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Arachidonic acid-activated Na^+-dependent Mg^{2+} efflux in rat renal epithelial cells

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Abstract

Arachidonic acid (AA), a metabolite of membrane phospholipids, and its metabolites are increased in Mg^{2+} deficiency. We examined whether the extracellular Mg^{2+} concentration affects AA production and whether AA regulates a putative Na^+-dependent Mg^{2+} efflux pathway in renal epithelial NRK-52E cells. We used the cells cultured in 5 mM Mg^{2+}-containing medium for 2 days because they enable us to detect Na^+-stimulated Mg^{2+} efflux that was not observed in normal culture medium. Removal of extracellular Mg^{2+} increased AA release both in the absence and presence of extracellular Na^+. This was inhibited by methyl arachidonyl fluorophosphonate (MAFP, 10 μM), an inhibitor of cytosolic phospholipase A_2 ($cPLA_2$) and Ca^{2+}-independent phospholipase A_2 ($iPLA_2$), and bromoenol lactone (BEL, 10 μM), an inhibitor of $iPLA_2$. However, LY-311727 (10 μM), a secretory phospholipase A_2 ($sPLA_2$) inhibitor, had no inhibitory effect. Reverse transcriptase-polymerase chain reaction (RT-PCR) showed that NRK-52E cells express $cPLA_2$ and $iPLA_2$ mRNAs, but not $sPLA_2$. In the mag-fura 2 fluorescence measurements, extracellular Mg^{2+} removal caused slight decrease in the intracellular free Mg^{2+} concentration ($[Mg^{2+}]_i$) in the Na^+-free condition. The addition of Na^+ caused a rapid decrease in $[Mg^{2+}]_i$, indicating the presence of a Na^+-dependent Mg^{2+} efflux pathway. The Na^+-dependent $[Mg^{2+}]_i$ decrease was suppressed by MAFP and BEL. On the other hand, AA metabolite inhibitors, nordihydroguaiaretic acid (NDGA) (50 μM), indomethacin (10 μM) and 17-octadecynoic acid (ODYA) (10 μM), enhanced the Na^+-dependent $[Mg^{2+}]_i$ decrease. Furthermore, the addition of exogenous AA (30 μM) enhanced the Na^+-dependent $[Mg^{2+}]_i$ decrease, which was significantly inhibited by imipramine (0.1 mM), a putative Na^+/Mg^{2+}-exchanger inhibitor. These results suggest that extracellular Mg^{2+} removal elevates AA release mediated mainly by $iPLA_2$ and that AA upregulates the Na^+-dependent Mg^{2+} efflux in NRK-52E cells.

Keywords: Arachidonic acid; Intracellular Mg^{2+}; Kidney; Mag-fura 2; Phospholipase A_2

1. Introduction

The Mg^{2+} concentration in the body is regulated by renal Mg^{2+} reabsorption. Most glomerular filtered Mg^{2+} is reabsorbed from the proximal tubule and thick ascending limb [1,2]. The Mg^{2+} transport pathways are divided into two types; a paracellular pathway and a transcellular pathway. The latter includes a putative Mg^{2+} channel, Mg^{2+} pump, and Na^+/Mg^{2+}-exchanger, which also regulate the intracellular free Mg^{2+} concentration ($[Mg^{2+}]_i$). $[Mg^{2+}]_i$ is an essential cofactor for many enzymes [3], a modulator of DNA and protein synthesis [4], and plays a role in cell differentiation and proliferation [4,5]. Recently, we reported that NRK-52E cells display both a Na^+-dependent and -independent Mg^{2+} efflux when incubated in the absence of extracellular Mg^{2+} and that the former is predominant [6,7]. The regulatory mechanisms of $[Mg^{2+}]_i$, however, have not been fully clarified. The Na^+-dependent Mg^{2+} transport is inhibited by amiloride, quinidine, and imipramine and may be regulated by protein kinase A, protein kinase C (PKC), and protein kinase G depending on cell type [7–11].

The concentration of free ionized Mg^{2+} in the blood is rigorously controlled within the physiological range (about 0.6 mM). A reduction of Mg^{2+} concentration increases the levels of prostanoids in the plasma [12]. Furthermore, the urinary excretion of arachidonic acid (AA) metabolites, including prostaglandin E_2, 6-keto-prostaglandin F1, and thromboxane B2, was increased in rats maintained on a low

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Mg^{2+} diet [13]. These reports suggest that there is a relationship between AA metabolism and Mg^{2+} homeostasis.

