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Involvement of ERK1/2 and p38 in Mg^{2+} accumulation in liver cells

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Abstract

Activation of PKC signaling induces Mg^{2+} accumulation in liver cells. To test the hypothesis that PKC induces Mg^{2+} accumulation via MAPKs activation, hepatocytes were incubated in the presence of PD98059 and SB202190 as specific inhibitors of ERK1/2 and p38, respectively, and stimulated for Mg^{2+} accumulation by addition of PMA or OAG. Accumulation of Mg^{2+} within the cells was measured by atomic absorbance spectrophotometry in the acid extract of cell pellet. The presence of either inhibitor completely abolished Mg^{2+} accumulation irrespective of the dose of agonist utilized while having no discernible effect on β-adrenoceptor mediated Mg^{2+} extrusion. A partial inhibition on α_1-adrenoceptor mediated Mg^{2+} extrusion was observed only in cells treated with PD98059. To confirm the inhibitory effect of PD98509 and SB202190, total and basolateral liver plasma membrane vesicles were purified in the presence of either MAPK inhibitor during the isolation procedure. Consistent with the data obtained in intact cells, liver plasma membrane vesicles purified in the presence of PD98509 or SB202190 lost the ability to accumulate Mg^{2+}in exchange for intra-vesicular entrapped Na^+ while retaining the ability to extrude entrapped Mg^{2+} in exchange for extra-vesicular Na^+. These data indicate that ERK1/2 and p38 are involved in mediating Mg^{2+} accumulation in liver cells following activation of PKC signaling. The absence of a detectable effect of either inhibitor on β-adrenoceptor induced, Na^+-dependent Mg^{2+} extrusion in intact cells and in purified plasma membrane vesicles further support the hypothesis that Mg^{2+} extrusion and accumulation occur through distinct and differently regulated transport mechanisms. (Mol Cell Biochem **288:** 191–199, 2006)

Key words: MAPKs, p42/p44, p38, hepatocyte, Mg^{2+} accumulation, PKC, PMA, OAG

Introduction

In mammalian cells, Mg^{2+} homeostasis and transport are tightly regulated [1, 2] to the point that cellular Mg^{2+} content remains virtually unchanged in the absence of metabolic or hormonal stimuli. Within the cell, endo-sarco-plasmic reticulum, mitochondria, and nucleus represent three major Mg^{2+} pools, with concentrations ranging between 14 to 20 mM in each of these compartments. A significant amount of Mg^{2+} is also present in the cytoplasm in the form of a complex with ATP and other phospo-metabolites (∼5mM, [3]). As a result of this distribution, less than 1 mM Mg^{2+} is free in the cytoplasm or within the mitochondrial matrix [1, 2]. Following the administration of hormones or agents that increase cellular cAMP level, mammalian cells extrude a sizeable amount of Mg^{2+} in the extracellular space [see refs. [1, 4] for a list], and ultimately in the blood [5]. Although the transporter(s) ultimately responsible for extruding Mg^{2+} out of the cell has not been cloned to date, pharmacological and experimental evidence in intact cells and purified liver plasma membrane vesicles [6–9] suggests that Mg^{2+} extrusion across the cell membrane mainly occurs via a putative Na^+/Mg^{2+} exchanger [reviewed in [10]]. Under the stimulatory conditions described above, a detectable decrease in total cellular

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Mg^{2+} content has been observed [11], which mainly affects Mg^{2+} level within cellular compartments [12] while cytosolic free Mg^{2+} concentration remains virtually unchanged [13].

As a result of hormonal stimuli, mammalian cells can also accumulate Mg^{2+} from the extra-cellular compartment. This accumulation is the result of protein kinase C activation by hormones (e.g. vasopressin) [14] or agents such as diacylglycerol analogs [14] or phorbol-myristate acetate derivates [14]. Under these conditions, total cellular Mg^{2+} content increases significantly [14] while cytosolic free Mg^{2+} concentration varies little, if at all [13]. It is presently undefined whether Mg^{2+} entry is mediated via the reverse operation of the Na^+/Mg^{2+} exchanger, as suggested by data obtained in liver plasma membranes [8, 9], or a distinct pathway, possibly a channel, as suggested by results in cardiac ventricular myocytes [15] or kidney cells [16].

It is also unclear whether protein kinase C activates the Mg^{2+} entry mechanism directly or indirectly. Experimental evidence from various laboratories [17–20] suggests a role of MAPKs in modulating cellular Mg^{2+} content in a variety of experimental models, although the underlaying mechanisms have not been elucidated. Further, Touyz and collaborators [19] have reported that changes in extra-cellular and/or intracellular Mg^{2+} content can modulate ERK1/2 level of activation and affect cell cycle progression and regulation.

In the present study, we tested the hypothesis that MAPKs operates downstream protein kinase C pathway to mediate Mg^{2+} accumulation in liver cells. To this purpose, suspensions of collagenase-dispersed hepatocytes incubated in the presence of specific inhibitors for ERK1/2, p38 and JNK kinases, and liver plasma membrane vesicles purified in the presence of these inhibitors were used. The results obtained in both experimental models support the hypothesis that ERK1/2 and p38 play an essential role in mediating protein kinase C-induced Mg^{2+} accumulation in liver cells.

