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Mg^{2+} release coupled to Ca^{2+} uptake: A novel Ca^{2+} accumulation mechanism in rat liver*

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Abstract

Isolated hepatocytes release 2–3 nmol Mg^{2+}/mg protein or ~10% of the total cellular Mg^{2+} content within 2 minutes from the addition of agonists that increase cellular cAMP, for example, isoproterenol (ISO). During Mg^{2+} release, a quantitatively similar amount of Ca^{2+} enters the hepatocyte, thus suggesting a stoichiometric exchange ratio of 1 Mg^{2+}:1Ca^{2+}. Calcium induced Mg^{2+} extrusion is also observed in apical liver plasma membranes (aLPM), in which the process presents the same 1 Mg^{2+}:1Ca^{2+} exchange ratio. The uptake of Ca^{2+} for the release of Mg^{2+} occurs in the absence of significant changes in $\Delta\psi$ as evidenced by electroneutral exchange measurements with a tetraphenylphosphonium (TPP^+) electrode or ^{3}H-TPP^+. Collapsing the $\Delta\psi$ by high concentrations of TPP^+ or protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) does not inhibit the Ca^{2+}-induced Mg^{2+} extrusion in cells or aLPM. Further, the process is strictly unidirectional, serving only in Ca^{2+} uptake and Mg^{2+} release. These data demonstrate the operation of an electroneutral Ca^{2+}/Mg^{2+} exchanger which represents a novel pathway for Ca^{2+} accumulation in liver cells following adrenergic receptor stimulation.

Key words: Ca^{2+} accumulation, Ca^{2+} transport, hepatocyte, liver plasma membranes, Mg^{2+} transport

Abbreviations: TPP^+, tetraphenylphosphonium; SCN^-, sodium thiocyanate; aLPM, apical liver plasma membranes; AAS, atomic absorption spectrophotometry; FCCP, protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

Introduction

Total Mg^{2+} concentration is approximately 20 mM in mammalian cell types and tissues [1, 2]. It is well-known that Mg^{2+} is essential for the regulation of many cellular functions and enzymes [3–5]. *Free* Mg^{2+} remains relatively stable despite rapid and massive changes in total Mg^{2+} concentration. It has been observed that very large quantities of Mg^{2+}, approximately 10% of the total cell/tissue content, are mobilized within minutes of stimulation by hormonal or non-hormonal stimuli [6–9]. For example, adrenergic agonists or agents that increase cellular cAMP levels [6–9] stimulate a massive Mg^{2+} efflux from hepatocytes, cardiac myocytes, as well as rat, chicken and human erythrocytes [6–10]. This evidence strongly suggests the operation of powerful Mg^{2+} transport machinery within the plasma membranes of mammalian cells. Findings from this laboratory [11, 12] have indicated the operation of three distinct Mg^{2+} exchange mechanisms in rat liver: two individual Na^+/Mg^{2+} transporters and one Ca^{2+}/Mg^{2+} transporter, which are located in distinct regions of the cell membrane and operate with distinct criteria [11, 12].

The operation of a Na^+-dependent and a Na^+-independent (Ca^{2+} dependent) Mg^{2+} extrusion mechanism is corroborated

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by various experimental evidences in intact cells [6, 9]. However, because these Mg^{2+} transporters have not been isolated, their basic kinetic properties remain unresolved. In fact, inconsistent results can be found in the literature for the exchange ratios [1, 13], the role of trans-membrane potential, and the effectiveness of various inhibitors [1, 5, 14] on these transporters. These inconsistencies are more pronounced for the Na^{+}-independent Mg^{2+} extrusion pathway whose presence has been proposed in human, chicken, and rat erythrocytes [14]. The requirement for a specific counter-ion to be transported in exchange for cellular Mg^{2+} is not well-defined. In different experimental models or conditions, cellular Mg^{2+} has been shown to exchange for extracellular Mn^{2+} [15], Ca^{2+} [9], HCO_3^{2-} [14], or other cations or anions [14], with various stoichiometries.

The operation of a putative Ca^{2+}/Mg^{2+} exchanger in isolated apical liver plasma membrane vesicles (aLPM) has been described by this laboratory in terms of inhibition, localization, dose-response, and exchange ratios with Mg^{2+} [11, 12]. This exchanger is localized in the apical domain of the hepatocyte is unidirectional and is inhibited by amiloride, imipramine, and quinidine [11, 12]. In the present study, we further characterize the hepatic Ca^{2+}/Mg^{2+} exchanger and demonstrate that in both cells and isolated membranes a large and rapid Ca^{2+} uptake is observed in exchange for Mg^{2+} release upon stimulation by the integrated β-adrenergic pathway.