The present study was undertaken to examine whether the extracellular Mg^{2+} concentration affects AA production and whether AA is involved in the regulation of Na^+-dependent Mg^{2+} efflux measured in NRK-52E cells incubated in the absence of Mg^{2+}. Our findings demonstrated that NRK-52E cells express cytosolic phospholipase A_2 ($cPLA_2$) and Ca^{2+}-independent phospholipase A_2 ($iPLA_2$) mRNAs and that the decrease in extracellular Mg^{2+} concentration increases AA production. After extracellular Mg^{2+} removal, the addition of Na^+ induced a potent $[Mg^{2+}]_i$ decrease which was mediated mainly by $iPLA_2$ activation. The Na^+-induced $[Mg^{2+}]_i$ decrease was enhanced by AA metabolite inhibitors and inhibited by imipramine, a putative Na^+/Mg^{2+}-exchanger inhibitor. This is the first report showing that AA is involved in the upregulation of the Na^+-dependent Mg^{2+} efflux pathway.

2. Materials and methods

2.1. Materials

Bromoenol lactone (BEL) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma (St. Louis, MO, USA). Indomethacin was from Wako Pure Chemical Industries (Osaka, Japan). Mag-fura 2/acetoxymethyl ester (AM) and Pluronic F-127 were obtained from Molecular Probes (Eugene, OR, USA). [^{3}H]-Arachidonic acid ([^{3}H]-AA) was from PerkinElmer Life Sciences (Boston, MA, USA). Methyl arachidonyl fluorophosphonate (MAFP), nordihydroguaiaretic acid (NDGA) and 17-octadecynoic acid (ODYA) were from Cayman Chemical Company (Ann Arbor, MI, USA). LY-311727 was a generous gift from Eli Lilly (Indianapolis, IN, USA).

2.2. Cell culture

The NRK-52E cells were obtained from American Type Culture Collection (Manassas, VA, USA). This cell line is derived from the proximal tubule epithelium of normal rat kidney. The cells were grown on 100-mm plastic culture dishes (Corning, NY, USA) in DMEM supplemented with 5% fetal bovine serum, 0.14 mg/ml streptomycin sulfate and 0.07 mg/ml penicillin G potassium in a humidified environment of 5% CO_2–95% air at 37 °C. All cells used in the present experiments were cultured in 5 mM Mg^{2+}-containing DMEM for 1–2 days before use as described previously [7].

2.3. Measurement of AA and its metabolites

NRK-52E cells were grown on 24-well plates (Corning) to sub-confluence for 2 days. The generation of AA and its metabolites was analyzed as the release of [^{3}H]-metabolites from the membrane phospholipids. The cells were preincubated with DMEM containing [^{3}H]-AA (0.5 μCi/well) for 24 h at 37 °C resulting in equilibrium incorporation of the [^{3}H]-AA into the phospholipids. The medium containing [^{3}H]-AA was removed from the cells, which were then washed three times with Na^+-free/Mg^{2+}-containing solution. We used four solutions; a Na^+-free/Mg^{2+}-containing solution contained (in mM): 140 choline-Cl, 20 HEPES, 5.4 KCl, 0.4 KH_2PO_4, 5 $MgCl_2$, and 10 glucose; a Na^+-containing/Mg^{2+}-containing solution was identical except for the replacement of choline-Cl with NaCl; a Na^+-containing/Mg^{2+}-free solution was identical except for the replacement of choline-Cl with NaCl and the omission of $MgCl_2$; and a Na^+-free/Mg^{2+}-free solution was identical except for the omission of $MgCl_2$. The pH of all solutions was adjusted to 7.4 with Tris. The cells were preincubated with the Na^+-free/Mg^{2+}-containing solution in the presence and absence of PLA_2 inhibitors for 30 min, followed by incubation with the indicated solutions. The [^{3}H]-AA release was measured with a liquid scintillation counter and the values are represented as dpm/μg protein.

