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PLASMALEMMAL TRANSPORT OF MAGNESIUM IN EXCITABLE CELLS

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1. ABSTRACT

In excitable cells, the concentration of intracellular free Mg^{2+} ($[Mg^{2+}]_i$) is several hundred times lower than expected if Mg^{2+} ions were at electrochemical equilibrium. Since Mg^{2+} is a permeant ion across the plasmalemma, it must be constantly extruded. An ATP-dependent Na/Mg exchanger has been proposed as the sole mechanism responsible for Mg^{2+} extrusion. However, this hypothesis fails to explain numerous observations including the fact that K^+ and Cl^- appear to be involved in Mg^{2+} transport. Until now three main limitations have hampered the studies of plasmalemmal Mg^{2+} transport: i) ^{28}Mg, the only useful radioactive isotope of Mg^{2+}, has a short half-life and is difficult to obtain; ii) squid giant axons, the ideal preparation to carry out transport studies under "zero-trans" conditions, are only available during the summer months; and iii) the ionic fluxes mediated by the Mg^{2+} transporter are very small and difficult to measure. The purpose of this manuscript is to review how these limitations have been recently overcame and to propose a novel hypothesis for the plasmalemmal Mg^{2+} transporter in squid axons and barnacle muscle cells. Overcoming the limitations for studying the plasmalemmal Mg^{2+} transporter has been possible as a result of the following findings: i) the Mg^{2+} exchanger can operate in "reverse", thus extracellular Mg^{2+}-dependent ionic fluxes (e.g., Na^+ efflux) can be utilized to measure its activity; ii) internally perfused, voltage-clamped barnacle muscle cells which are available all year long can be used in addition to squid axons; and iii) phosphoinositides (e.g., PIP_2) produce an 8-fold increase in the ionic fluxes mediated by the Mg^{2+} exchanger. The hypothesis that we postulate is that, in squid giant axons and barnacle muscle cells, a 2Na+2K+2Cl:1Mg exchanger is responsible for transporting Mg^{2+} across the

plasmalemma and for maintaining $[Mg^{2+}]_i$ under steady-state conditions.

2. INTRODUCTION

Intracellular magnesium (Mg_i) is the second most abundant intracellular cation (1). It plays an essential role in protein biosynthesis and it is a key cofactor for hundreds of enzymes (1), especially enzymes involved with transfer of phosphate groups (e.g., ATPases, phosphatases, kinases, etc.). Mg_i modulates membrane receptors (2), ionic channels (3-5) and transporters (6-9). Activation of FAS on B-cell lymphomas causes an increase in $[Mg^{2+}]_i$ that appears to be required for apoptosis (10).

Homeostasis of plasma concentration of Mg^{2+} in humans is achieved via renal conservation mechanisms (reviewed by Quamme and de Rouffignac, this volume. Ref. 11) and hormonal control of magnesium absorption (reviewed by Schweigel and Martens, this volume, Ref. 12). Changes in the Mg^{2+} plasma concentration occur during alcoholism (13), central nervous system injury (reviewed by Vink and Cernak, this volume, Ref. 14) and diabetes mellitus (15,16). In fact, a primary defect in $[Mg^{2+}]_I$ handling may be a critical effector of non-insulin dependent diabetes mellitus (16,17). In addition, hypomagnesemia may produce nervous hyperexcitability (see, 18,19), tetanic syndrome (20), and meningo encephalic syndrome (21).

In excitable cells, the intracellular free Mg^{2+} concentration ($[Mg^{2+}]_i$) is several hundred times lower than expected if distributed passively (e.g., 600 times lower for squid axons and barnacle muscle cells). Since plasma membranes are permeable to Mg^{2+} (22), constant extrusion of this ion occurs. Hormones induce massive efflux of Mg_i from cells (reviewed by Romani and Scarpa, this volume, Ref. 23) but the underlying mechanisms of plasmalemmal transport of Mg^{2+} remain largely unknown.

3. PREVIOUS KNOWLEDGE ABOUT PLASMALEMMAL Mg^{2+} TRANSPORT IN EXCITABLE CELLS

Several biological preparations have been used to study plasmalemmal Mg^{2+} transport in cells. These preparations include the following: liver and cardiac cells (reviewed by Romani and Scarpa, this volume, Ref. 23); squid giant axons (e.g., 24-28), helix aspersa neurons (29), frog skeletal muscle (30,31), red blood cells (32), epithelial secretory cells (reviewed by Yago, *et al.*, this volume, Ref. 33); kidney cells (reviewed by Quamme and de Rouffignac, and by Beyenbach, this volume, Refs. 11 and 34); epithelial gastrointestinal cells (reviewed by Schweigel and Martens, this volume, Ref. 12); and barnacle muscle cells (22,35-40). Among these preparations, barnacle muscle cells and squid giant axons offer the advantage that, owing to their large size, they can be internally perfused or dialyzed, and voltage-clamped. Thus, all the relevant parameters of plasmalemmal Mg^{2+} transport (i.e., composition of the intracellular environment, membrane potential) can be measured and controlled. Furthermore, intracellular perfusion/dialysis allows study of the function of plasmalemmal ionic transporters under "zero-trans" conditions (see below).

3.1. Intracellular perfusion or dialisis of barnacle muscle cells or squid giant axons

Operation of the Na^+/Mg^{2+} exchanger requires that Na^+ binds to the "cis"-side of the transporter protein while Mg^{2+} binds to the "trans"-side. Subsequently, the ions are translocated to the opposite side of the membrane. Demonstration that the putative Na^+/Mg^{2+} exchanger is the mechanism responsible for transporting Mg^{2+} across the plasmalemma can be provided by showing that the flux (influx or efflux) of each ion involved is a function of the electrochemical gradient of the other ion. This can be determined by measuring the unidirectional influx or efflux of Na^+, simultaneously with the unidirectional flux of Mg^{2+} going in the opposite direction. Ideally, experiments should be conducted under conditions in which the ions to be translocated are only present in the side of the membrane where they bind to the transporter (i.e., "zero-trans" condition). This permits avoidance of the following possible errors (41): i) there is no contamination of the fluxes mediated by the Mg^{2+} exchanger by fluxes mediated by other mechanisms; ii) there is no possibility of exchange fluxes mediated by the Mg^{2+} exchanger; and iii) any possible regulatory effects of the ions to be transported acting on the trans-side of the membrane are prevented. These ideal conditions can be attained in giant cells using intracellular perfusion or dialysis techniques.

The methods for intracellular perfusion and dialysis in barnacle muscle cells and squid giant axons have been described in detail (42-48). Unidirectional fluxes under "zero-trans" conditions are attained by adding the ion (and its radioactive isotope) whose transport is to be measured, only at either the intracellular or extracellular fluids. Subsequently, effluxes or influxes of the labeled ion are measured accomplished by taken aliquots from the fluid at the opposite side of the plasma membrane where the ion was originally added, and measuring their radioactive content.