Materials and methods

Hepatocytes isolation

Hepatocytes were isolated by collagenase digestion according to the procedure of Seglen [21]. After isolation, the hepatocytes were resuspended in a medium having the following composition (mM): NaCl 120, KCl 3, $CaCl_2$ 1, $MgCl_2$ 1, K_2HPO_4 1.2, $NaHCO_3$ 12, glucose 15, Hepes 10, pH 7.2 at 37°C, equilibrated with an O_2:CO_2 (95:5 v/v) gas mixture, and kept at room temperature under constant flow of O_2:CO_2 until used. Cell viability, assessed by Trypan Blue exclusion test, was found to be 90 ± 3, $n = 9$, and did not change significantly over the course of 4 hours (88 ± 4, $n = 8$).

Mg^{2+} extrusion

To measure Mg^{2+} extrusion, aliquots of cell suspension (1 mL) were transferred to microfuge tubes, and the cells were rapidly sedimented at 600 g for 30 seconds. The cell pellets were washed with 1 mL of a nominal Mg^{2+}-free medium having a composition similar to that described above, with the exception that $MgCl_2$ was omitted from the medium. Magnesium contaminant in the medium was measured by atomic absorbance spectrophotometry (AAS) in a Perkin Elmer 3100, and accounted for ~ 5 μM. After the washing, the cells were transferred to 8mL of Mg^{2+}-free incubation medium, pre-warmed at 37°C, and incubated under continuous O_2:CO_2 flow and stirring. After 2 min of equilibration, 5 μM phenylephrine or 10 μM isoproterenol were added to the incubation system to activate α_1- and β-adrenergic receptor, respectively. At 2 min intervals, 0.7 mL of incubation mixture were withdrawn in duplicate, and the cells sedimented in microfuge tubes. The supernatants were removed and their Mg^{2+} content determined by AAS. Pharmacological doses of α_1- or β-adrenergic receptor agonist were used to exclude that a decrease in Mg^{2+} extrusion could depend on altered receptor responsiveness.

Mg^{2+} accumulation

To measure Mg^{2+} accumulation, 1 ml aliquots of cell suspension were washed as indicated above and resuspended in with 1 mL of the Mg^{2+}-containing medium previously described. The cells were then transferred to 8 mL of Mg^{2+}-containing incubation medium, pre-warmed at 37°C, and incubated under continuous O_2:CO_2 flow and stirring. After 2 min of equilibration, 20 nM 1-oleyl-2-acetyl-glycerol (a diacyl-glycerol analog) or 1 μM phorbol myristate acetate (PMA) were added to the incubation system to activate protein kinase C signaling. At 2 min intervals, 0.7 mL of incubation mixture were withdrawn in duplicate, and the cells sedimented in microfuge tubes. To remove excess extracellular Mg^{2+}, hepatocytes were sedimented trough a dibutyl-phthalate:dioctylphthalate (2:1 v/v) oil layer. The supernatant and the oil layer were aspirate by vacuum suction and the cell pellets resuspended in 0.5 mL 10% HNO_3, and digested overnight. The Mg^{2+} content of the acid extract was measured by AAS after sedimentation of the denaturated protein in microfuge tube at 8,000 *g* for 5 min.

To calculate the net Mg^{2+} movement in and out of the cell, the amount of Mg^{2+} present in the pellet or in the extracellular compartment at time $t = 0$ min and $t = 2$ min was calculated, averaged and subtracted from the later time points of incubation.

Purification of total (tLPM) and basolateral (bLPM) liver plasma membrane vesicles

Total liver plasma membrane (tLPM) vesicles were isolated and stored as described in detail elsewhere [8]. To assess a possible role of ERK1/2 and p38 on Mg^{2+} transport in plasma membrane vesicles, tLPM were purified in the presence of 10 μM PD98059 or 10 μM SB202190 added at the time of homogenization. 5′-nucleotidase, cytochrome-*c* oxidase and glucose 6-phosphatase activities were used as markers for plasma membrane, mitochondria and endoplasmic reticulum, respectively [8], to assess plasma membrane purity. Preparations of tLPM were then used to isolate apical (aLPM) and basolateral (bLPM) vesicles as described in detail by Cefaratti *et al*. [9]. Na^+/K^+-ATPase, alkaline phosphatase, and 5′-nucleotidase were used to assess purity and orientation of aLPM and bLPM [9].

Mg^{2+} Loading

Aliquots of tLPM, aLPM or bLPM were loaded with 20 mM Mg^{2+}, or 20 mM Na^+ according to our published protocols [8, 9], and the orientation of the vesicles determined by measuring Na^+/K^+-ATPase and 5′-nucleotidase activities [8, 9]. The enzymatic activity of these assays indicated that $\geq$ 90% of Mg^{2+}-loaded vesicles were in the '*inside-in*' configuration, consistent with previous reports from our laboratory [8]. The loaded vesicles were resuspended in 5 ml of 250 mM sucrose, 25 mM K-Hepes, pH 7.4 (*Mg^{2+}-free medium*) and stored in ice until used. Loading efficiency was assessed by treating the vesicles with ionophore (A23187) or detergent (Triton X-100), and measuring by AAS the amount of Mg^{2+} extruded in the extravesicular space or retained within the vesicle pellet [2]. Irrespective of the absence or presence of the MAPK inhibitors during the isolation procedure, tLPM aLPM and bLPM contained ~1200 nmol Mg^{2+}/mg protein.