2.4. RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

Cells were grown on six-well plates (Corning) to sub-confluence for 2 days. Total RNA was isolated using a QuickPrep Total RNA Extraction Kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). RT-PCR was performed with a TaKaRa RNA PCR Kit (AMV) Ver. 2.1 (Takara Bio, Tokyo, Japan). Total RNA (1 μg) was oligo-dT-primed in the presence of the AMV reverse transcriptase XL (42 °C for 30 min and heated at 99 °C for 5 min). PCR reactions were performed in a 20-μl reaction volume of 3 μl cDNA mixture, 1× PCR buffer containing 1.5 mM $MgCl_2$, 200 μM of each dNTP, 400 nM of each primer, and 1 unit of the TaKaRa Taq DNA polymerase. The DNA was denatured at 94 °C for 3 min prior to PCR cycling (30 cycles) at 94 °C for 0.5 min (denaturation), 58 °C for 0.5 min (annealing), and 72 °C for 1 min (extension). Following the last cycle, elongation was extended for an additional 7 min at 72 °C before refrigeration. The following primers were used: $cPLA_2$ (type IVA), 5′-GATTGTGCGACCTACGTTGC-3′ and 5′-CTACCCAATCGGCAAACATC-3′; $iPLA_2$ (type VIA), 5′-AGAAGGGGTGTGCTGAATG-3′ and 5′-TGGGGCATGAGATCCTGAAGC-3′; and secretory PLA_2 ($sPLA_2$, type IIA), 5′-TGGCTCAATACAGGTCCAAG-3′ and 5′-TCATGAGTAACACAGCACCG-3′. The PCR products were visualized with ethidium bromide after electrophoretic separation on a 2% agarose gel. The $cPLA_2$, $iPLA_2$, and $sPLA_2$ primer pairs were designed to amplify fragments of 387, 417, and 166 bp, respectively.

2.5. Measurement of intracellular free Mg^{2+} concentration ($[Mg^{2+}]_i$)

Cells were grown on glass slides to sub-confluence for 1–2 days. $[Mg^{2+}]_i$ was determined using a Mg^{2+}-sensitive

fluorescent dye, mag-fura 2. Cells were incubated with the Na^+-free/Mg^{2+}-containing solution in the presence of 2 μM mag-fura 2 acetoxymethylester (mag-fura 2/AM, Molecular Probes) and a detergent, Pluronic F127 (0.025%, w/v, Molecular Probes), at 37 °C for 30 min. Then, the mag-fura 2-loaded cells were washed twice with the dye-free Na^+-free/Mg^{2+}-containing solution and placed in a glass cuvette. The mag-fura 2 fluorescence was monitored at 1-s intervals using a dual-excitation wavelength spectrofluorometer (Hitachi F-2000, Tokyo, Japan) with excitation at 340 and 380 nm and emission at 495 nm. The ratio of the two fluorescence emission intensities was stored for further analysis. Bathing solution was perfused continuously at a speed of 1.5 ml/min. Experiments were performed at 37 °C. The $[Mg^{2+}]_i$ was calculated according to the formula of Grynkiewicz et al. [14], using a dissociation constant (K_d) of 1.45 mM for the Mg^{2+}-mag-fura 2 complex. The maximum (R_{max}) and minimum (R_{min}) ratios were determined for the cells permeabilized with 5 μM ionomycin in the presence of 20 mM $MgCl_2$ and 50 mM EDTA, respectively. The initial rate of $[Mg^{2+}]_i$ decrease (μM/s) was estimated following substitution from the Na^+-free/Mg^{2+}-free solution to the Na^+-containing/Mg^{2+}-free solution.