3.2. Evidence for Na^+/Mg^{2+} exchange in barnacle muscle cells and squid giant axons

Numerous observations in injected and dialyzed (or perfused) squid axons and barnacle muscle cells have led to the suggestion that a Na/Mg exchanger is responsible for extruding Mg^{2+} from these cells. The evidence shows that Mg^{2+} efflux is:

- Largely dependent on extracellular Na^+ (Na_o) (25,26,28);
- Reduced by metabolic poisoning (25,26);
- Highly sensitive to temperature (26);
- Unaffected by changes in membrane potential (Vm) (26); and
- Promoted by the presence of extracellular K^+ (K_o) (39,40)

DiPolo and Beaugé (24) have confirmed and extended these observations reporting that:

- Unidirectional Mg^{2+} efflux from dialyzed squid axons is completely dependent on ATP and external Na^+ (Na_o/Mg_i exchange).
- ATP-γ-S substitutes for ATP for activation of this exchanger. This suggests that ATP is a substrate for kinases rather than for ATPases;
- In the presence of ATP and the absence of Na_o, extracellular Mg^{2+} (Mg_o) stimulates (tracer) Mg^{2+} efflux (Mg/Mg exchange); and
- Mg^{2+} efflux is insensitive to membrane potential (from –80 to –30 mV)

There is also a (tracer) Na/Na exchange in squid axons that is ouabain insensitive and is not mediated by the Na/Ca exchanger (since it does not require the presence of activating intracellular Ca^{2+}) (49). Since this exchange has an absolute requirement for ATP (49), it is unlikely to be mediated by the Na/H exchanger and is therefore, likely to be mediated by the (ATP-dependent) Na/Mg exchanger. In summary, the published results suggest that Na/Mg exchange:

- Is the only mechanism responsible for extruding Mg^{2+} from squid axon and barnacle muscle;
- Has an absolute requirement for ATP;
- Like Na/Ca exchange, seems to be able to operate in several modes of exchange: Na_o/Mg_i; intracellular Na^+ (Na_i)/extracellular Mg^{2+} (Mg_o); (tracer) Mg/Mg, and (tracer) Na/Na exchange modes; and
- It is voltage-insensitive;

Several critical observations, however, remain unexplained by the Na/Mg exchanger hypothesis:

a. Extracellular Na^+ (Na_o) activates Mg^{2+} efflux (25) and extracellular Mg^{2+} (Mg_o) activates Na^+ efflux (50) with Michaelis-Menten kinetics suggesting a stoichiometry of $1Na^+:1Mg^{2+}$. However, Na_o-dependent Mg^{2+} efflux is insensitive to membrane potential (24,26) suggesting that either: (i) the stoichiometry of the exchanger is $2Na^+:1Mg^{2+}$; (ii) another cation is co-transported with 1 Na^+ in exchange for Mg^{2+} ($1Na^+$ + cation/Mg^{2+} exchange); or (iii) the transporter itself has a negative charge that is neutralized by Na^+ ($1Na^+:1Mg^{2+}$ exchange) and the return half-cycle carries a different cation than Na^+ (e.g., K^+).

b. If the electrochemical potential of Na^+ is responsible for maintaining $[Mg^{2+}]_I$ under steady-state conditions, $[Mg^{2+}]_I$ would be governed by the following equations (26):

For an electroneutral exchange (i.e. $2Na^+:1Mg^{2+}$):

$$\text{(Eq. 1)}\quad [Mg^{2+}]_I = [Mg^{2+}]_o \frac{[Na^+]_I^2}{[Na^+]_o^2}$$

where the suffixes i and o represent the intra and extracellular compartments, respectively.

$$\text{(Eq. 2)}\quad [Mg^{2+}]_I = [Mg^{2+}]_o \frac{[Na^+]_I^2}{[Na^+]_o^n} e^{(z-n)(z\,Vm\,F/RT)}$$

For an electrogenic exchange (i.e. a stoichiometry other than $2Na^+:1Mg^{2+}$):

where n is the number of Na^+ ions exchanged per Mg^{2+} ion, Vm, F, R, z and T have their usual meanings.

- For the electrogenic $3Na^+:1Mg^{2+}$ stoichiometry, $[Mg^{2+}]_o$ = 44 mM (25,26), $[Na^+]_o$ = 461 mM, and $[Na^+]_I$ = 27 mM (27), membrane potential (Vm) = -60 mV, at 16°C, $[Mg^{2+}]_I$ would be about 2 μM at equilibrium. This value is at least 1000 times smaller than the measured free $[Mg^{2+}]_I$, which is 2 – 3.5 mM (25,51).
- For the electroneutral $2Na^+:1Mg^{2+}$ stoichiometry, $[Mg^{2+}]_i$ at equilibrium would be about 0.15 mM which is at least 13 times smaller than the measured free $[Mg^{2+}]_i$.
- For the electrogenic $1Na^+:1Mg^{2+}$ stoichiometry, the expected $[Mg^{2+}]_i$ at equilibrium would be about 9 mM which is at least 2.6 times larger than the measured $[Mg^{2+}]_i$.

These results are important since they indicate that 2 or more extracellular Na^+ ions are required to account for the observed steady-state distribution of $[Mg^{2+}]_i$.

Unfortunately, in spite of the great interest in understanding how Mg^{2+} is regulated in excitable cells, studies of Mg^{2+} fluxes have been hindered by three major limitations:

1. ^{28}Mg, the only useful radioisotope of Mg^{2+} has a very short half-life (21 h) and is currently being produced on only 1 day each year in the United States;

2.The use of squid axons is limited by their seasonal availability (3-4 months/year) and by the fact that, due to the fragility of the squid, the experiments have to be carried out at the place where they are captured (e.g., Marine Biological Laboratory, Woods Hole, MA);

3.Na^+-dependent Mg^{2+} fluxes are very small ($\leq$ 5 pmoles cm^{-2} sec^{-1}) (24, and see below). Thus, ability to carry out systematic studies of these fluxes is limited by their intrinsic low signal/noise ratio.

As shown below, our laboratory has developed strategies to overcome these limitations.

4. RECENT CONTRIBUTIONS TO THE UNDERSTANDING OF PLASMALEMMAL Mg^{2+} TRANSPORT IN EXCITABLE CELLS

The following is a summary of two contributions that our laboratory has made to the field of Mg^{2+} transport in excitable cells. The first one is technical and consists of overcoming the limitations to study ionic fluxes mediated by the Mg^{2+} transporter, the second is scientific and consists of showing that, besides the electrochemical gradient of Na^+, other ions are also involved in Mg^{2+} transport:

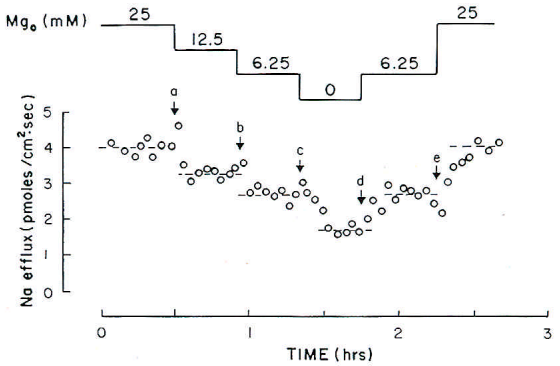

Figure 1. Effect of various concentrations of extracellular Mg^{2+} (Mg_o) on Na^+ efflux in an internally dialyzed squid axon. Concentration of Mg_o was diminished in 3 steps: from 25 to 12.5 mM (at a), then to 6.25 mM (at b), and finally to 0 (at c). Subsequently, Mg_o was restored in two steps: first to 6.25 mM (at d) and then to 25 mM (at e). Internal fluid contained very low [Ca^{2+}] (~10^{-10} M) to prevent activation of the Na/Ca exchanger. Discontinuous horizontal lines on graph represent average flux values. Reproduced with permission from the American Physiological Society. (Gonzalez-Serratos & Rasgado-Flores, 1988).