Materials and methods

Isolation of collagenase dispersed cells

Fed male Sprague–Dawley rats (230–250 g body weight) were anesthetized by i.p. injection of saturated sodium-pentobarbital solution. The portal vein was cannulated and collagenase-dispersed hepatocytes were isolated according to the procedure of Seglen [16]. After isolation, hepatocytes were resuspended as previously described [17].

Plasma membrane isolation

Total liver plasma membrane (tLPM) vesicles were isolated and stored as described in detail elsewhere [11, 12]. The purity of plasma membrane vesicles was assessed by using 5′-nucleotidase, cytochrome-*c* oxidase, and glucose 6-phosphatase activities as markers for plasma membrane, mitochondria, and endoplasmic reticulum, respectively. Negligible amounts of endogenous carryover cations and adenine phosphonucleotides were detected by atomic absorbance spectrophotometry and HPLC, respectively.

Isolation of aLPM fraction from total LPM population, purity and orientation

Apical liver plasma membrane vesicles were isolated and stored as described in detail elsewhere [11, 12]. Alkaline phosphatase, Na^{+}/K^{+}-ATPase and 5′-nucleotidase were used to assess purity and orientation of aLPM.

Plasma membrane loading

Loading of membranes are described in detail elsewhere [11, 12]. Efficiency of loading was assessed by treating the vesicles with the ionophore A23187, and measuring the amount of Mg^{2+} extruded in the extra-vesicular space or retained into the vesicle pellet by AAS, as reported previously [11, 12].

^{45}Ca accumulation

To quantify the amount of Ca^{2+} accumulated by the plasma membranes the membranes were incubated in the presence of 1.2 mM $CaCl_2$ labeled with 0.5 μCi/ml $^{45}Ca^{2+}$. After 3 min equilibration, 0.5 ml of the incubation mixture was withdrawn in duplicate before and 1 min after cation administration and filtered onto glass fiber filters (Whatman, 0.25-μm pore size) under vacuum suction [18]. The filters were rapidly washed with 5-ml ''ice-cold'' sucrose (250 mM) [18]. The radioactivity retained onto the filters was measured by β-scintillation counting in a Beckman LS 7000. Similar procedures were used for the measurement of ^{3}H-TPP^{+}.

Measurement of cation content cells or vesicles by Atomic Absorbance Spectroscopy (AAS)

Determination of Mg^{2+} and Ca^{2+} content in cells and aLPM was carried out as previously described in detail [11].

Determination of membrane potential

Membrane potential was monitored with the use of a tetraphenylphosphonium (TPP^{+}) electrode. The TPP^{+}-sensitive electrode was made by preparing a solution of 100-mg sebacic acid bis-2-ethylhexyl ester or dioctylpthalate, 97.5-mg polyvinylchloride (PVC) and 20-mg Na-tetraphenylborate (TPB) in 1-ml tetrahydofuran (THF). All the components were added and solubilized in the PVC–THF solution, which was poured into a 2.5-cm diameter ring plate and left to evaporate for 48 h. A piece of membrane

was then glued onto 2.5-mm-diameter PVC tubing and an Ag–AgCl electrode was added. The TPP^+ electrode was filled with 1–100-μM TPP^+ in 10-mM KCl. The reference was an Ag–AgCl electrode filled with 3 M KCl salt bridge. The response of the electrode in the nano or μmolar range was logarithmic with a slope of 63 mV.

Animal ethics

Animals were maintained and handled in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Science, National Research Council, 1996), as approved by the Animal Resource Center at Case Western Reserve University, Cleveland, Ohio.

Other experimental procedures

Protein content was determined using the procedure of Bradford *et al.* [19], using BSA as standard.

Chemical

Collagenase (CLS-2) was from Worthington (Lakewood, NJ). All other reagents were of the purest analytical grade, from Sigma (St. Louis, MO).

Statistical analysis

Data are presented as means ± SE. Data were first analyzed by one-way ANOVA. Multiple means were then compared by Tukey's multiple comparison test with $p \leq 0.05$ designated as statistically significant.