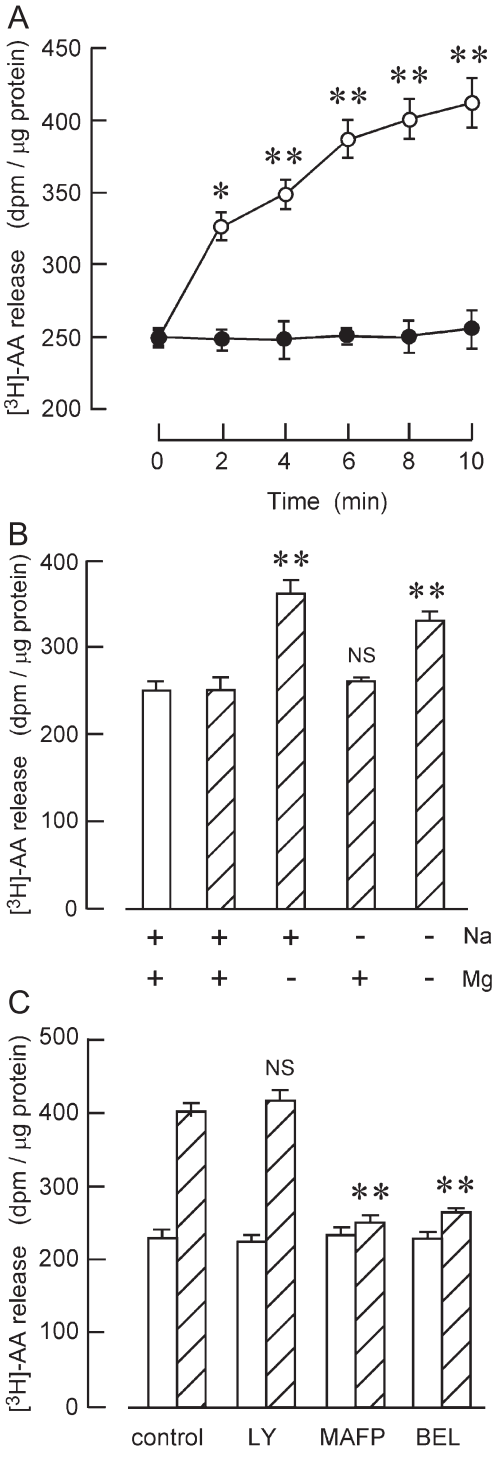

Fig. 1. Removal of extracellular Mg^{2+} induces AA release. (A) NRK-52E cells were cultured in DMEM containing [^{3}H]-AA (0.5 μCi/well) for 24 h. The cells were preincubated with Na^+-free/Mg^{2+}-containing solution for 30 min, then incubated with Na^+-containing/Mg^{2+}-free solution (open circle) or Na^+-free/Mg^{2+}-containing solution (closed circle) for the indicated times. Extracellular release of AA and its metabolites was measured with a liquid scintillation counter. $n=4$. *$P<0.05$ and **$P<0.01$, significantly different from 0 min. (B) The cells were incubated for 4 min in the presence of Na^+ and Mg^{2+}, in the presence of Na^+ and absence of Mg^{2+}, in the absence of Na^+ and presence of Mg^{2+}, and in the absence of Na^+ and Mg^{2+} (hatched columns). Open column shows the solution that was collected just before the replacement with the indicated solution. **$P<0.01$, significantly different from the value in the Na^+-containing/Mg^{2+}-containing solution. NS $P>0.05$. (C) The cells were preincubated with the Na^+-free/Mg^{2+}-containing solution in the absence (control) and presence of PLA_2 inhibitors, 10 μM LY-311727 (LY), 10 μM MAFP, or 10 μM BEL for 30 min, then incubated with either the Na^+-free/Mg^{2+}-containing solution (open columns) or the Na^+-free/Mg^{2+}-free solution (hatched columns) without (control) or with the indicated PLA_2 inhibitor for 4 min. $n=4$. **$P<0.01$, significantly different from control. NS $P>0.05$.

2.6. Statistics

Results are presented as the means ± S.E. Differences between groups were analyzed by one-way analysis of variance (ANOVA), and correction for multiple comparison was made using Tukey's multiple comparison test and Dunnett's multiple comparison test. Where appropriate, Student's *t*-test was used. Significant differences were assumed at $P<0.05$.

3. Results

3.1. Elevation of the release of AA and its metabolites by extracellular Mg^{2+} removal

A low Mg^{2+} diet causes a decrease in the serum Mg^{2+} concentration and increase in AA and prostanoid concentrations in the rat [12,13]. In the first part of this study, we investigated the effect of extracellular Mg^{2+} removal on the production and release of AA metabolites in NRK-52E cells. Cells were cultured in 5 mM Mg^{2+}-containing DMEM for 2 days to correspond with the conditions of

the Mg^{2+} efflux measurement (see Section 3.3). [^{3}H]-AA-labeled cells were preincubated in Na^+-free/Mg^{2+}-containing solution for 30 min. A subsequent change to Na^+-containing/Mg^{2+}-free solution induced the release of AA and its metabolites in a time-dependent manner (Fig. 1A). AA release remained unaltered upon continued incubation in Na^+-free/Mg^{2+}-containing solution. Mg^{2+} removal also increased the AA metabolites release in the Na^+-free condition, whereas Na^+ removal was without effect in the presence of Mg^{2+} (Fig. 1B). The Mg^{2+} removal-induced release of AA metabolites was virtually abolished by MAFP (10 μM), a $cPLA_2$ and $iPLA_2$ inhibitor, and BEL (10 μM), an irreversible $iPLA_2$ inhibitor (Fig. 1C). On the other hand, LY-311727 (10 μM), a selective $sPLA_2$ inhibitor, did not inhibit the Mg^{2+} removal-induced AA release. These inhibitors did not affect AA release in the presence of extracellular Mg^{2+}.

3.2. Determination of $cPLA_2$ and iPLA2 transcripts in NRK-52E cells by RT-PCR

In the RT-PCR experiment, we used three sets of primers that amplify partial cDNA fragments of $cPLA_2$, $iPLA_2$ and $sPLA_2$. Fig. 2 shows that $cPLA_2$ and $iPLA_2$ transcripts exist in NRK-52E cells. However, a specific band for $sPLA_2$ was not detected in the cells. These results correspond to the inhibitory effects (Fig. 1C), indicating that $cPLA_2$ and $iPLA_2$ are mainly involved in Mg^{2+} removal-induced release of AA metabolites.