Measurement of Mg^{2+} fluxes in plasma membrane vesicles

Mg^{2+}-loaded tLPM, aLPM or bLPM were incubated in the Mg^{2+}-free medium mentioned above, at 37 °C, under continuous stirring, at the final concentration of ~300 μg protein/ml. After 2 min of equilibration, aliquots of the incubation mixture were withdrawn in duplicate (t = 0 min), and the vesicles sedimented in microfuge tubes at 7,000 *g* for 45 s. Total Mg^{2+} content in the supernatants was measured by AAS. The residual Mg^{2+} content within the vesicles was also measured by AAS after overnight digestion of the vesicle pellets in 0.5 mL 10% HNO_3, and sedimentation of denaturated proteins. Two time points after the equilibration period (t = 0 and t = 2 min) were used to assess basal vesicular and extravesicular Mg^{2+} level. Following the withdrawal in duplicate of the sample t = 2 min, Mg^{2+} transport was stimulated by addition of 25 mM Na^+ or 500 μM Ca^{2+} to the incubation mixture. The incubation was continued for 6 additional minutes, and samples were withdrawn in duplicate at 2 min intervals. Because Mg^{2+} content in the supernatant could vary considerably among preparations as a result of the loading procedure and carry-over, the data are reported as the net variation in extravesicular (or vesicular) Mg^{2+} content, normalized per mg of protein, for simplicity. To determine net Mg^{2+} extrusion, Mg^{2+} content in the supernatant at t = 0 min and t = 2 min was calculated, averaged and subtracted from the values of the subsequent time points of incubation.

Similar experimental procedures were used for Na^+ loaded Mg^{2+}-stimulated bLPM vesicles. We have previously reported the presence of Mg^{2+} accumulation in exchange for intravesicular entrapped Na^+ exclusively in this plasma membrane sub-population [9]. For these experiments, bLPM vesicles were loaded with 20 mM Na^+ and stimulated by extravesicular addition of 10 mM Mg^{2+} [9]. The amount of Na^+ extruded from the vesicles was measured in the supernatant by AAS following sedimentation of the vesicles at 7000 g for 45 s. For comparison purposes, the amount of Mg^{2+} accumulated into the vesicles was measured by AAS following acid extraction of the vesicle pellet.

Statistical analysis

The data are reported as mean ± SEM. Data were first analyzed by one-way ANOVA. Multiple means were then compared by Tukey's multiple comparison test performed with a q value established for statistical significance of $P < 0.05$.

Results

Figure 1 shows a typical incubation experiment, which indicates that no extrusion or uptake of Mg^{2+} occurred in non-stimulated hepatocytes irrespective of the absence or the presence of 10 μM SB202190, a specific p38 inhibitor, in the incubation system. In the absence of the inhibitor, the administration of 10 μM isoproterenol resulted in a marked extrusion of Mg^{2+} from the hepatocytes into the extracellular space whereas the administration of 1 μM PMA resulted in an accumulation of Mg^{2+} into the cells. Comparable results in terms of Mg^{2+} extrusion and Mg^{2+} accumulation were observed in hepatocytes stimulated by 5 μM phenylephrine, and 20 nM OAG or vasopressin, respectively (data not shown, [14]). We have previously reported that Mg^{2+} accumulation is independent of extracellular Mg^{2+} concentration and can still occur in the presence of minimal concentrations of Mg^{2+} in the external milieu [22]. In contrast, the presence