Results

Mg^{2+} release coupled to Ca^{2+} uptake in isolated hepatocytes

Figure 1 shows that stimulation by selective α_1 (10-μM phenylephrine), β (10-μM isoproterenol), or mixed (10-μM epinephrine) adrenergic receptor agonists, or by 250-μM 8Br-cAMP induces a marked Ca^{2+} accumulation into rat liver hepatocytes. The net amount of Ca^{2+} uptake by the cell is approximately 2 nmol/mg protein above the basal control values. This value is similar to the amount of Mg^{2+} released under the same experimental conditions (Fig. 1) and suggests that equivalent amounts of Mg^{2+} are released from the cells in exchange for Ca^{2+} accumulation. Hepatocytes stimulated by adrenergic agonists release Mg^{2+} and increase their total Ca^{2+} content with a stoichiometry 1:1.

Net $^{45}Ca^{2+}$ accumulation into apical liver membranes (aLPM)

Figure 2 shows purified, isolated apical liver plasma membranes loaded with 20-mM $MgCl_2$ and stimulated by 500-μM $CaCl_2$ labeled with $^{45}Ca^{2+}$. The amount of Mg^{2+} released is quantitatively similar to the amount of $^{45}Ca^{2+}$ accumulated within the aLPM at 1 min after addition of Ca^{2+} (i.e., 128.41 ± 3.18 nmol Mg^{2+}/mg protein for 128.4 ± 0.18 nmol Ca^{2+}/mg protein). We have previously reported that the Ca^{2+}/Mg^{2+} exchanger is not present in the basolateral membrane of the hepatocytes [12]. Consistent with this observation, when 20-mM Mg^{2+} loaded bLPM were stimulated by 500-μM $CaCl_2$ labeled with $^{45}Ca^{2+}$, no Ca^{2+} uptake was observed (data not shown).

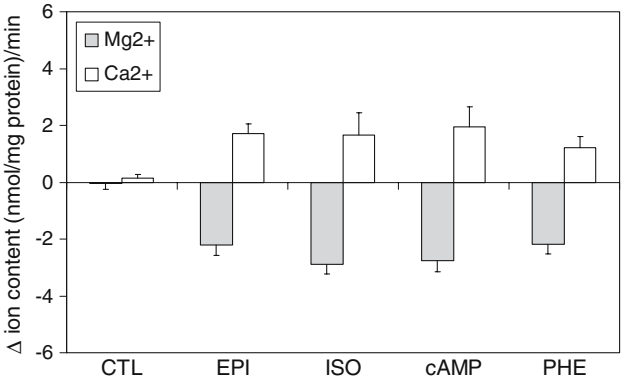

Fig. 1. Net Mg^{2+} extrusion and Ca^{2+} uptake from collagenase-dispersed hepatocytes stimulated by different adrenergic agonist *in vitro.* The amount of Mg^{2+} extruded into the extracellular compartment or Ca^{2+} accumulated in the cell was determined as described under Material and Methods. After a few minutes of equilibration 10-μM isoproterenol (ISO), 5-μM phenylephrine (PHE), 5-μM epinephrine (EPI) or 250-μM cAMP was added to the incubation mixture. The total amount of Mg^{2+} extruded (or Ca^{2+} accumulated) from the cells after 6-min stimulation with the different agonists was then plotted as net amount of respective cation. After 2-min equilibration, an aliquot corresponding to 500 μl of incubation media was withdrawn and rapidly sedimented in microfuge tubes. The supernatant was assayed for Mg^{2+} or Ca^{2+} content by AAS. The figure represents the net change in Mg^{2+} or Ca^{2+} content in the supernatant with respect to that present prior to addition of agonist. Data are means ± SEM of 4 different cell preparations, each of them performed in quadruplicate for all the experimental conditions. All points are statistically significant ($p < 0.05$ vs. control) for Tukey's test vs. control. At each time point, the amount of Mg^{2+} released vs. the corresponding Ca^{2+} uptake is not statistically significant.