1. Overcoming the limitations of investigating Mg^{2+} fluxes:
a. We have demonstrated that Mg-dependent ^{22}Na fluxes can be used to study the Mg^{2+} exchanger (50)
b. We have shown that, in addition to squid axons, barnacle muscle cells can reliably be used to study Mg^{2+} fluxes (36,37,39)
c. We have increased the signal/noise ratio of ionic fluxes mediated by the Mg^{2+} exchanger by increasing the magnitude of the measured fluxes by ~ 8 fold (52).

2. Demonstrating that, in addition to Na^+, the Mg^{2+} transporter also exchanges Mg^{2+} for K^+ and Cl^- (53)

4.1. Overcoming the limitations of investigating Mg^{2+} transport

4.1.1. Demonstration that Mg-dependent Na^+ fluxes can be used to study the Mg^{2+} exchanger (50

To overcome the inherent problems of working with ^{28}Mg, experiments were designed to assess whether the Na/Mg exchanger, like other gradient-driven transport systems (e.g., Na/Ca exchange and Na-K-Cl cotransporter) (46,54), is able to operate in "reverse", i.e., can mediate a Mg_o-dependent Na^+ efflux (i.e., Na_i/Mg_o exchange). Assessment of this possibility was initially accomplished in internally dialyzed squid giant axons. The experimental strategy consisted of increasing the intracellular concentration of Na^+ ($[Na^+]_i$), removing extracellular Na^+ (Na_o) to prevent Na/Na exchange and comparing the efflux of Na^+ in the presence and absence of Mg_o. Mg_o was isosmotically replaced with Tris or better yet, to maintain the extracellular concentration of divalent cations constant, with Ba^{2+} or Ca^{2+}.

Figure 1 shows a representative experiment in which the effect of various concentrations of extracellular Mg^{2+} were studied on Na^+ efflux. The external solution was free of Na^+ and contained ouabain (to inhibit the Na/K pump) and tetrodotoxin (TTX, to block Na^+ efflux *via* Na^+ channels). Solving for Michaelis-Menten equation, the kinetic data from this figure indicate that, the apparent K_{Mgo} = 23 ± 2 mM and $J_{Na(max)}$ = 4.6 ± 0.3 pmol•cm^{-2}•sec^{-1}.

Additional characterization of the Mg_o-dependent Na^+ efflux indicated the following (50):

- In these experiments Mg_o was replaced with Ba^{2+}, however, the reduction in Na^+ efflux was not due to the increase in [Ba^{2+}] but was due instead to the removal of Mg_o. Furthermore, replacement of Mg_o with Ca^{2+} or Tris produces similar results (see below);
- Changes in membrane potential were not responsible for the reduction in Na^+ efflux (see below);
- Mg_o-dependent Na^+ efflux was blocked by amiloride (IC_{50}= 1.8 mM), but was largely insensitive to bumetanide (5 µM) which blocks the Na+K+Cl co-transporter (a discussion about similarities and differences between the Mg^{2+} transporter and the Na+K+Cl co-transporter is presented below).
- activation of the Mg_o-dependent Na^+ efflux by Mg_o followed Michaelis-Menten kinetics.

In sum, these results indicate that, in squid giant axons, the Na/Mg exchanger can operate in "reverse" mediating a Mg_o-dependent Na^+ efflux.

4.1.2. Demonstration that barnacle muscle cells can reliably be used to study Mg^{2+} fluxes (36,37,39)

At the present time, the internally perfused, voltage-clamped barnacle muscle cell constitutes the only reasonable alternative to the squid giant axon to carry out transport studies under conditions in which the relevant parameters for membrane transport can be measured and controlled (i.e., voltage-clamp and intracellular perfusion or dialysis). As has been demonstrated, plasmalemmal transporters in squid and barnacle are very similar (e.g., Na/Ca exchanger) (55). Furthermore, recent experiments (see below) indicate that the Mg^{2+} transporter is also very similar for both preparations. Of particular importance is the fact that, barnacle muscle cells are available all year long and can survive indefinitely in an appropriate aquarium. Therefore, an ideal situation to achieve the most rapid and efficient progress on plasmalemmal Mg^{2+} transport would be if the squid could be used during the summer months and the barnacle during the rest of the year. However, the barnacle muscle preparation has the disadvantage that the measured fluxes are not as stable as in the squid. Therefore, to make the barnacle preparation as useful as the squid, it would be necessary to improve the signal/noise ratio of the measured fluxes in the former preparation. Fortunately, during the past few months our laboratory has been able to activate much larger ionic fluxes mediated by the Mg^{2+} transporter in barnacle muscle cells thereby increasing the signal/noise ratio (see below).

Two strategies have been used to demonstrate that a putative Na/Mg exchanger is present in internally

perfused barnacle muscle cells:

I. measurement of the activity of the Na/Mg exchanger operating in "reverse". This was accomplished by following a protocol identical to the one used in squid axons (see Figure 1, above), i.e., by measuring the counter ion-dependency of the efflux of Na^+ activated by extracellular Mg^2; and

II. measurement of the activity of the exchanger operating in the "forward" mode of operation. This was accomplished by measuring a Na_o-dependent Mg^{2+} efflux. As mentioned above, these experiments were limited by the difficulties in obtaining ^{28}Mg and by the fact that the production of this isotope has to be purchased in its entirety since there are no other users.

4.1.2.1. Assessment of activity of the Na/Mg exchanger operating in "reverse" mode of exchange (i.e., Na_i/Mg_o) in barnacle muscle cells

Experiments have been performed in which the effect on Na^+ efflux of isosmotic replacement of extracellular Mg^{2+} by either Ba^{2+}, Ca^{2+} or Tris was studied. The results indicate that, like in squid axons (see Fig. 1), a "reversed" Na_i/Mg_o exchange can be readily measured in barnacle muscle cells (37,56). The decreases in Na^+ efflux in response to Mg_o removal were not due to the addition of the cation substituting for Mg^{2+} because similar results were obtained when this cation was Ca^{2+}, Ba^{2+} or Tris. The reductions in Na^+ efflux were accompanied with hyperpolarizations of 2.3 to 4 mV. These hyperpolarizations, however, were not responsible for reductions in Na^+ efflux because similar experiments performed under voltage-clamp conditions yielded virtual identical results.

