be assumed that the efflux is such that the total Mg^{2+} is decreasing faster than the measured decrease in ATP. Be that as it may, it has to be emphasised that a decrease in $[ATP]_i$ in heart cannot be regarded as physiological, and under physiological conditions there does not appear to be a change in the $[Mg^{2+}]_i$.

Attempts to measure changes in $[Mg^{2+}]_i$ using Mg^{2+}-microelectrodes in heart trabeculae on the application of carbachol or insulin were also unsuccessful (McGuigan, unpublished data) However, the effect of insulin was studied in Tyrode using pyruvate as a substrate and Romani et al. [153] only observed the increased uptake with glucose as a substrate, so the microelectrode experiments carried out with pyruvate as a substrate, cannot be regarded as conclusive. Despite this proviso, it can be concluded that despite marked changes in the $[Mg]_t$ there are no, or at most minimal changes in cytosolic $[Mg^{2+}]$ on the application of either catecholamines or carbachol.

Mechanisms of Uptake and Loss of Mg^{2+}

Under catecholamines heart cells lose Mg^{2+}, ostensibly from both cytosol and mitochondria, but how can Mg^{2+} ions pass through the cytosol to the cell membrane without increasing the cytosolic $[Mg^{2+}]$? It has been suggested that Mg^{2+} is in the form of Mg-ATP^{2-} in the cytoplasm. However, adding Mg-ATP^{2-} to a Mg^{2+}/ATP^{4-} buffer increases the $[Mg^{2+}]_i$ (see Figure 6B of [65]). There are other buffers in the cytosol but calculation shows that the additional buffers together with ATP dampen the changes in the cytosolic $[Mg^{2+}]$, but the changes in $[Mg^{2+}]_i$ are still present.

Another possibility is that the main action of catecholamines is to increase the V_{max} of the cell membrane transporter, this increases the efflux and a decrease in the $[Mg^{2+}]_i$ is prevented by intracellular buffering and release of Mg^{2+} from the mitochondria. In this case the mitochondria would be playing a pivotal role in maintaining the cytosolic $[Mg^{2+}]_i$ more or less constant, despite a large net loss from the cell. The efflux of Mg^{2+} from the cell could, under these circumstances be likened to a wave of Mg^{2+} spreading from the centre of the cell to the surface membrane, in which the difference in the $[Mg^{2+}]_i$ between the cell centre and the membrane although present, would be minimal.

Carbachol causes an increase in total cellular Mg^{2+} and as no, or only minimal changes in cytosolic $[Mg^{2+}]$ have been measured, a similar problem to that of the action of catecholamines exists; as for catecholamines similar solutions can be proposed. However, it must be admitted that how the myocytes gain/lose Mg^{2+} while maintaining the $[Mg^{2+}]_i$ constant is still uncertain.

Maintenance of Steady State Concentrations of Magnesium

If Mg^{2+} in heart is to remain in a steady state, then there must be A) some mechanism to limit the actions of the hormones and B) after the hormonal action there has to be a net reversal of the hormone-induced flux otherwise the cells would be depleted of, or gradually gain, Mg^{2+}. Neither of these aspects has been studied in heart, although Günther et al. [159] did find a dual action of isoproterenol in hepatocytes, namely an initial net efflux within the first few minutes followed by a net influx. At present little can be said about such mechanisms in heart except to say they have to be present.

Physiological Functions

While there is no longer any debate concerning the action of these hormones on the total cellular Mg concentrations, the physiological function of these changes is unclear. In view of the fact that no, or only minimal changes in cytosolic $[Mg^{2+}]$ have been measured, it may be concluded that any physiological function of hormonal action is not in the cytosol. If an action in the cytosol is excluded, then an action of changes in $[Mg^{2+}]$ in intracellular organelles, specifically mitochondria, has to be considered. Another possibility is that the induced changes in the $[Mg^{2+}]$ of plasma have a modulating effect on specific tissues or organs (cf. [33]).