3.3. Effects of PLA_2 inhibitors on Na^+-dependent $[Mg^{2+}]_i$ decrease

In the second part of this study, we investigated the involvement of AA (metabolites) in the Na^+-induced $[Mg^{2+}]_i$ decrease in NRK-52E cells incubated in the absence of extracellular Mg^{2+}. After loading with mag-fura 2, the cells were incubated in a Na^+-free/Mg^{2+}-free solution and used for fluorescence measurements. Maintaining the cells in DMEM containing 5 mM Mg^{2+} for 1–2 days increased $[Mg^{2+}]_i$ from 0.37 ± 0.03 to 0.78 ± 0.02 mM ($n = 8$–10). Mg^{2+}-loaded cells enabled us to detect Na^+-induced Mg^{2+} efflux that was not observed in control cells presumably because of detection limitations. Although the $[Mg^{2+}]_i$ slightly decreased in the nominally Na^+- and Mg^{2+}-free conditions, the addition of Na^+ caused a rapid decrease in $[Mg^{2+}]_i$, resulting in a new steady state after 10 min (Fig. 3A and B). Calculation of initial rate of $[Mg^{2+}]_i$ decrease following the change of solution revealed that the effect of Na^+ was significant. (Fig. 3C). On the other hand, Na^+ did not alter $[Mg^{2+}]_i$ when added in the presence of Mg^{2+} (initial rate of $[Mg^{2+}]_i$ decrease: -0.02 ± 0.01 μM/s, $n = 6$). Fig. 4A shows that the Na^+-stimulated $[Mg^{2+}]_i$

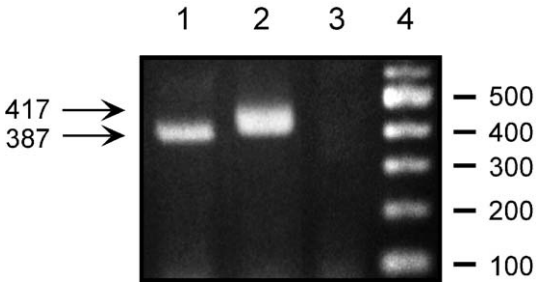

Fig. 2. Detection of PLA_2 mRNA by RT-PCR in NRK-52E cells. Total RNA was isolated from the NRK-52E cells cultured in 5 mM DMEM for 2 days. In lanes 1, 2 and 3, the primers for $cPLA_2$, $iPLA_2$ and $sPLA_2$ were used, respectively. Lane 4 shows a 100 bp DNA ladder.

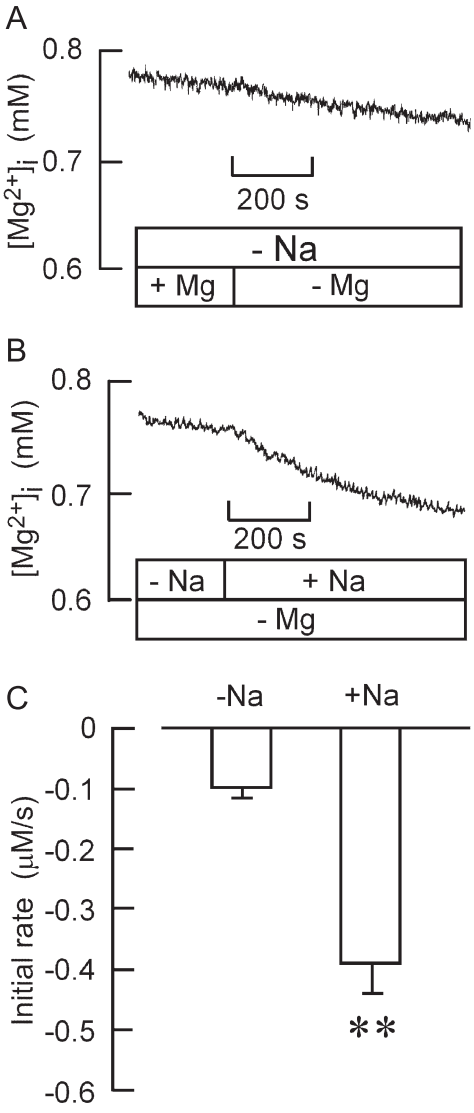

Fig. 3. Na^+-dependent $[Mg^{2+}]_i$ decrease in NRK-52E cells. (A, B) Representative traces of $[Mg^{2+}]_i$ measurements are shown. The cells were loaded with mag-fura-2, then placed in a glass cuvette containing the Na^+-free/Mg^{2+}-containing or the Na^+-free/Mg^{2+}-free solution. The Na^+-free/Mg^{2+}-containing solution was changed to the Na^+-free/Mg^{2+}-free solution (A) or the Na^+-free/Mg^{2+}-free solution was changed to the Na^+-containing/Mg^{2+}-free solution (B). (C) The initial rate of $[Mg^{2+}]_i$ decrease was estimated following perfusion with the Na^+-free/Mg^{2+}-free solution ($-$Na) or the Na^+-containing/Mg^{2+}-free solution ($+$Na). $n = 6$. ** $P < 0.01$, significantly different from the value in the Na^+-free/Mg^{2+}-free solution.