4.1.2.2. Measurement of activity of the Na/Mg exchanger operating in "forward" mode of exchange (i.e., Na_o/Mg_i) in barnacle muscle cells

Experiments have been performed in which extracellular Na^+-dependent Mg^{2+} efflux was measured in internally perfused barnacle muscle cells (37,56). The results show that, removal of Na_o (replaced by Tris) in the absence of Mg_o produced reductions in Mg^{2+} efflux of 0.2 to 0.3 pmoles/cm^2 sec. Likewise, addition of Na_o (replacing Tris) in the presence of Mg_o produced increases of 0.25 to 0.35 pmoles/cm^2 sec. Interestingly, removal of Mg_o (replaced by Ca_o) in the presence of Na_o produced an increase in Mg^{2+} efflux of 0.3 pmoles/cm^2 sec while addition of Mg_o in the absence of Na_o produced an increase in Mg^{2+} efflux of 0.17 pmoles/cm^2 sec. These results indicate the presence of Na_o-dependent Mg^{2+} efflux both in the presence and absence of Mg_o. Likewise, they indicate that Mg/Mg exchange is manifested only in the absence of Na_o. In the presence of Na_o, removal of Mg_o produces an increase in Mg^{2+} efflux, which may be due to activation of Mg^{2+} efflux by Na_o. In this case, Mg_o would appear to act as an inhibitor of Na_o-dependent Mg^{2+} efflux as a consequence of its stimulation of Mg/Mg exchange.

Experiments performed under voltage-clamp conditions indicate that the Na_o-dependent reductions in Mg^{2+} efflux were not due to changes in V_m. However, these results do not rule out the possibility that activity of the Mg^{2+} exchanger may be electrogenic. Analysis of the V_m changes associated with Mg_o-dependent Na^+ efflux and with the Na_o-dependent Mg^{2+} efflux show that this possibility is very unlikely:

- the hyperpolarizations that accompanies the reduction in the efflux of Na^+ (when Mg_o is removed) could be explained by the transfer of a net positive charge carried by Na^+; and
- the hyperpolarization that accompanies the reduction in Mg^{2+} efflux (when Na_o is removed) requires that the net positive charge be carried by Mg^{2+} instead.

Consequently, these changes in V_m cannot be attributed to the activity of the exchanger but instead may result from some other effect of the ionic substitution. These results are consistent with the hypothesis that the Mg^{2+} exchanger is electroneutral.

In sum, the results demonstrate that the internally perfused barnacle muscle cell can be used to study ionic fluxes mediated by the putative plasmalemmal Na/Mg exchanger operating in either the "forward" or "reverse" mode of exchange.

4.1.3. Enhancement of the signal/noise ratio of ionic fluxes mediated by the Mg^{2+} exchanger in barnacle muscle cells

Figure 1 shows that, in squid axons, the signal/noise ratio for the Mg_o-dependent Na^+ efflux is 2.0/2 = 1.0. Conversely, in barnacle muscle cells, this ratio is from 5/17=0.3 to 5/30=0.16, respectively. To improve the signal/noise ratio in barnacle muscle cells we attempted to enhance the ionic fluxes mediated by the Mg^{2+} transporter. Based on the arguments listed below, we reasoned that Phosphatidylinositol-4,5-bisphosphate (PIP_2) and/or phosphoarginine (P-Arg) could be strong candidates for activating the Mg^{2+} transporter:

- The Mg^{2+} transporter has an absolute requirement for ATP (24);
- ATP may activate transporters because it may work (reviewed in Ref. 57): i) as a substrate for ATPases; ii) as a substrate for protein kinases; iii) as a substrate for lipid kinases generating second messengers (e.g., Phosphatidylinositol phosphates); iv) by directly binding to the transporter inducing allosteric effects; iv) by inducing changes of actin cytoskeleton; v) by chelating polyvalent cations; and vi) by activating ATP-dependent phospholipases;

- In squid axons, the non-hydrolyzable ATP analog, ATP-γ-S, can substitute for ATP for activating the Mg^{2+} transporter. Thus, modulation by this nucleotide does not appear to involve an ATPase but may instead involve a kinase; and

- In squid axons, the Na/Ca exchanger, which is modulated by ATP, can be activated by PIP_2 (58) or P-Arg (59) in the absence of ATP.

Figure 2 shows a diagram depicting several of the possible pathways by which ATP could activate the putative Na/Mg exchanger:

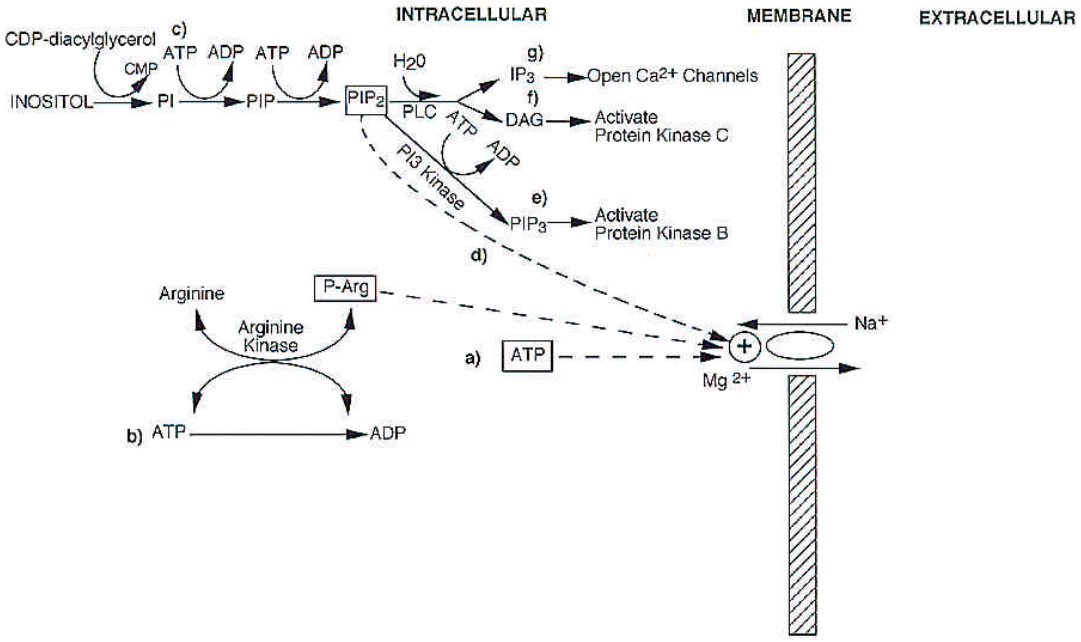

Figure 2. Diagram showing several of the possible mechanisms by which ATP could activate the Mg^{2+} transporter: a) via a direct effect; b) by being a precursor for P-Arg; or c) by being a precursor for PIP_2. PIP_2 could in turn activate the transporter by: d) a direct effect; e) being a precursor of PIP_3; f) being a precursor of diacylglycerol (DAG); or g) being a precursor of IP_3. See text for further details.

a. ATP could be working directly either by being a substrate for an ATPase, by directly producing an allosteric modification of the transporter or another cellular component related to the transporter, or by chelating polyvalent cations;

b. ATP could work by being a substrate for arginine kinase yielding phosphoarginine (P-Arg) which would in turn activate the transporter;

c. ATP could work by being a substrate of phophatidylinositol (PI) kinase yielding PIP_2;

d. PIP_2 could directly activate the transporter;

e. PIP_2 could work by being a substrate of Phosphatidylinositol 3'-kinase (PI3 kinase) yielding Phosphatidylinositol-3,4,5-trisphosphate (PIP_3) which would in turn activate the transporter

f. PIP_2 could work by being a substrate of phospholipase C (PLC) yielding diacylglycerol (DAG) which would in turn activate the transporter

g. PIP_2 could work by being a precursor of inositol 1,4,5-trisphosphate (IP_3) which would in turn activate the transporter

The experimental strategy followed to test whether PIP_2 and/or P-Arg, could substitute for ATP in activating the Mg^{2+} transporter consisted of comparing the Mg_o-dependent Na^+ fluxes in cells perfused with ATP-free solutions containing either 0.1 mM PIP_2 or 5 mM P-Arg. To ascertain the absence of ATP in nominal ATP-free perfusates, 10 U/ml of the ATP-degrading enzyme apyrase (42) were added to the internal solutions.