Mitochondria $[Mg^{2+}]$: The key enzymes of the Krebs cycle in the mitochondria are up-regulated by Ca^{2+} (reviewed in [160, 161]) and it has been suggested that changes in intramitochondrial $[Ca^{2+}]$ modulates ATP production (see discussion in [162]). However, mitochondrial enzymes are not only regulated by Ca^{2+}, but by Mg^{2+}. The activities of pyruvate dehydrogenase phosphate dehydrogenase, NAD^+-isocitrate dehydrogenase and inorganic pyrophosphatase are dependent on the $[Mg^{2+}]$. The first two are crucial for the citric acid cycle and the last may be involved in volume control (see [163]). It is unclear if 2-oxoglutarate dehydrogenase is regulated by Mg^{2+} ions. McCormack and Denton [164] and Rutter et al. [163] found no evidence of such an action but Rodriguez-Zavala and Moreno-Sanchez [165] with isolated mitochondria and measuring $[Mg^{2+}]_i$ in the mitochondria with mag-fura, suggested that the actions of 2-oxoglutarate dehydrogenase and ATP synthase were modulated via the $[Mg^{2+}]_i$. Panov and Scarpa [166, 167] have also found that the a ketoglutarate dehydrogenase complex is dependent on both Mg^{2+} and Ca^{2+}. The measured $K_{0.5}$ for Mg^{2+} was 25 μmol/l but since the resting $[Mg^{2+}]$ in mitochondria lies between 0.5 and 0.7 mmol/l (see [168]) it is questionable if Mg^{2+} ions in mitochondria would influence the enzyme.

Despite the fact that *in vitro* experiments have demonstrated a regulatory effect of Mg^{2+} on mitochondrial enzymes there are no studies on putative changes in mitochondrial $[Mg^{2+}]$ due to the actions of hormones. The possible intra-mitochondrial alteration in the $[Mg^{2+}]$ and its effects on metabolism have still to be investigated.

Plasma $[Mg]_t$: Catecholamines induce a release of Mg^{2+} not only from heart, but also from liver [169] and bone [170] and, to prevent excessive loss of tissue Mg^{2+}, some mechanism is required to dampen the action of the catecholamines. A negative feedback between plasma $[Mg^{2+}]$ and catecholamines has been postulated, plasma $[Mg^{2+}]$ inhibiting both the release and the peripheral action of catecholamines [171–173]. Indeed experiments on dogs [174] showed that an increase of $[Mg]_t$ in plasma from 0.8 to 1.0 mmol/l decreased the catecholamine output from the adrenals by some 26 %. Other evidence of a connection between $[Mg^{2+}]$ and catecholamine release comes from clinical studies. In patients undergoing intubation for anaesthesia, Mg^{2+} infusion sufficient to increase the plasma $[Mg]_t$ from 0.85 to 2.95 mmol/l significantly reduced both the concentrations of adrenaline and noradrenaline in plasma [175]. In patients with phaeochromocytoma Mg^{2+} infusion has been used successfully to prevent catecholamine secretion during resection of the tumour [176].

While these results suggest a negative feedback between plasma $[Mg^{2+}]$ and catecholamine release there is evidence against it. Infusion of Mg^{2+} to approximately double the $[Mg]_t$ had no effect on resting plasma catecholamines concentrations [177]. Deppeler [178] found that doubling the plasma $[Mg]_t$ in human subjects did not significantly reduce catecholamines during exercise. However, the infused Mg^{2+} doubled plasma $[Mg]_t$, a concentration high enough to cause side effects which could have influenced the results.

Since catecholamines cause an increased loss of tissue Mg^{2+}, it would be expected that their infusion would cause an increase in the plasma $[Mg]_t$. Indeed in rats Keenan et al.

[179] have demonstrated a dose dependent increase in plasma $[Mg]_t$ on infusion of isoproterenol, but the tissue source of the Mg^{2+} could not be determined in these experiments. This action was due to a β_2-action of the drug and there was evidence that a β_1-action might increase uptake. The authors suggest that this increased plasma $[Mg]_t$ could regulate catecholamine release.