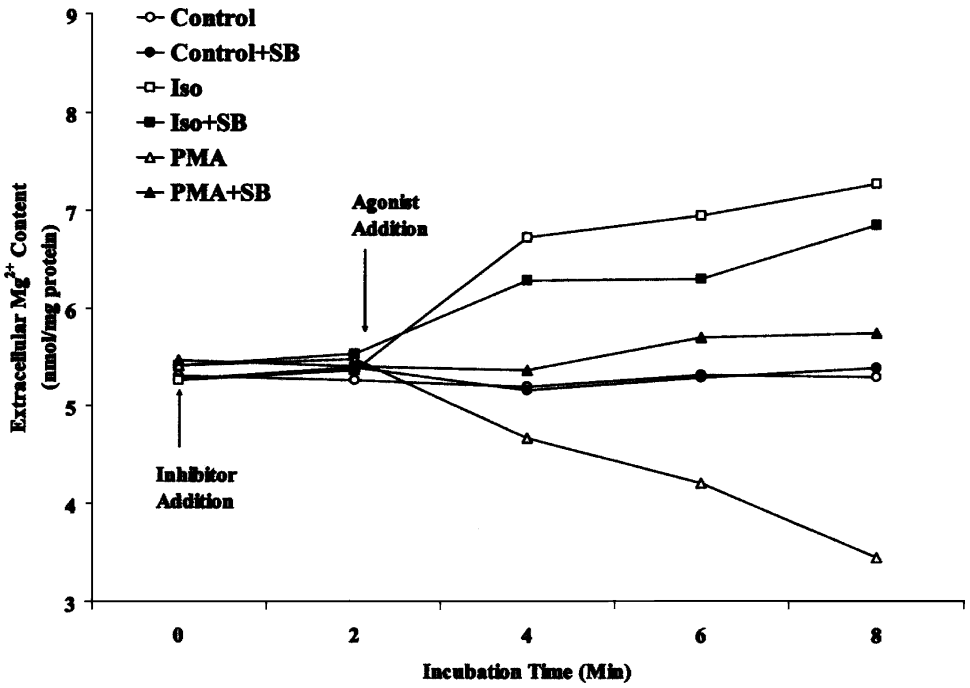

Fig. 1. Mg^{2+} extrusion and accumulation in collagenase-dispersed hepatocytes. Hepatocytes were isolated and incubated as reported under Materials and Methods. SB202190 (10 μM) and PD98059 (10 μM) as selective inhibitors for p38 and ERK1/2, respectively, were added at time $t = 0$ min together with the cells. Phenylephrine (5 μM), isoproterenol (10 μM), phorbol-myristate acetate (PMA, 1 μM), or oleyl-acetyl-glycerol (OAG, 20 nM) were added after withdrawal of time = 2 min. A typical experiment out of 5 is reported.

of SB202190 in the incubation system completely abolished PMA-stimulated Mg^{2+} accumulation while having no effect on isoproterenol-induced Mg^{2+} extrusion (Fig. 1). Similar results on isoproterenol-induced Mg^{2+} extrusion, and PMA or OAG-induced Mg^{2+} accumulation were observed in hepatocytes incubated in the presence of 10 μM PD98059, a specific ERK1/2 inhibitor. Incubation of liver cells in the presence of 1 μM SP600125 as JNK inhibitor did not alter the amplitude of Mg^{2+} accumulation or extrusion (not shown).

The net amounts of Mg^{2+} extruded or accumulated by the cells in the absence or in the presence of SB202190 or PD98059 are reported in Figs. 2A and 2B, respectively. Because Mg^{2+} movement in and out of the cell reached the maximum at time = 8 min (i.e. 6 min after agonist addition), not changing significantly at later time points [7], the net increase in cellular Mg^{2+} content (accumulation) and extracellular Mg^{2+} content (extrusion) at this time point are reported in the figure for simplicity. As Figs. 2A and 2B show, the presence of either inhibitor prevented Mg^{2+} accumulation in hepatocytes stimulated by PMA or OAG, or vasopressin (not shown). In contrast, isoproterenol-induced Mg^{2+} extrusion was not affected by the presence of the p38 (Fig. 2A) or ERK1/2 (Fig. 2B) inhibitor. As for the Mg^{2+} extrusion elicited via α_1-adrenergic stimulation (phenylephrine), no inhibitory effect was observed in hepatocytes pre-incubated with SB202190 whereas Mg^{2+} mobilization decreased by ~70% in hepatocytes incubated in the presence of PD98059 (2.7 ± 0.3 vs. 0.8 ± 0.6 nmol/mg protein/6 min for hepatocytes incubated in the absence and in the presence of the inhibitor, respectively, $n = 5$, $p < 0.02$).

The inhibitory effect of SB202190 or PD98059 on Mg^{2+} transport was further investigated in tLPM vesicles purified in the absence or in the presence of either SB202190 or PD98059. The plasma membrane model presents the advantage that Mg^{2+} transport can be assessed under conditions in which the incubation milieu can be manipulated as desired, in the absence of significant buffering or interference from cellular compartments or signaling components [8, 9]. Yield, purity, orientation and Mg^{2+} loading of tLPM isolated in the presence of PD98059 or SB202190 were completely super-imposable to those of tLPM isolated in the absence of either inhibitor, and consistent with previous reports [8, 9] (not shown).

As Fig. 3A shows, total liver plasma membrane vesicles purified in the absence of PD98059 or SB202190 and loaded with Mg^{2+} mobilized ~142 and ~214 nmol Mg^{2+}/mg protein at time = 6 min after the addition of 50mM NaCl and 500 μM $CaCl_2$, respectively, to the extravesicular milieu. We have previously reported that these concentrations are optimal to elicit maximal Mg^{2+} mobilization from the vesicles