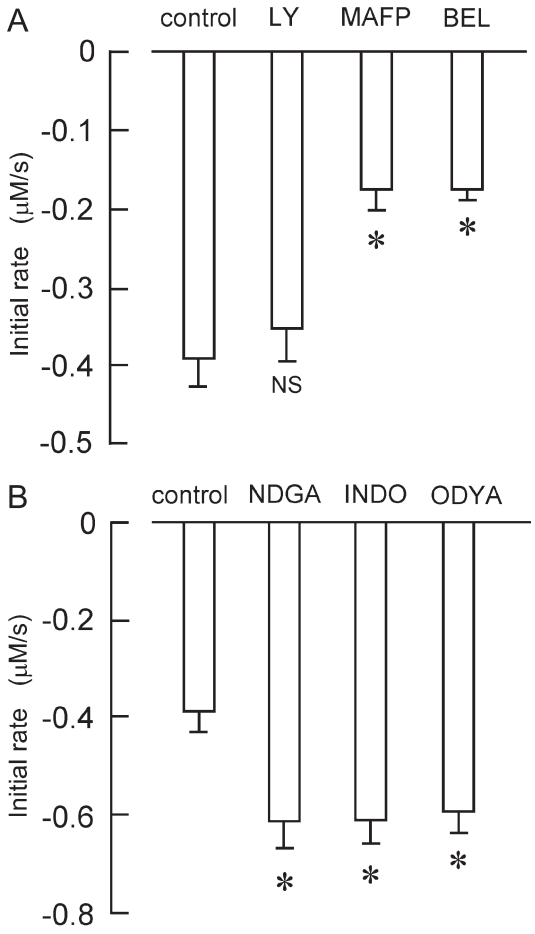

Fig. 4. Effects of PLA_2 and AA metabolite inhibitors on Na^+-dependent $[Mg^{2+}]_i$ decrease. (A) The cells were preincubated with the Na^+-free/Mg^{2+}-containing solution in the absence (control) and presence of 10 μM LY-311727 (LY), 10 μM MAFP, or 10 μM BEL for 30 min. The initial rate of $[Mg^{2+}]_i$ decrease was estimated following perfusion with the Na^+-containing/Mg^{2+}-free solution. $n=6-16$. $^*P<0.05$, significantly different from control. NS $P>0.05$. (B) The cells were preincubated with the Na^+-free/Mg^{2+}-containing solution in the absence (control) and presence of AA metabolite inhibitors, 50 μM NDGA, 10 μM indomethacin (INDO), or 10 μM ODYA for 30 min. The initial rate was estimated following perfusion with the Na^+-containing/Mg^{2+}-free solution. $n=5-12$. $^*P<0.05$, significantly different from control.

decrease in the absence of Mg^{2+} was significantly inhibited following pretreatment with MAFP (10 μM) and BEL (10 μM), but not LY-311727 (10 μM). These inhibitors did not significantly affect the basal $[Mg^{2+}]_i$ measured immediately before changing to Na^+-containing/Mg^{2+}-free solution (control: 0.76 ± 0.03 mM; MAFP: 0.76 ± 0.02 mM; BEL: 0.77 ± 0.03 mM, and LY-311727: 0.76 ± 0.02 mM, $n=4-9$, $P>0.05$ vs. control). There were no synergistic or additive effects by co-treatment with MAFP and BEL (initial rate of $[Mg^{2+}]_i$ decrease of MAFP: -0.18 ± 0.02, BEL: -0.18 ± 0.01, and co-treatment of MAFP and BEL: -0.16 ± 0.03 μM/s, respectively, $n=4-8$, $P>0.05$). The inhibitory effects of MAFP and BEL are consistent with effects on AA release (Fig. 1C), indicating that $iPLA_2$-mediated AA production is involved in upregulation of the Na^+-dependent Mg^{2+} efflux.