In exercise in humans, the changes in $[Mg]_t$ depend on both the duration and severity of the exercise; short intensive exercise causes an increase in the $[Mg]_t$, longer less intensive exercise will eventually cause a decrease [180, 181]. The changes in $[Mg^{2+}]$ can either mimic the changes in $[Mg]_t$ [182, 183], go in opposite directions [184, 185] or depend on training status [186]. Changes in plasma Mg^{2+} occurring during stress (parachuting, bungee jumping, aerobatics) have shown increases in $[Mg^{2+}]$ at the expense of $[Mg]_t$ [184, 187]. However, detailed studies of the changes in $[Mg]_t$ and $[Mg^{2+}]$ before, during and after exercise, as well as in stress, are lacking.

In contrast to results in rats, infusion of adrenaline in human volunteers causes a decrease in $[Mg]_t$ in the plasma [188–190] and in $[Mg^{2+}]_i$ [182]. This suggests uptake sites for Mg^{2+} under adrenaline influence but the sites are unknown. Infusion of noradrenaline had no effect [188]. Adrenaline acts via both α- and β-agonist [191] and Joborn et al. [188] found that propranolol inhibited the decrease suggesting it was β-effect. Whyte et al. [190] found the effect could be mimicked by salbutamol a β_2-agonist, again supporting a β-action for adrenaline.

The results can only be described as confusing. There is no good explanation for the difference between the action of catecholamine infusion in rats and humans and it is surprising that in human subjects, exercise and stress cause increases in the plasma $[Mg^{2+}]$ but infusion of catecholamines does the opposite. Whether the changes in plasma $[Mg^{2+}]$ have a physiological function or are simply a by-product of hormonal action is still very much an open question and invites re-investigation. However, in any future studies care should be taken to correctly interpret the measurements. The total Mg concentration in plasma is in two fractions, one bound and one ionised and it is the ionised that is the physiological parameter [192]. Moreover, the ionised fraction is distributed between plasma and interstitial fluid, the concentration in the interstitial fluid being slightly less than in the plasma because of the Donnan factor of 0.925 [193]. Plasma Mg^{2+} can also exchange, although slowly, with erythrocyte Mg (see [194]). Taking normal values for plasma volume, interstitial volume, haematocrit and Mg concentrations in plasma and erythrocytes, gives a total Mg in plasma of around 3 mmol and 4.8 mmol in interstitial fluid and a total of 7.8 mmol in the extracellular volume. Erythrocytes contain around 12.8 mmol. Thus interstitial fluid and erythrocytes represent reservoirs for Mg in the plasma and have to be taken into account when considering the plasma $[Mg]_t$ changes. This has not always been done in the published literature and the consequences can be very misleading. A further complicating factor is that during exercise plasma is concentrated due to water loss. This has in many cases, not been allowed for.

General Conclusions

Present State of the Art

An overview of magnesium homeostasis was illustrated in Figure 4. With this Figure in mind, it would seem worthwhile to give a short summary of how we regard the present state of Mg^{2+} homeostasis in the heart. It is unclear how Mg^{2+} enters heart cells. This could be carrier or channel mediated. An efflux mechanism must be present as at equilibrium the $[Mg^{2+}]_i$ would be over 200 mmol/l. One such mechanism is most probably, a Na^+/Mg^{2+} exchanger, since the microelectrode experiments [1, 124] have now been reinterpreted as not denying the presence of such an exchanger. However, the stoichiometry, characteristics and ATP dependence, if any, of this Na^+/Mg^{2+} mechanism are unknown. Other efflux mechanisms probably also exist but, like the Na^+/Mg^{2+} exchanger, have not been characterised. When investigating efflux mechanisms, the membrane potential dependence or lack of it, is a useful criterion to exclude or include possible antiport or symport mechanisms. When considering influx and efflux it is the concentration directly under the cell membrane that is of physiological importance. No systematic changes in the $[Mg^{2+}]$ have been described in the "fuzzy space" although such changes have been measured for Ca^{2+} and Na^+. If such changes did occur, they would have important consequences for Mg^{2+} transport.