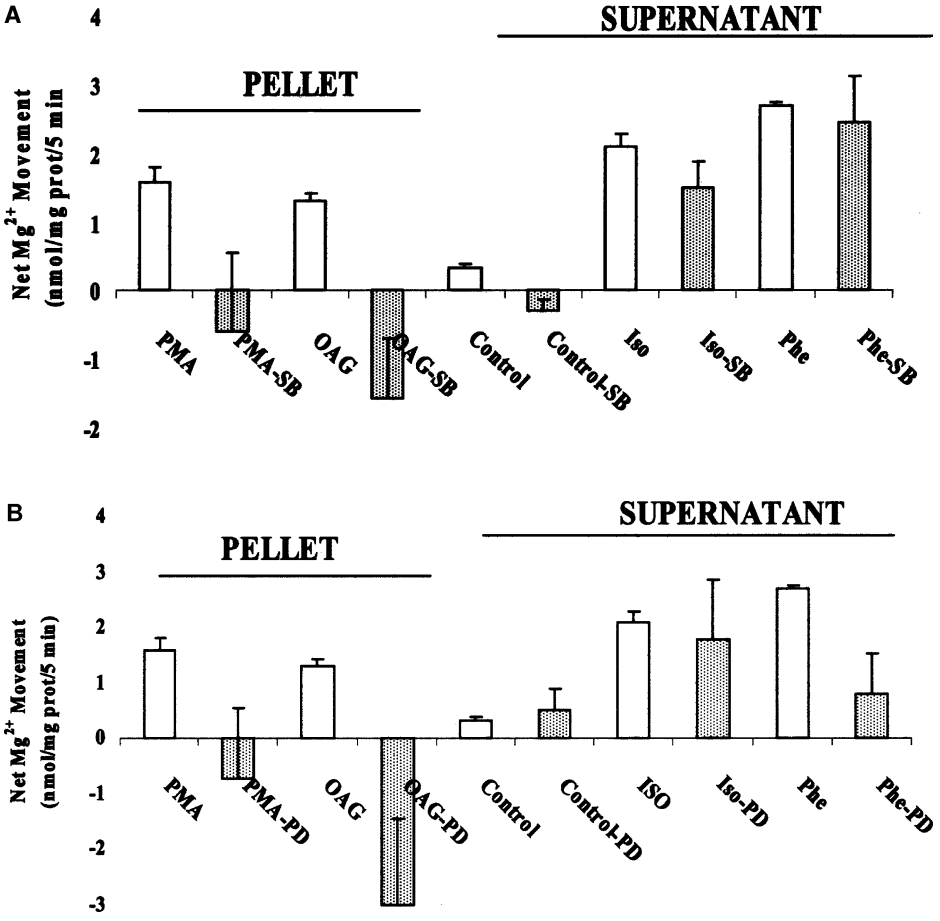

Fig. 2. Net change in cellular and extracellular Mg^{2+} content in hepatocytes incubated in the presence of p38 (Fig. 2A) or ERK1/2 (Fig. 2B) inhibitor.Hepatocytes were isolated and incubated as reported under Materials and Methods, in the presence of SB202190 and PD98059. Cells were stimulated by addition of phenylephrine (5 μM) or isoproterenol (10 μM) to elicit Mg^{2+} extrusion, and by PMA (1 μM) or OAG (20 nM) to induce Mg^{2+} accumulation. Net change in cellular (for the uptake) and extra-cellular Mg^{2+} content (for the extrusion) was calculated as indicated under Materials and Methods. For simplicity, only the net changes at time = 6 min for all the various stimulatory conditions in the absence and in the presence of MAPKs inhibitors are reported. Data are means ±S.E. of 5 different preparations, each performed in duplicate.

[8, 9]. Plasma membrane vesicles purified in the presence of PD98059 or SB202190 did not show a significant change in the amplitude of Na^+-induced Mg^{2+} extrusion (~150 nmol Mg^{2+}/mg protein/6 min in the presence of either inhibitor, Fig. 3B). When extravesicular Ca^{2+} was added to mobilize entrapped Mg^{2+}, however, vesicles purified in the presence of PD98059 or SB202190 presented a reduced Mg^{2+} extrusion (64.2 and 128.9 nmol Mg^{2+}/mg protein/6 min, respectively, vs. 216.8 nmol Mg^{2+}/mg protein/6 min mobilized from untreated plasma membrane vesicles, $n = 4$ for all experimental conditions, $p < 0.001$) (Fig. 3B). This represent ~70% and ~40% decrease, respectively, in the amplitude of Mg^{2+} extrusion as compared to untreated vesicles. A similar inhibitory effect by PD98059 or SB202190 was observed in aLPM, in which the Ca^{2+}-induced Mg^{2+} extrusion mechanism is specifically located [9]. In aLPM purified in the presence of PD98059 or SB202190, Ca^{2+}-induced Mg^{2+} extrusion accounted for 65.8 ± 7.6 vs. 152.2 ± 9.5 nmol Mg^{2+}/mg protein/6 min, respectively, as compared to 205.7 ±14.3 nmol Mg^{2+}/mg protein/6 min in aLPM purified in the absence of either inhibitor, $n = 5$, $p < 0.01$). Purification of tLPM or aLPM in the presence of both inhibitors did not results in an enlarged inhibition of Ca^{2+}-dependent Mg^{2+} extrusion (not shown).