Control experiments consisted of cells perfused with the ATP-free perfusate containing Apyrase; experimental cells were also perfused with a similar perfusate than experimental cells, but in this instance, the fluid also contained PIP_2 or P-Arg.

The results using PIP_2 show (52,26) that, in control cells, Mg_o removal produced no effect on Na^+ efflux. The efflux of Na^+ under this condition is very steady and low (average 10 pmoles$\bullet cm^{-2}\bullet sec^{-1}$) because it mainly represents a Na^+ "leak" in the absence of Na_o and presence of ouabain, bumetanide and verapamil. In contrast, in the presence of PIP_2, Mg_o removal produced a large reduction in Na^+ efflux (i.e., 30-75 pmol$\bullet cm^{-2}\bullet sec^{-1}$) that became significantly different than control cells ($P<0.05$) 2 minutes following the removal.

The results using P-Arg show (52,26) that removal of Mg_o in cells perfused with P-Arg produced a large reduction in Na^+ efflux (i.e., 45-75 pmol$\bullet cm^{-2}\bullet sec^{-1}$) that became significantly different than control cells 2 minutes after Mg_o removal. In control solutions (0-ATP and Apyrase) Mg_o removal did not affect Na^+ efflux.

However, in the presence of PIP_2 or P-Arg, this manipulation produced a reversible reduction in Na^+ efflux of 30-75 (n=7) and of 45-75 (n=6) pmol•cm^{-2}•sec^{-1}, respectively.

These results demonstrate that PIP_2 and P-Arg promote an 8-fold activation in Na_o-dependent Mg^{2+} efflux in barnacle muscle cells. Clearly, it is expected that similar results would be obtained in squid giant axons.

Our laboratory is currently attempting to dissect the metabolic pathway by which P-Arg and PIP_2 activate the Mg^{2+} exchanger. Preliminary studies have been performed assessing the ability of the metabolic products of PIP_2 (i.e., IP_3 and DAG) to activate the transporter. The results show that DAG but not IP_3, activates the exchanger. This suggests that Protein Kinase C may be involved in activating the Mg^{2+} exchanger *via* a likely phosphorylation. In any event, the new signal/noise ratio of the PIP_2- or P-Arg-stimulated Mg_o-dependent Na^+ efflux in barnacle is about 75/90= 0.8. This value is a great improvement over the 0.16-0.3 values in the absence of the stimulator and is very close to the one obtained using squid axons. Thus, barnacle muscle cells can now reliably be used to measure ionic fluxes mediated by the Mg^{2+} transporter. Furthermore, making the reasonable assumption that barnacle muscle cells and squid giant axons possess similar transporters (as is the case for the Na/Ca exchanger), both preparations can be used to study the plasmalemmal Mg^{2+} transporter.

4.2 Demonstration that, in addition to Na^+, the Mg^{2+} tranporter also exchanges mg^{2+} for K^+ and Cl^-

4.2.1. Introduction

Numerous observations indicate that there is a strong relationship between the transport of Mg^{2+} and K^+ in cells. Some examples of these observations are the following:

- There is a direct correlation between the concentrations of intracellular free K^+ and Mg^{2+} in skeletal muscle (31);
- There is a strong correlation between the total intracellular concentrations of Mg and K in red cells, lymphocytes, and skeletal muscle (reviewed in Ref. 60);
- Extracellular Mg^{2+} induces K^+ efflux in barnacle muscle cells (40);
- Mg^{2+} affects K^+ transport in red blood cells and muscle cells (6,60-63);

There is a direct correlation between the changes in plasma Mg and K in pathological conditions such as Bartter's syndrome (64) and Alzheimer disease (65).

Likewise, some evidence, though indirect, suggests that there is a relationship between the fluxes of Cl^- and Mg^{2+} across the plasma membrane of cells:

- Extracellular Mg^{2+} and Cl^- interact to modulate the tone and contractility of vascular muscle (9);
- Intracellular Mg^{2+} inhibits Cl^- efflux in barnacle muscle cells (66);
- Net Mg^{2+} efflux is dependent on net Cl^- efflux in Mg^{2+}-loaded human erythrocytes (67);
- Ingestion of supplementary dietary Cl^- produces a reduction in plasma Mg^{2+} in rats (68).

In sum, given the fact that K^+ and Cl^- appear to be involved in regulating $[Mg^{2+}]_I$, attention should be given to the possibility that the electrochemical gradients of these ions could be involved in Mg^{2+} transport.

Using internally dialyzed squid giant axons and intact barnacle muscle cells we have explored the possibility that, in addition to the electrochemical gradient of Na^+, the electrochemical gradients of K^+ and Cl^- may be involved in the regulation of $[Mg^{2+}]_I$ (39,53). A summary of the results is the following:

4.2.2. Involvement of K^+ in the regulation of $[Mg^{2+}]_I$

We have found that, in squid giant axons and barnacle muscle cells, the electrochemical gradient of K^+ is coupled to Mg^{2+} transport. Three main observations described below support this:

I. There is an absolute requirement of intracellular K^+ for the Mg_o-dependent Na^+ efflux in squid axons;
II. Removal of extracellular Mg^{2+} produces a simultaneous and equimolar reduction in Na^+ and K^+ efflux in squid axons; and;
III. Removal of extracellular K^+ (K_o) produces an increase in the free and total intracellular Mg concentration in intact barnacle muscle cells.

A more detailed description of these observations is as follows:

4.2.2.1. There is an absolute requirement of intracellular K^+ for the Mg_o-dependent Na^+ efflux in squid axons

We have assessed the role of intracellular and extracellular K^+ for operation of the Mg_o/Na_i exchanger (53). To perform these experiments, the dialyzed squid axons were incubated in the absence of both intra- and extracellular K^+, in the absence of extracellular Na^+ (replaced by Tris) and presence of phenylpropyltriethylammonium bromide (PPTEA) to block K^+ channels (69). Under these conditions, replacement of extracellular Mg^{2+} by Ba^{2+} produced no effect on Na^+ efflux. This indicates that either intra- and/or extracellular K^+ are necessary for activation of the Na/Mg exchanger. Addition of extracellular K^+ promoted a ouabain-insensitive Na^+ efflux. This activation, however, was not due to activation of the Na/Mg exchanger since removal of external Mg^{2+} produced no effect on Na^+ efflux. On the other hand, the effect of intracellular K^+ (K_i) on the Na/Mg exchanger was tested by adding K_i to the dialysis solution. This manipulation also produced an increase in Na^+ efflux but in this instance, this effect is attributed to activation of the Na/Mg exchanger because under this condition, removal of extracellular Mg^{2+} produced a reversible reduction in Na^+ efflux of 0.8 pmoles•cm^{-2}•sec^{-1}. This indicates that activity of the Na/Mg exchanger operating in "reverse" (Na_i/Mg_o exchange) requires intracellular K^+.