Cytosolic $[Mg^{2+}]$ is difficult to change due to cytosolic buffering and influx and efflux from mitochondria and possibly SR. However, much remains to be investigated. From the cytosolic buffers for Mg^{2+} only one, namely ATP has been characterised; actin probably acts as a cytosolic Mg^{2+} buffer, but its role in heart has not yet been determined in detail; the others remain unknown. The exact role of mitochondria and the SR in intracellular muffling has still to be determined. While an inwardly directed Mg^{2+} transporter has been cloned for mitochondria no efflux system has as yet, been cloned. The detailed characteristics of the influx and efflux mechanisms in mitochondria are not known. If mitochondria and SR play a role in intracellular muffling there could be changes in $[Mg^{2+}]$ in either mitochondria and/or SR. The function of any such changes in $[Mg^{2+}]$ remain obscure.

Hormones influence both influx and efflux in heart, but much remains obscure. While catecholamines cause a net loss of Mg^{2+}, carbachol and insulin cause a net gain, the physiological function of any of these changes is unclear. Despite these net changes in total magnesium, no changes in cytosolic $[Mg^{2+}]$ have been measured. Catecholamines reputedly increase the net loss of Mg^{2+} from the mitochondria and carbachol is purported to increase the uptake into the SR. Whither these changes influence the ionised concentrations in these organelles has not been investigated, nor has the physiological function of any such putative changes. It is not known how the lost or gained Mg^{2+} is replaced or removed by or from the cell and the mechanisms for limiting hormonal action have still to be investigated. Increase in the $[Mg]_t$ in plasma can occur with catecholamines, exercise and stress but the physiological function of this is also unclear.

Strategies for the Future

The above review paints a dismal picture of the present state of knowledge of Mg^{2+} regulation and homeostasis in heart. However, two major advances now make a reinvestigation of Mg^{2+} homeostasis in heart most opportune. The first is conceptual in that there is now a better understanding of what has to be measured, ie there can be large changes in $[Mg]_t$ with little or no change in $[Mg^{2+}]_i$, cf. the effects of the catecholamines and carbachol. Thus both have to be measured, as well as the putative changes in the intracellular distribution of Mg^{2+}. Moreover, a clear distinction has to be made between cytosol and intracellular organelles. The second advance is methodological, in that during recent years new and improved methods for investigation of Mg^{2+} have been introduced. These new methods can be applied to suspensions of myocytes, single myocytes, membrane vesicles and intrac-

ellular organelles either isolated or *in situ*. Molecular methods will no doubt give the opportunity of investigating directly the physiological properties of magnesium transport systems.

It is difficult to predict the direction of future studies, but fundamental questions have still to be answered. These include, the membrane transport system or systems and importantly, their stoichiometry. If the stoichiometry was known it would exclude many of the transport systems listed in Table 3. The extent of intracellular buffering and the role of mitochondria and SR in muffling could provide answers to the short term regulation of cytosolic [Mg^{2+}]. Moreover, what is the role of the restricted sub-membrane space in cytosolic Mg^{2+} regulation? The study of hormonal action has to include putative changes in mitochondrial and SR [Mg^{2+}], the factors leading to the limitation of hormonal action and the mechanisms necessary for the restitution of the steady state. Included in hormonal action is the intriguing question of the role of the changes in the plasma $[Mg]_t$. Is this part of a feedback loop limiting the secretion of catecholamines in exercise and stress? Do the changes in plasma $[Mg]_t$ have other functions? Answers to these questions, would at least provide a framework for an understanding of magnesium regulation and homeostasis in heart. With the changing face of magnesium research, we are confident it will now be only a matter of time before these questions can be answered.

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