We have reported that Mg^{2+} accumulation only occurs in bLPM vesicles, in which intravesicular entrapped Na^+ is exchange for extravesicular Mg^{2+} with ratio ~1 [9]. Consistent with this observation, untreated basolateral liver plasma membrane vesicles extruded ~300 nmol Na^+/mg protein/6 min (Fig. 4) while accumulating an equivalent amount of Mg^{2+}(289 ± 15 nmol/mg protein/6 min, $n = 4$). Purification of basolateral plasma membrane vesicles in the

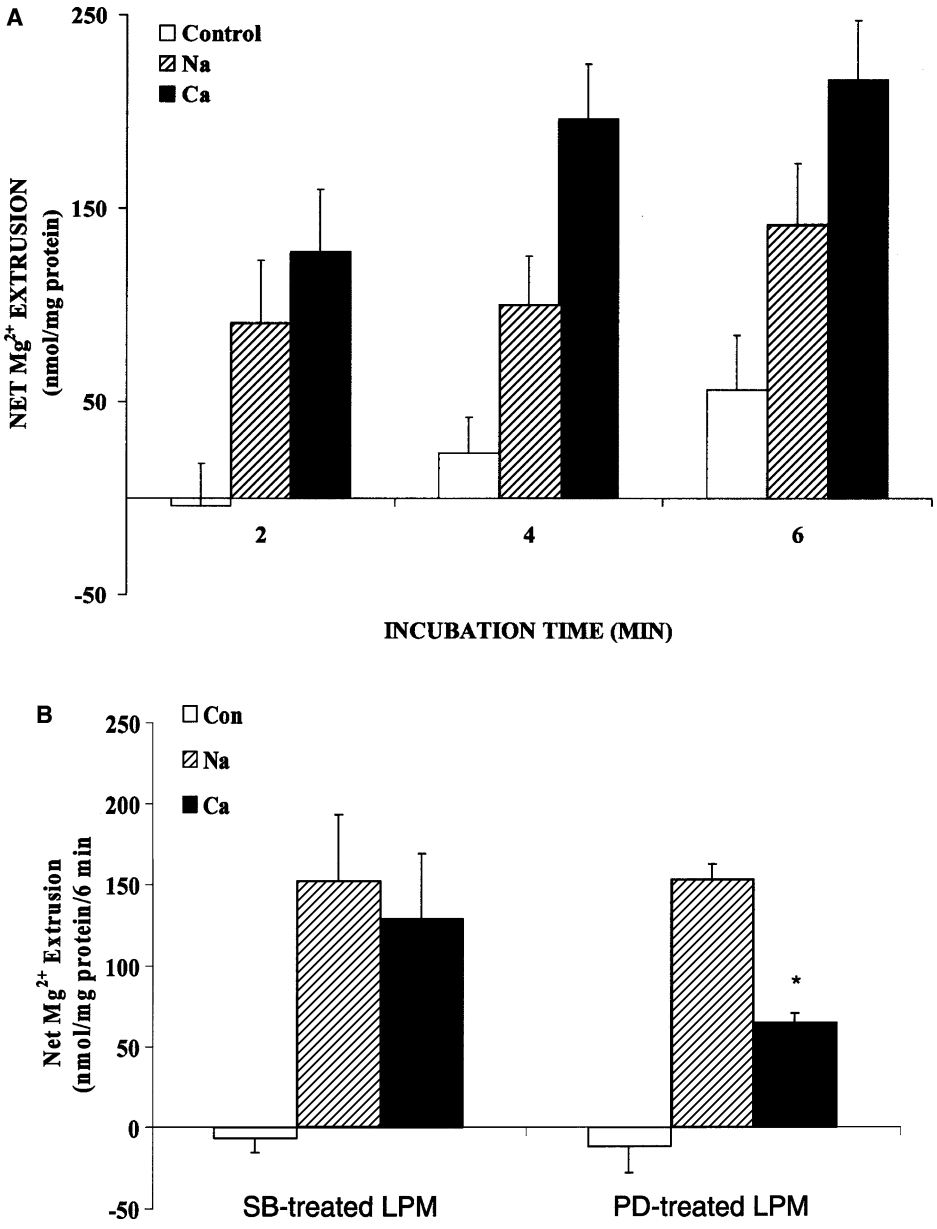

Fig. 3. Net Mg^{2+}extrusion from untreated liver plasma membrane vesicles (Fig. 3A) and from vesicles purified in the presence of p38 or ERK1/2 inhibitor (Fig. 3B).Total liver plasma membrane vesicles were purified as described under Materials and Methods in the absence (Fig. 3A) and in the presence of SB202190 and PD98059 (Fig. 3B). Extrusion of Mg^{2+} from the vesicles was induced by addition of 50mM NaCl or 500 μM$CaCl_2$. In Fig. 3A, the time course of net change in extra-vesicular Mg^{2+} content for untreated plasma membrane is reported. In Fig. 3B, the net change in extra-vesicular Mg^{2+} content at time = 6 min for plasma membrane isolated in the presence of SB202190 or PD98059 inhibitor and stimulated by addition of similar doses of NaCl or $CaCl_2$ is reported for simplicity. Data are means ± S.E. of 4 different preparations for both untreated and treated plasma membrane vesicles, each performed in duplicate.

presence of either PD98059 or SB202190 resulted in the complete abolishment of Mg^{2+} accumulation in exchange for intra-vesicular Na^+. Concentrations of Mg^{2+} higher than the 10mM tested in the figure were similarly ineffective at restoring Mg^{2+} accumulation (not shown).