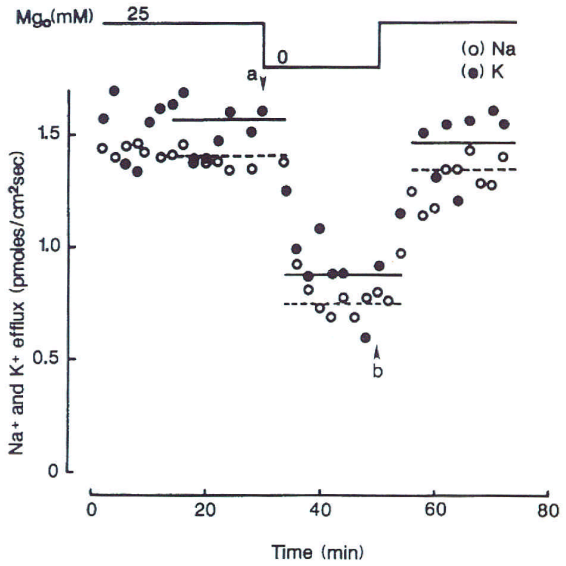

Figure 3. Effect of Mg_0 on simultaneous efflux of Na^+ (O) and K^+ (●) in an internally dialyzed squid axon. The external solution was Na^+-free (replaced by Tris) and contained (among other components) 25 mM Mg^{2+} and 10 K^+, 0.1 mM ouabain, 0.2 mM TTX and 10 μM bumetanide. The dialysis solution contained among other components (in mM): 39 Na^+, 99 K^+, 4 ATP-Mg, 5 EGTA, 1 Mg^{2+}, and 20 PPTEA. Fluxes were allowed to equilibrate for 1 hr before sample collection. Solid and dashed lines represent the average of K^+ and Na^+ efflux in the presence and absence , respectively of Mg_o. Reproduced with permission from the American Physiological Society (Rasgado-Flores & Gonzalez-Serratos, 1994).

4.2.2.2. Removal of extracellular Mg^{2+} produces a simultaneous and equimolar reduction in Na^+ and K^+ efflux in squid axons

Requirement of K_i for operation of the Na_i/Mg_o exchange could be due to two possibilities: i) K_i is a necessary co-factor for Na_i/Mg_o exchange; or ii) K_i is co-transported with Na_i in exchange for Mg_o. To test these possibilities we determined the effect of removal of Mg_o on the simultaneous efflux of K^+ and Na^+.

Figure 3 is an example of the simultaneous reduction of Na^+ and K^+ efflux on removal of extracellular Mg^{2+}. The external solution was Na^+-free and contained ouabain, TTX and bumetanide. The dialysis solution contained ATP-Mg, EGTA, no added Ca^{2+}, and PPTEA.

To simultaneously measure the Mg_o-dependent efflux of K^+ and Na^+, ^{42}K and ^{22}Na were used as tracers because these isotopes have very different half-lives (12.4 hours and 2.6 years, respectively). The magnitude of the fluxes of each ion was determined by subtracting the radioactive content of aliquots of the superfusate and counted immediately (furnishing the counts of both isotopes) from the counts obtained as a result of re-counting the same aliquots after the isotope of short half-life has decayed (giving the counts of the isotope with long half-life, i.e. ^{22}Na). Under these conditions, the steady-state efflux of Na^+ (open circles) and K^+ (closed circles) were similar (1.6 and 1.4 pmoles•cm^{-2}•sec^{-1}, respectively). Removal of Mg^{2+} (from **a** to **b**) produced a reversible reduction in the efflux of both Na^+ and K^+ (0.7 and 0.65 pmoles•cm^{-2}•sec^{-1}, respectively).

The average ratio of the Mg^{2+}-dependent Na^+ efflux over the Mg^{2+}-dependent K^+ efflux from two independent experiments was 0.95 ± 0.2. This experiment, therefore, indicates that extracellular Mg^{2+} activates the co-transport of stoichiometrically equal amounts of Na^+ and K^+.

4.2.2.3. Removal of extracellular K^+ (K_o) produces an increase in the free and total intracellular Mg^{2+} concentration in intact barnacle muscle cells

We have observed that in intact barnacle muscle cells:

I. There is an inverse relationship between the concentrations of total intracellular Mg content and extracellular K measured using flame photometry and atomic absorption spectroscopy (39); and

II.Extracellular K^+ (K_o) stimulates Mg^{2+} efflux, measured with differential absorption spectroscopy using eriochrome blue as a $[Mg^{2+}]_i$ indicator (39).

These results suggest that K^+ influx is coupled to Mg^{2+} efflux via a K^+/Mg^{2+} exchange.

Involvement of K^+ for Mg^{2+} transport may not be limited to invertebrate cells: in frog skeletal muscle, for example, it has been observed that there is a direct correlation between the levels of $[Mg^{2+}]_i$ and intracellular free K^+ (see Fig. 7 in Alvarez-Leefmans et al., 1986, Ref. 31). This is consistent with K^+/Mg^{2+} exchange similar to the well-documented direct relationship between the intracellular concentrations of Na^+ and Ca^{2+} in excitable cells resulting from activity of the Na/Ca exchanger (70).

One piece of information apparently contradicts the involvement of K^+ on the regulation of $[Mg^{2+}]_i$ in excitable cells: In injected squid giant axons, removal of extracellular K^+ (K_o) does not affect unidirectional Mg^{2+} efflux (25). This observation does not rule out a role for K_o because K_o removal (in the presence of K_i) may not completely eliminate the possibility that intracellular K^+ could leak from the cell and activate the exchanger from the external surface of the membrane. It has been reported that K^+ efflux from the axon (through resting K^+ channels) to the extracellular restricted-diffusion space (71) raises K_o to about 0.5 mM above the nominal K^+ concentration in the seawater (72). Therefore, if K_o stimulates Mg^{2+} efflux with high affinity, the lack of effect of K_o removal on Mg^{2+} efflux is not adequate to rule out a role for K_o in the promotion of Mg^{2+} efflux.