3.4. Effects of AA metabolite inhibitors on Na^+-dependent $[Mg^{2+}]_i$ decrease

The cells were preincubated with NDGA (50 μM), a lipoxygenase (LOX) inhibitor, indomethacin (10 μM), a cyclooxygenase (COX) inhibitor, or ODYA (10 μM), a cytochrome P450-dependent epoxygenase and ω-hydroxylase inhibitor. All these chemicals enhanced the Na^+-dependent $[Mg^{2+}]_i$ decrease (Fig. 4B) without affecting the basal $[Mg^{2+}]_i$ (control: 0.76 ± 0.03 mM; NDGA: 0.79 ± 0.01 mM; indomethacin: 0.78 ± 0.01 mM and ODYA: 0.75 ± 0.02 mM, $n=5-7$, $P>0.05$ vs. control). These results suggest that an increase in AA concentration caused by inhibition of AA metabolism enhanced the Na^+-dependent $[Mg^{2+}]_i$ decrease.

3.5. Effects of exogenous AA and Na^+/Mg^{2+}-exhanger inhibitors on Na^+-dependent $[Mg^{2+}]_i$ decrease

So far, Na^+-dependent Mg^{2+} transport has been shown to be inhibited by the putative Na^+/Mg^{2+}-exchanger inhibitors, imipramine and quinidine in MDCK cells [15] and ferret red cells [16], imipramine in rat hepatocytes [8] and human red

Table 1
AA-sensitive Na^+-dependent $[Mg^{2+}]_i$ decrease is inhibited by imipramine in NRK-52E cells

	Basal $[Mg^{2+}]_i$ (mM)	Initial rate (μM/s)	Control (%)
Control	0.76 ± 0.03 (7)	0.37 ± 0.02	100
+ quinidine (0.1 mM)	0.77 ± 0.02 (4)	0.39 ± 0.01	105.9
+ imipramine (0.1 mM)	0.75 ± 0.01 (5)	0.13 ± 0.01**	36.2
AA (30 μM)	0.76 ± 0.01 (6)	0.55 ± 0.03**	148.2
+ imipramine (0.1 mM)	0.75 ± 0.02 (6)	0.28 ± 0.02§§	76.4

Values are means ± S.E.; number of experiments is in parenthesis. The cells were preincubated with the Na^+-free/Mg^{2+}-containing solution containing quinidine or imipramine for 10 min. After the cells were placed in a glass cuvette containing the Na^+-free/Mg^{2+}-free solution, the solution was changed to the Na^+-containing/Mg^{2+}-free solution as shown in Fig. 3B. The basal $[Mg^{2+}]_i$ was measured just before changing to the Na^+-containing/Mg^{2+}-free solution. Initial rate of $[Mg^{2+}]_i$ decrease was estimated following perfusion with the Na^+-containing/Mg^{2+}-free solution. The effects of chemicals on the initial rate are represented as percentage of control.

** $P<0.01$, significantly different from control.

§§ $P<0.01$,significantly different from AA.

cells [17], and quinidine in rat sublingual acinar cells [18]. We found that the Na^+-dependent $[Mg^{2+}]_i$ decrease was inhibited by imipramine (0.1 mM), but not by quinidine (0.1 mM) in NRK-52E cells (Table 1). On the other hand, the addition of exogenous AA (30 μM) enhanced the Na^+-dependent $[Mg^{2+}]_i$ decrease. The AA-induced $[Mg^{2+}]_i$ decrease was significantly inhibited by imipramine. Wolf et al. [19] reported that an AA-activated and imipramine-inhibited Mg^{2+} efflux pathway exists in mouse ascites cells. However, they showed that the Mg^{2+} efflux pathway is activated by AA metabolites, but not by AA itself. Our results indicate that a putative Na^+/Mg^{2+}-exchanger in NRK-52E cells is upregulated by AA and inhibited by imipramine.

4. Discussion

About 80% of the serum Mg^{2+} is filtered through the glomerular membrane (for review see Ref. [20]) and most is reabsorbed in the proximal tubule, including the convoluted and straight portions, and the thick ascending limb. The regulatory mechanisms of Mg^{2+} transport have been well studied in the thick ascending limb. The reabsorption of Mg^{2+} within the proximal tubule and the thick ascending limb is suggested to be mainly mediated by a paracellular pathway. Some investigators postulated an additional transcellular pathway that is regulated by hormones and non-hormonal factors [1,2]. To confirm the functions of transcellular Mg^{2+} transporters in physiological and pathological conditions, regulatory mechanisms of $[Mg^{2+}]_i$ were studied. Recently, we reported that Na^+-dependent Mg^{2+} efflux is observed in renal epithelial NRK-52E cells incubated in Mg^{2+}-free conditions. In the present study, we examined the effect of extracellular Mg^{2+} removal on AA production and the involvement of AA in the regulation of the Na^+-dependent Mg^{2+} efflux pathway.