Discussion

In the last fifteen years, incontrovertible evidence has accumulated about the operation of hormonal controlled mechanisms that transport Mg^{2+} in and out of mammalian cells. In the majority of mammalian cells and tissues tested, Mg^{2+} is

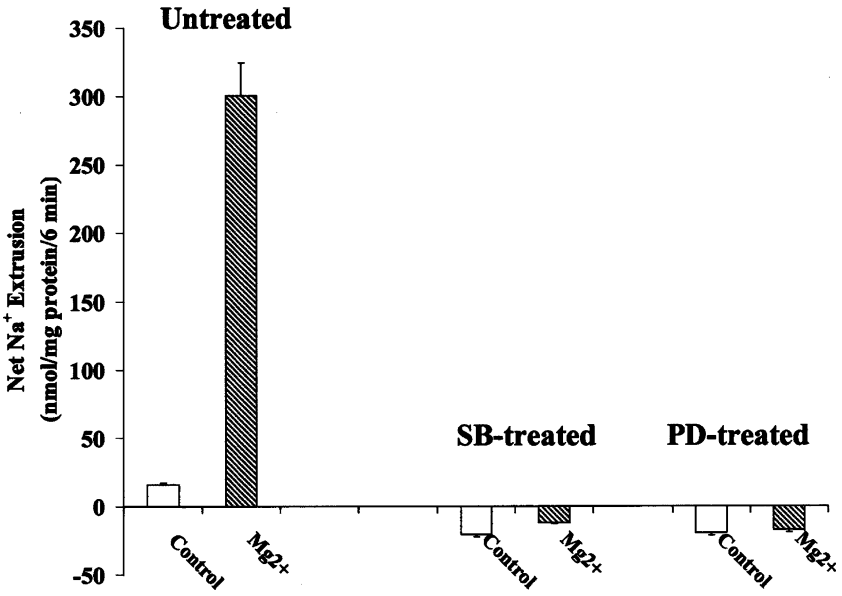

Fig. 4. Net Na^+ extrusion in basolateral liver plasma membrane vesicles purified in the absence and in the presence of p38 or ERK1/2 inhibitor. Basolateral liver plasma membrane vesicles were purified as described under Materials and Methods in the absence and in the presence of SB202190 and PD98059 inhibitors. Vesicles were loaded with 20 mM NaCl, and Na^+ extrusion from the vesicles was induced by addition of 20 mM $MgCl_2$. For simplicity, the net change in extravesicular Na^+ content at time = 6 min after $MgCl_2$ addition is reported. Data are means ± S.E. of 4 different preparations for both untreated and treated plasma membrane vesicles, each performed in duplicate.

extruded via distinct Na^+-dependent and Na^+-independent mechanisms [reviewed in [1, 2]]. These two pathways appear to be activated via the increase in cellular cAMP level [23] and cytosolic Ca^{2+} [24] that follow the activation of β- and α_1-adrenergic receptors, respectively. The structure and intrinsic regulation of the Mg^{2+} extrusion mechanisms operating at the cell membrane level, however, still remains to be fully elucidated.

The same cells types and tissues also accumulate Mg^{2+} following the activation of protein kinase C (PKC) by hormones (e.g. vasopressin [14], bombesin [25] or insulin [26]), phorbol myristate acetate and derivates [14] or diacyl-glycerol analogs [14]. However, it is still controversial which mechanism is ultimately responsible for Mg^{2+} accumulation as the operation of a Na^+/Mg^{2+} exchange and a channel have been reported in liver plasma membrane vesicles [8, 9], and cardiac [15] and kidney cells [16], respectively. While acceptable to some extent in purified plasma membrane vesicles, the operation in reverse of the putative Na^+/Mg^{2+} exchange is conceptually inconsistent with the trans-membrane Na^+ gradient present in intact cells. On the other hand, support for the operation of a channel has been provided by the observation that TRPM6 [27] and TRPM7 [28], two members of the TRP channel family [29], can transport Mg^{2+} under well defined conditions. More recently, new Mg^{2+} transport mechanisms have been cloned [30–33] but it is still undefined whether SLC41, MagT1 or ACDP2 are channels or exchangers, and how are they regulated.

In addition to protein kinase C, recent evidence suggests the involvement of MAPKs in mediating Mg^{2+} accumulation under physiological and pathological conditions [17–20], at least in smooth muscle cells [19, 20] and neuronal cells [17, 18], following stimulation by angiotensin-II or other agents. The results reported in the present study are consistent with these observations as they indicate that ERK1/2 and p38, but not JNK, are involved in mediating Mg^{2+} accumulation in liver cells. This effect is observed in intact hepatocytes following the activation of protein kinase C signaling by PMA or OAG, as well as in basolateral-enriched liver plasma membrane vesicles, which accumulate extravesicular Mg^{2+} in exchange for intravesicular entrapped Na^+ (Fig. 4, and also [9]).