In sum, the electrochemical gradients of Na^+ and K^+ appear to be coupled to regulate intracellular Mg^{2+}. Consistently with this, an electroneutral $1Na^+ + 1K^+ : 1Mg^{2+}$ exchanger could be postulated to regulate $[Mg^{2+}]_i$. In this case, the equilibrium $[Mg^{2+}]_i$ is predicted by:

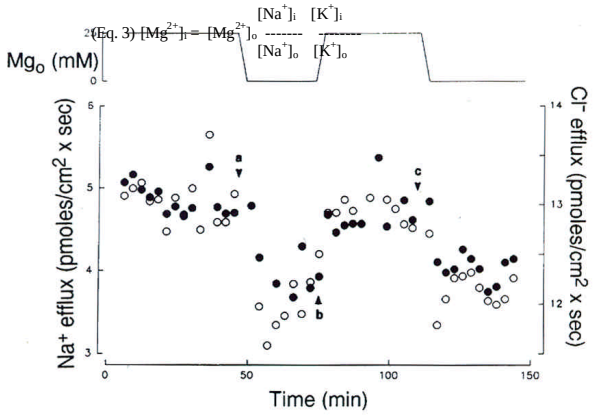

Figure 4.. Effect of removal of Mg_0 on simultaneous efflux of Cl^- (O) and Na^+ (●) in an internally dialyzed squid axon. Among other components, the external solution contained (in mM): 25 Mg^{2+}, 567 Cl^-, 10 K^+, 0.1 mM ouabain, 0.2 mM TTX and 10 µM bumetanide. Among other components, the dialysis solution contained (in mM): 40 Na^+, 52 Cl^-, 100 K^+, 1 Mg^{2+}, 20 PPTEA, 8 EGTA, no added Ca^{2+}, and 4 ATP-Mg. Fluxes were allowed to equilibrate for 1 hr before collection of the samples. Reproduced with permission from the American Physiological Society (from Rasgado-Flores & Gonzalez-Serratos, 1994). See text for further details.

Substituting $[K^+]_i$=325 and $[K^+]_o$=17 (73,74) and for the other terms as in equation 1, equation 3 gives $[Mg^{2+}]_i$ = 49 mM. This value is about 19 times larger than the measured $[Mg^{2+}]_i$ (25,51). Consequently, we are led to conclude that an electroneutral 1 Na^++ 1 K^+: 1 Mg^{2+} exchanger is unlikely to be responsible for maintaining $[Mg^{2+}]_i$ under equilibrium conditions. Following this conclusion, the question arises then as to whether an additional ion may be involved in Mg^{2+} transport.

4.2.3. Involvement of Cl^- in the regulation of $[Mg^{2+}]_i$

We have found that, in squid axons, in addition to the electrochemical gradients of Na^+ and K^+, the electrochemical gradient of Cl^- is coupled to Mg^{2+} transport. Two main observations support this:

a. there is an absolute requirement of intracellular Cl^- for the Mg_o-dependent Na^+ efflux; and
b. removal of extracellular Mg^{2+} produces a simultaneous equimolar reduction in Na^+ and Cl^- efflux. The following is a more detailed description of these observations:

4.2.3.1. There is an absolute requirement of intracellular Cl^- for the Mg_o-dependent Na^+ efflux

We have studied the effect of intracellular Cl (Cl_i) on the Mg_o-dependent Na^+ efflux (53). To carry out these studies, the dialyzed squid axon was incubated in the absence of both intra- (substituted with Trizma-base and aspartate) and extracellular (substituted with methanesulfonate) Cl^-. The results show that, under these conditions, removal of extracellular Mg^{2+} failed to induce a reduction in Na^+ efflux. However, when the internal fluid was substituted for a solution containing 52 mM Cl^-, this manipulation activated the efflux of Na^+. That this was due to activation of the Na/Mg exchanger was shown by the fact that removal of external Mg^{2+} produced reversible reductions in Na^+ efflux of 0.85-1.2 pmoles•cm^{-2}•sec^{-1}.

4.2.3.2. Removal of extracellular Mg^{2+} produces a simultaneous equimolar reduction in Na^+ and Cl^- efflux

Figure 4 is an example of the simultaneous reduction of Na^+ and Cl^- efflux upon removal of Mg^{2+}. To measure the efflux of Cl^- and Na^+ simultaneously, a similar strategy to the one used for the simultaneous measurement of K^+ and Na^+ fluxes was followed (see Fig. 3). In this case, the the radioisotopes used as tracers were ^{36}Cl and ^{24}Na. The half-lives of ^{36}Cl and ^{24}Na are 3 x 10^5 years and 14.9 hours, respectively.

Figure 4 shows that, in the presence of Mg_o, the steady-state efflux of Na^+ (closed circles) and Cl^- (open circles) were 4.8 and 13 pmoles•cm^{-2}•sec^{-1}, respectively. Removal of Mg_o (from a to b and at c) produced a reversible reduction in the efflux of both Na^+ and Cl^- (1.02 ± 0.2 and 1.13 ± 0.2 pmol•cm^{-2}•sec^{-1}, respectively). The ratio of the Mg^{2+}-dependent Na^+ efflux over the Mg^{2+}-dependent Cl^- efflux was 0.9 ± 0.1 (n=2). This suggests that extracellular Mg^{2+} activates the co-transport of stoichiometrically equal amounts of Na^+ and Cl^-.

Consistent with these results, an electrogenic 1Na^++ 1K^++ 1Cl^-:1 Mg^{2+} exchanger could be postulated to regulate $[Mg^{2+}]_i$. Under steady-state conditions, $[Mg^{2+}]_i$ would be predicted by the following equation:

$$\text{(Eq. 4)}\ [Mg^{2+}]_i = [Mg^{2+}]_o \frac{[Na^+]_i}{[Na^+]_o} \frac{[K^+]_i}{[K^+]_o} \frac{[Cl^-]_i}{[Cl^-]_o} e^{(z-n)(z\,Vm\,F/RT)}$$

By substituting in the above equation the values (in mM) of $[Cl^-]_i$=120, and $[Cl^-]_o$=470 (75) and the rest of the terms as in equations 2 and 3, equation 4 gives an expected value of $[Mg^{2+}]_i$ of 44 mM. This value is about 17 fold larger than the measured $[Mg^{2+}]_i$ (25,51). Thus, thermodynamically, this stoichiometry is not feasible.

On the other hand, if an electroneutral 2Na^++2K^++2Cl^-:1Mg^{2+} exchanger is postulated, the predicted value of $[Mg^{2+}]_i$ under steady-state conditions would be determined by the following equation:

$$\text{(Eq. 5)}\ [Mg^{2+}]_i = [Mg^{2+}]_o \frac{[Na^+]_i^2}{[Na^+]_o^2} \times \frac{[K^+]_i^2}{[K^+]_o^2} \times \frac{[Cl]_i^2}{[Cl]_o^2}$$

Substituting in this equation the appropriate values used before, gives an expected value of $[Mg^{2+}]_i$ at equilibrium of 3.6 mM which is precisely in the range of measured $[Mg^{2+}]_i$ (2-3.5 mM) (25,51). Thus, this stoichiometry is possible if $[Mg^{2+}]_i$ is distributed nearly at equilibrium.

5. CONCLUSIONS AND FUTURE EXPERIMENTS

In an attempt to explain our results and other available information about Mg^{2+} transport, we suggest that the electrochemical potentials of Na^+, K^+, Cl^- and Mg^{2+} are coupled through an electroneutral ATP-dependent

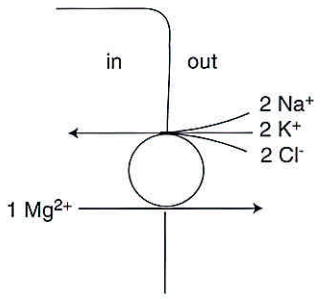

Figure 5. Schematic representation of the proposed 2 Na^+ + 2K^+ + 2Cl^-: 1Mg^{2+} exchanger. See text for details.