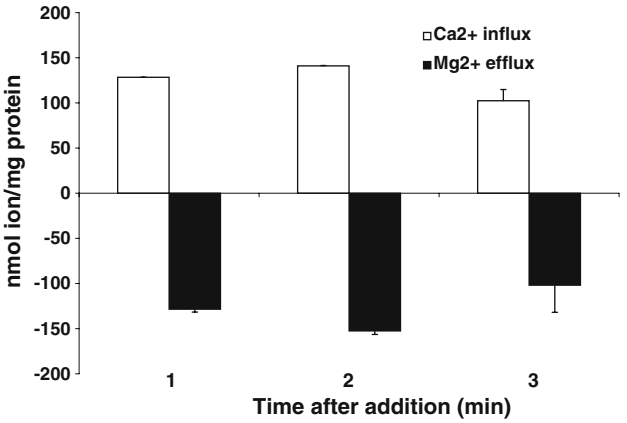

Fig. 2. Net Mg^{2+} extrusion and $^{45}Ca^{2+}$ uptake from purified aLPM. The amount of Mg^{2+} extruded into the extravesicular compartment or $^{45}Ca^{2+}$ accumulated into the vesicles was determined as described under Material and Methods. Data are means ± SEM of 4 different membrane preparations. *$p < 0.05$ vs. Mg^{2+} efflux. At each time point, the amount of Mg^{2+} released vs. the corresponding Ca^{2+} uptake is not statistically significant.

Directionality of the Ca^{2+}/Mg^{2+} transporter in aLPM and hepatocytes

Figure 3 shows aLPM loaded with either 20-mM $CaCl_2$ (Fig. 3A) or 20-mM $MgCl_2$ (Fig. 3B). The addition of 500-μM $MgCl_2$ to Ca^{2+} loaded vesicles does not induce an efflux of Ca^{2+} from (Fig. 3A) or an uptake of Mg^{2+} into the vesicles (not shown). However, addition of 500-μM $CaCl_2$ to Mg^{2+} loaded vesicles released ~65% of the loaded Mg^{2+}. Most of the Mg^{2+} releasable by Ca^{2+} could also be released by detergent or cation ionophore treatment demonstrating that the vesicles were loaded (Figs. 3A and B) and not spontaneously leaky [see refs. 11, 12].

The Mg^{2+}/Ca^{2+} exchanger is not dependent upon membrane potential

Figure 4 shows that when membrane potential is collapsed in cells (Fig. 4A) or aLPM (Fig. 4B) using a high concentration of the lipophilic cation, TPP^+ chloride, Mg^{2+} can still be released in exchange for Ca^{2+}. Stimulation of cells (not shown) or vesicles by TPP^+ alone (Fig. 4B), even up to a concentration of 10 mM does not inhibit the efflux of Mg^{2+} from or a Ca^{2+} uptake into cells or aLPM. Similar results were observed in cells treated with ouabain (OUB) (Fig. 4A), the lipophilic anion, sodium thiocyanate (SCN^-) (Fig. 4A) or the protonophore, protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (not shown). Even when vesicles were loaded with TPP^+ and Mg^{2+}, there were no changes in the operation of the Ca^{2+}/Mg^{2+} exchanger (data not shown).

The Mg^{2+}/Ca^{2+} exchange is electroneutral

Figure 5 demonstrates changes in membrane potential using a TPP^+ electrode. The time course of accumulation of TPP^+ by aLPM loaded with Mg^{2+} was kinetically measured. Fig. 5A (control) shows the changes in the electrode with subsequent addition of 125-nM TPP^+ and addition of 500-μM Ca^{2+}. Figure 5B demonstrates that upon addition of 500-μM Ca^{2+} to Mg^{2+} loaded vesicles, there are no detectable changes in membrane potential (i.e., no accumulation of TPP^+). Similar results were obtained with ^{3}H-TPP^+ (data not shown). The Ca^+/Mg^{2+} antiport is charge balanced as there are no more or less charge equivalents transported by out by Mg^{2+} efflux than carried in by Ca^{2+} influx that required a compensation by TPP^+ accumulation.

Discussion

Characteristics of the Mg^{2+}/Ca^{2+} Exchange Mechanism

Stoichiometry

In hepatocytes (Fig. 1) and aLPM (Fig. 2), the Mg^{2+}:Ca^{2+} ratio is 1:1. The Mg^{2+} efflux mechanism in aPLM is dependent upon extravesicular Ca^{2+} and operates only in terms of Mg^{2+} release and Ca^{2+} uptake. This mechanism, which is observed consistently in membranes as well as isolated hepatocytes, could be seen as an additional mechanism for cellular Ca^{2+} uptake during catecholamine stimulation.