AA is a *cis*-polyunsaturated fatty acid and ubiquitously present in the plasma membrane. It is normally linked covalently to other molecules in the membrane to form phospholipids, but can be liberated by activation of cellular phospholipases. We report for the first time that extracellular Mg^{2+} removal elevates AA release in NRK-52E cells. Alzola et al. [21] reported that ATP activates AA release that is inhibited by 5 mM Mg^{2+} in rat submandibular glands. They suggested that the activation of nonspecific cation channels coupled to purinoceptors increases the AA release. In our study, extracellular Mg^{2+} removal might have cancelled the inhibition of the nonspecific cation channels to increase AA release.

Three types of phospholipase A_2, $iPLA_2$, $cPLA_2$, and $sPLA_2$, are present in the rat kidney [22]. We showed that $iPLA_2$ and $cPLA_2$ exist in NRK-52E cells by RT-PCR (Fig. 2). $iPLA_2$ is an 85-kDa protein that is inhibited by BEL [23,24]. The functions of $iPLA_2$ have not been fully explored and its physiological and pathological relevance remains under investigation. On the contrary, $cPLA_2$ has been reported to require micromolar Ca^{2+} concentrations in order to translocate to the plasma membrane on activation but does not require Ca^{2+} for its catalytic activity. In $cPLA_2$-knockout mice, basal urinary prostaglandin E_2 excretion was reduced, suggesting that $cPLA_2$ is involved in the regulation of Na^+ and water reabsorption under physiological conditions [25]. In our experimental conditions, however, Mg^{2+} removal did not significantly elevate the intracellular free Ca^{2+} concentration [7]. Furthermore, the Na^+-dependent $[Mg^{2+}]_i$ decrease was inhibited by BEL and MAFP (Fig. 4) and because the combination of BEL and MAFP did not give additive or synergistic effects, both inhibitors most probably act on the same target. Therefore, although we cannot completely exclude the involvement of $cPLA_2$ and other molecules, $iPLA_2$ appears to be the predominant AA-producing enzyme activated in response to a decrease in the extracellular Mg^{2+} concentration in NRK-52E cells.

Unesterified or free AA has been shown to modulate the activity of a variety of ion transporters (for review see Ref. [26]). AA modulation can occur by both direct and indirect mechanisms. Many indirect effects are apparently mediated by AA metabolites generated by COX, LOX, or cytochrome P450-dependent epoxygenase and ω-hydroxylase [27–29]. In other cases, however, AA has also been shown to act directly on channel proteins [30,31]. We found that the Na^+-dependent $[Mg^{2+}]_i$ decrease occurred in the absence of extracellular Mg^{2+}, a condition that also increased the production of AA and its metabolites. Furthermore, the Na^+-dependent $[Mg^{2+}]_i$ decrease is upregulated by AA itself, but not by its metabolites (Fig. 4 and Table 1). At present, we do not know how AA upregulates Na^+-dependent Mg^{2+} efflux. AA has been reported to regulate PKC in rat hippocampal [32] and chondrocytes [33]. We recently reported that a PKC inhibitor diminishes the Na^+-dependent $[Mg^{2+}]_i$ decrease in NRK-52E cells [7], suggesting that AA-elicited PKC activation may be involved in upregulation of the Na^+-dependent Mg^{2+} efflux pathway.

In isolated porcine thick ascending limb cells, Dai and Quamme [34] reported that Mg^{2+} uptake was inhibited by Ca^{2+} channel blockers, although it was not inhibited by quinidine, suggesting that the Mg^{2+} influx pathway is a channel with close homology to known Ca^{2+} channels. On the other hand, the presence of a putative Na^+-dependent Mg^{2+} transporter has been reported in various mammalian cells [16,17,35,36]. This transporter is commonly inhibited by amiloride, quinidine and imipramine. Our results show that the Na^+-dependent $[Mg^{2+}]_i$ decrease is inhibited by imipramine, but not by quinidine in NRK-52E cells. Therefore, we suggest that the Na^+-dependent Mg^{2+} efflux pathway in NRK-52E cells is a novel pathway that is sensitive to the extracellular Na^+ concentration and imipramine.

In conclusion, we have found that extracellular Mg^{2+} removal elevated AA production by the activation of $iPLA_2$

in renal epithelial NRK-52E cells, and that AA upregulates Na^+-dependent Mg^{2+} efflux which is inhibited by imipramine. There is a possibility that the Na^+-dependent Mg^{2+} efflux pathway is involved in reabsorption of Mg^{2+} as well as in control of intracellular Mg^{2+} homeostasis because it is activated by changing the extracellular Mg^{2+} concentration. Also, AA may play an important role in regulating transcellular Mg^{2+} reabsorption in Mg^{2+} deficiency.

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