Na+K+Cl/Mg exchanger with a stoichiometry of $2Na^+$+$2K^+$+$2Cl^-$:$1Mg^{2+}$ that operates near its thermodynamic equilibrium. This hypothesis is consistent with the observations that:

I. intracellular K^+ and Cl^- are required for operation of Na_i/Mg_o exchange (see above);

II.K_i and Cl_i are co-transported with Na_i in stoichiometrically equal amounts in exchange for Mg_o (Figs. 3 and 4);

III. the activation curves of extracellular Mg^{2+} for Mg_o-dependent Na^+ efflux follow Michaelis-Menten kinetics (50);

IV. Na_o-dependent Mg^{2+} efflux is voltage-insensitive (24,26);

V. Na_o-dependent Mg^{2+} efflux is ATP-dependent (24); and

VI. The steady-state $[Mg^{2+}]_i$ is predicted by such exchanger.

This model is shown in Figure 5. The diagram shows that 2 Na^+, $2K^+$ and $2Cl^-$ ions bind to the exchanger at the extracellular side of the membrane while 1 Mg^{2+} binds to the intracellular side. This model is consistent with all the major observations on Mg^{2+} transport in excitable cells. Thus, we believe that this proposed exchanger is a reasonable working hypothesis that should be tested. The following is a list of experiments that should be performed to advance our understanding of the plasmalemmal Mg^{2+} transporter:

1. Demonstration that in barnacle muscle cells, presence of intracellular K^+ and Cl^- are essential for operation of the Mg_o-dependent Na^+ efflux, and that intracellular K^+ and Cl^- are co-transported with Na_i in exchange for Mg_o. A positive result will indicate that the postulated $2Na^+$+$2K^+$+$2Cl^-$:$1Mg^{2+}$ exchanger is not unique for squid axons.

2. Confirmation that P-Arg and PIP_2 stimulate the plasmalemmal Mg^{2+} transporter in squid axons. A positive result will indicate that the transporter in squid and barnacle share basic characteristics;

3. Demonstration that the fluxes of Na^+,K^+,Cl^- going in one direction across the plasmalemma are coupled with the flux of Mg^{2+} going in the opposite direction. This can be determined by showing that the flux (influx or efflux) of each of the four ions involved is a function of the electrochemical gradient of the other three ions;

4. Testing if the stoichiometry of the Mg^{2+} exchanger is: $2Na^+$+$2K^+$+$2Cl^-$:$1Mg^{2+}$. This can be accomplished by measuring the stoichiometric ratio of the Mg^{2+}-dependent unidirectional fluxes of Na^+, K^+ and Cl^- as well as of the Na^++K^++Cl^--dependent unidirectional fluxes of Mg^{2+} under "zero-trans" conditions;

5. Assessing whether the electrochemical gradients of Na^+, K^+ and Cl^- determine the intracellular free and total Mg^{2+} concentrations under steady-state conditions. This can be accomplished by measuring the effect of changes in the electrochemical gradients of Na^+, K^+, or Cl^- and of combinations of these three ions on the total and free intracellular concentrations of Mg, Na, K and Cl in either intact or voltage-clamped cells which have been perfused/dialyzed only during the first hour of the experiment in order to establish a desired composition of the intracellular environment.

6. Evaluation of whether the $2Na^+$+$2K^+$+$2Cl^-$:$1Mg^{2+}$ exchanger operates as a result of a simultaneous or consecutive binding of the ionic species involved. This information could be assessed by studying the specific requirements for the occurrence of each of the possible unidirectional exchanges mediated by the Mg^{2+} exchanger between any combination of the three ionic species transported (i.e. Na_o/Mg_i; Na_i/Mg_o; K_o/Mg_i; K_i/Mg_o; Cl_o/Mg_i; Cl_i/Mg_o; Na_o/K_i; Na_i/K_o; Na_o/Cl_i; Na_i/Cl_o; K_o/Cl_i; K_i/Cl_o and (tracer) Na/Na, Mg/Mg, K/K, and Cl/Cl exchange). These experiments could be performed providing that specific intra and extracellular ionic requirements are met for each exchange mode (76); and

7. Assessment of whether the putative $2Na^+$+$2K^+$+$2Cl^-$:$1Mg^{2+}$ exchanger and the Na+K+Cl co-transporter are either the same protein entity operating in different "modes" of operation or are members of the same gene family. Discussion of this subject merits special attention and is presented below.

6. COMPARISON BETWEEN THE ELECTRO-NEUTRAL Na+K+2Cl (OR 2Na+1K+3Cl, IN THE SQUID) COTRANSPORTER AND THE POSTULATED 2Na+2K+2Cl/1Mg ELECTRONEUTRAL EXCHANGER

Postulation of the putative $2Na^+$+$2K^+$+$2Cl^-$:$1Mg^{2+}$ exchanger may raise the question of whether a protein transporting all these ions could in fact exist in the plasma membrane. Answering this question brings to mind the existence of another, well established and already cloned transporter: the Na+K+Cl cotransporter (NKCC) (reviewed in Ref. 41). This cotransporter operates with a Na+K+2Cl stoichiometry in most cells but with a 2Na+K+3Cl stoichiometry in squid giant axons (77). There are some striking similarities between the putative $2Na^+$+$2K^+$+$2Cl^-$:$1Mg^{2+}$ exchanger and the NKCC including the fact that both transporters:

- effect the translocation of Na, K, Cl from the extracellular to the intracellular side of the membrane;

- are electroneutral;
- can operate in various modes of exchange;
- involve intracellular Mg^{2+} (Mg_i) for their operation: NKCC is activated by Mg_i (independent of ATP) (78) while the $2Na^+$+$2K^+$+$2Cl^-$:$1Mg^{2+}$ exchanger transports this divalent cation; and
- have an absolute dependence on intracellular ATP

Having so many similarities, the possibility could be raised that both transporters are in fact the same protein moiety with the ability to "switch" modes of operation. Although this possibility cannot be discarded at present, there is an important difference between both transporters: NKCC is highly sensitive to bumetanide (i.e., $K_{0.5}$= 10^{-7} M) (54) while the $2Na^+$+$2K^+$+$2Cl^-$:$1Mg^{2+}$ exchanger appears to be much less sensitive to this loop diuretic since it is able to operate in the presence of this concentration of the loop diuretic (53).

For many years it was thought that the Na+Cl cotransporter, the K+Cl cotransporter and the NKCC constituted different "modes" of operation of a single transport moiety. However, it has now been established that all three of these cotransporters are separate proteins encoded by the same gene family (79-81). In fact, it has been proposed that this gene family be termed the cation chloride cotransporter gene family (82). These three cotransporters share numerous characteristics but are somewhat distinguishable by pharmacological means. Bumetanide inhibits with much lower potency the K+Cl cotransporter (i.e., $K_{0.5}$= 10^{-4} M) (83) as compared to the NKCC (see above) and does not appear to inhibit the Na+Cl cotransporter (84).

Clearly, it is very tempting to speculate that the putative $2Na^+$+$2K^+$+$2Cl^-$:$1Mg^{2+}$ exchanger and the NKCC are either the same protein entity or are members of the same gene family. Answer to these question will be vigorously pursued.

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