Directionality

The presence of a physiological concentration of extracellular Ca^{2+} is required for agonist-stimulated Mg^{2+} extrusion (Fig 1 and refs. 8 and 9). However, the role of Ca^{2+} in Mg^{2+} influx is negligible [17]. It has been shown that Mg^{2+} *uptake* in isolated hepatocytes is independent of Ca^{2+}concentration [17]. In addition, in the absence of extracellular Ca^{2+}, Mg^{2+} uptake is unaffected and cellular Ca^{2+} content remains unchanged [17]. In contrast, Mg^{2+} extrusion is completelty inhibited in the absence of extracellular Ca^{2+} at least when selective α_1 agonists, such as phenylephrine (PHE), are used further suggesting the presence and operation of the unidirectional Ca^{2+}/Mg^{2+} exchanger in isolated cells during adrenergic stimulation [18].

Ca^+/Mg^{2+} transporter is not dependent on membrane potential and is electroneutral

The Ca^+/Mg^{2+} transporter operates with a stoichiometry of 1:1, consistent with the above data (Figs. 1 and 2) as well as the TPP^+ electrode data presented. The data further demonstrate that the Ca^+/Mg^{2+} transporter in aLPM operates in

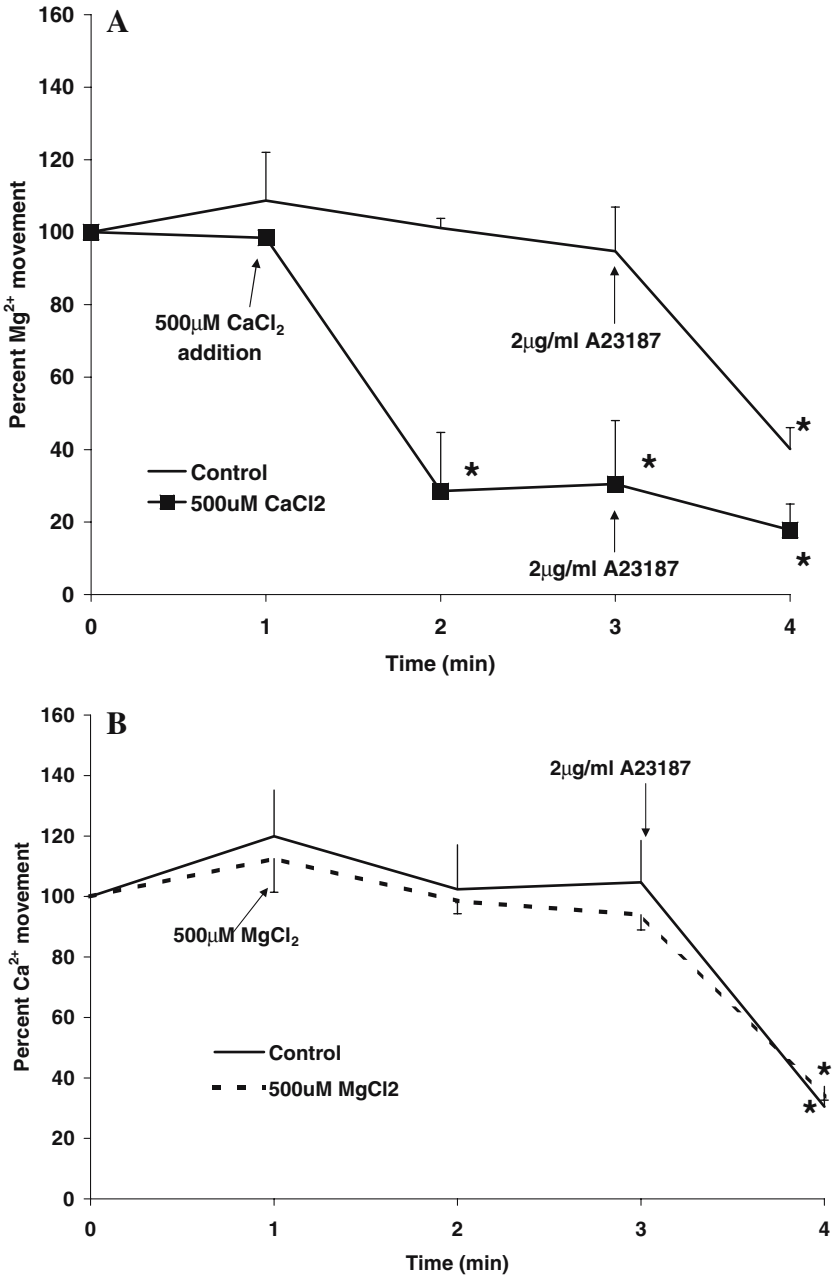

Fig. 3. Unidirectionality of the Mg^{2+}:Ca^{2+} exchanger in aLPM. Percent movement of Mg^{2+} from 20-mM $CaCl_2$ loaded vesicles (A) or Ca^{2+} movement from 20-mM $MgCl_2$ loaded vesicles (B). After 1-min equilibration, 500-μl aliquots were withdrawn and rapidly sedimented through an oil layer (see Materials and Methods). The supernatant and the oil layer were removed and the pellets were digested overnight in 500-μl 10% HNO_3. The Mg^{2+} or Ca^{2+} content in the acid extract was assayed by AAS. After the second withdrawal, either 500-μM Ca^{2+} (A) or 500 Mg^{2+} (B) or 2-μg/ml A23187 were added. Aliquots of the incubation media were withdrawn at the reported time points and processed as above. The Mg^{2+} or Ca^{2+} content retained in the vesicles following addition or A23187 is expressed as a percent of the initial ion content present at time 0. Data are means ± SEM of 4 different preparations. Statistically significant vs. control (*$p < 0.05$).

an electroneutral fashion. As an additional control, the addition of Na^+ to Mg^{2+} loaded aLPM demonstrated a time dependent decrease in concentration of extravesicular TPP^+ indicating a TPP^+ uptake by the Mg^{2+} loaded aLPM vesicles (Fig. 5B). This would be consistent with the previous report that the Na^+/Mg^{2+} exchanger in the aLPM is electrogenic [12]. In addition, even in the presence of a collapsed membrane potential, the Ca^{2+}/Mg^{2+} exchanger is functional.