The observation that MAPKs operate downstream protein kinase C is not novel as activation of ERK1/2 by PKCα [34] or PKCε [35] and activation of p38 by PKCδ [36] have been reported. Because the PKC isoform(s) involved in Mg^{2+} accumulation in hepatocytes has(have) not yet been identified, we cannot presently correlate the activation of ERK1/2 and p38 to a specific protein kinase C isoenzyme. Further studies are therefore necessary to identify the PKC isoform(s) involved in mediating Mg^{2+} accumulation under our experimental conditions as well as the modality by which this(these) isoform(s) trans-activate ERK1/2 and p38. Also, the time

course of our experimental protocol does not allow us to confirm whether the inhibition of Mg^{2+} accumulation has long-term effects on hepatocyte functionality or cell cycle, similar to those observed by Touyz and collaborators in cultures of smooth muscle cells [19]. The involvement of ERK1/2 in Mg^{2+} accumulation, however, rises some interesting questions in terms of possible changes in nuclear functions as ERK2 depends on Mg^{2+} level to properly dimerize, translocate and activate specific nuclear targets [37]. A similar dependence on Mg^{2+} for p38 has not been described. It is possible, however, that the increase in cellular Mg^{2+} mediated via this MAPK can modulate the activity state of various (p38-related) enzymes and proteins in the cytoplasm or within cellular organelles.

The involvement of both ERK1/2 and p38 in Mg^{2+} accumulation also rises the possibility that the two MAPKs play a differential role in regulating the Mg^{2+} entry pathway or modulate distinct sites of the transport mechanism. Also, the limited information currently available about the mechanism by which Mg^{2+} enters the cell membrane and its regulation does not allow us to exclude that Mg^{2+} entry and Na^{+} extrusion in bLPM vesicles occur trough two separate but coupled mechanisms differently regulated by p38 and ERK1/2. Additional studies are necessary to discriminate among these possibilities, as well as to determine whether the two MAPKs modulate a differential cellular Mg^{2+} distribution.

Lastly, PD98059 appears to have a partial inhibitory effect of on α_1-adrenoceptor mediated Mg^{2+} extrusion in intact hepatocytes and Ca^{2+}-mediated Mg^{2+} extrusion in liver plasma membrane vesicles. This suggests that ERK1/2 plays a yet undefined role also in regulating Mg^{2+} extrusion trough these two processes. This result confirms the recent report by Shang-Jin *et al.* [38], published during the preparation of this manuscript, that PD98509 administration blocks phenylephrine-induced, Ca^{2+}-mediated Mg^{2+} extrusion in cardiac cells. Hence, it would appear that ERK1/2 has a dual function as it is involved in mediating both Mg^{2+} accumulation and extrusion in liver cells. This effect appears to be restricted to the α_1-adrenoceptor signaling pathway since Mg^{2+} extrusion via the β-adrenoceptor activated, Na^{+}-dependent mechanism is unaffected. Considering together the effect of PD98059 on isolated cells and tLPM vesicles, it would appear that the α_1-adrenoceptor induced, Ca^{2+}-mediated Mg^{2+} extrusion operating in hepatocytes [24] and the Ca^{2+}-induced Mg^{2+} extrusion in tLPM cells [8, 9] are but two aspects of the same transport mechanism. To out knowledge, it is the first time that a direct connection between the activating signaling pathway and the effector transport mechanism can be established with certainty in liver cells. At variance of the well established signaling-effector connection between activation of β-adrenoceptor and operation of the putative Na^{+}/Mg^{2+} exchanger in cells [7, 22] and plasma membrane vesicles [8, 9], it was still uncertain which signaling pathway was responsible for activating the Ca^{2+}-mediated Mg^{2+} extrusion, at least in hepatocytes, since α_1-adrenoceptor mediated Mg^{2+} extrusion in liver cells appear to require the presence of physiological concentrations of both Na^{+} and Ca^{2+} in the extra-cellular milieu [23]. Whether p38 has also a dual role in regulating both Mg^{2+} accumulation and Mg^{2+} extrusion is not completely clear. In fact, our data would indicate an involvement of p38 in Ca^{2+}-mediated Mg^{2+} extrusion in plasma membrane vesicles but not intact hepatocytes. Because the inhibitory effect of SB202190 in tLPM is $\sim$50% lower than that observed with PD98509 and accounts for $\sim$30% decrease in the amplitude of the Ca^{2+}-mediated Mg^{2+} extrusion as compared to that observed in untreated plasma membrane vesicles in the absence of detectable changes in yield, purity and marker enzymes activity, it cannot be excluded that it represents a non-specific effect, possibly due to some interference during tLPM isolation.

Conclusions

Our results provide evidence that p38 and ERK1/2 play a key role in modulating PKC-induced Mg^{2+} accumulation in liver cells. ERK1/2 also appears to be involved in mediating α_1-adreno-ceptor mediated Mg^{2+} extrusion. Because of the relevance of these kinases in regulating gene expression, these results provide a new framework to understand the short- and long-term implications of changes in cellular Mg^{2+} level for various cell functions, including cell cycle following various hormonal stimuli.

Acknowledgments

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