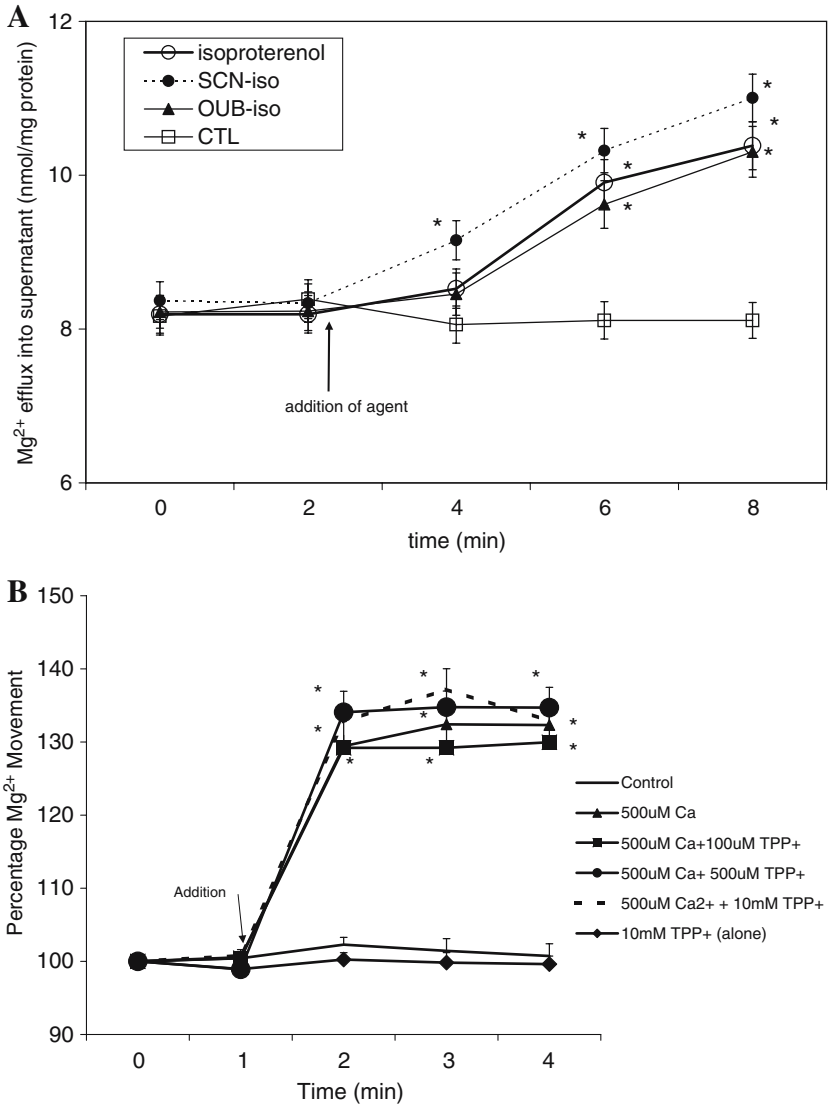

Fig. 4. Mg^{2+} /Ca^{2+} exchanger and dependence on membrane potential in cells and aLPM. Figure 4A shows the amount of Mg^{2+} extruded into the extracellular compartment was determined as described under Material and Methods. After a few minutes of equilibration 10-μM isoproterenol (ISO) alone or 10-mM isoproterenol (ISO) and ouabain and/or SCN were added to the incubation mixture. The total amount of Mg^{2+} extruded from the cells was measured every 2 min. Figure 4B shows percent movement of Mg^{2+} from 20-mM $MgCl_2$ loaded vesicles stimulated with Ca^{2+} and/or increasing membrane collapsing concentration of TPP^+. After 1 min equilibration, 500-μl aliquots were withdrawn and rapidly sedimented through an oil layer (see Materials and Methods). For both figures data are means ± SEM of 4 different membrane preparations. For Fig. 4A $*p < 0.05$ vs. control. None of the individual agonist points were statistically significant from each other at each time point. For Fig. 4B, $*p < 0.05$ vs. control or for 10-mM TPP^+. None of the individual agonist points were statistically significant from each other.

Physiological significance of the Ca^{2+}/Mg^{2+} exchanger

Data in the literature indicate that the concentration of total Ca^{2+} in the bile ranges from 2 to 16 mM [20]. It is well known that concentration of Ca^{2+} in the biliary duct contributes to the formation of gallstones [20–24]. High concentrations of Ca^{2+} in the bile decrease biliary flow [24] which ultimately increases the formation of calcium salts. The physiological role of the Ca^{2+}/Mg^{2+} exchanger in hepatocytes may play a role in the prevention of bile stones. The unidirectional Ca^{2+}/Mg^{2+} exchange could be responsible for decreasing biliary Ca^{2+} thereby preventing the formation of bile stones, or be an additional mechanism contributing to the overall reabsorption of Ca^{2+} [20–23]. In addition, the Ca^{2+}/Mg^{2+} exchanger described here is novel in the respect that it is not sensitive to membrane potential as many of the Na^+-dependent apical bile acid transporters and the cAMP dependent K^+ channels are [25, 26]. This transporter may be constitutively active in the apical region

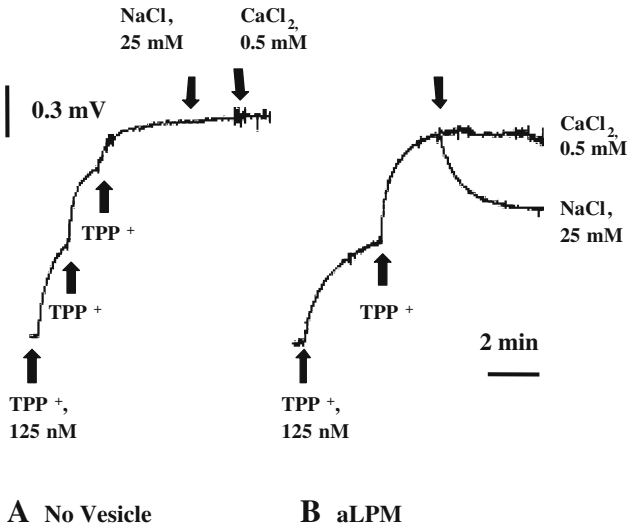

Fig. 5. TPP^+ electrode measurements. Panel A (control) shows sequential addition of 125-nM TPP^+ and then addition of 10-mM NaCl or 500-μM $CalCl_2$ to incubation buffer in the absence of aLPM. Panel B shows sequential addition of TPP^+ to 20-mM $MgCl_2$ loaded aLPM and consequential addition of either 25-mM NaCl or 500-μM $CaCl_2$ performed in separate experiments. Both time and mV are indicated in the figure. Accumulation of TPP^+ into aLPM suggests that, upon exchange, the net positive charge carried out by Mg^{2+} is greater than the positive charge carried into the vesicles by Na^+. A typical experiment for both experimental conditions is shown. A total of 10 similar experiments was performed.

of the liver and may be significant in both bile and liver Ca^{2+} homeostasis during adrenergic stimulation and thus represents a novel Ca^{2+} uptake mechanism in hepatocytes.

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