Introduction

From both clinical and epidemiologic aspects, Mg$^{2+}$-deficiency is related to cardiovascular diseases [1–3]. More Mg$^{2+}$ intake is recommended to prevent arteriosclerosis and hypertension. It is important, however, to point out that the serum Mg$^{2+}$ level does not reflect Mg$^{2+}$-deficiency of the entire body [4]. Cellular Mg$^{2+}$-deficiency is caused by a malfunction of Mg$^{2+}$ transporters across the plasma membrane, as well as a fall in the extracellular Mg$^{2+}$ concentration. It is therefore of great interest to investigate mechanisms driving Mg$^{2+}$ transport into the cell, especially into vascular smooth muscle cells.

Na$^+$-independent Mg$^{2+}$ transport sensitive to 2-aminoethoxydiphenyl borate (2-APB) in vascular smooth muscle cells: involvement of TRPM-like channels

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Abstract

Magnesium is associated with several important cardiovascular diseases. There is an accumulating body of evidence verifying the important roles of Mg$^{2+}$-permeable channels. In the present study, we estimated the intracellular free Mg$^{2+}$ concentration ([Mg$^{2+}$]i) using $^{31}$P-nuclear magnetic resonance ($^{31}$P-NMR) in porcine carotid arteries. pH$_i$ and intracellular phosphorus compounds were simultaneously monitored. Removal of extracellular divalent cations (Ca$^{2+}$ and Mg$^{2+}$) in the absence of Na$^+$ caused a gradual decrease in [Mg$^{2+}$]i to ~60% of the control value after 125 min. On the other hand, the simultaneous removal of extracellular Ca$^{2+}$ and Na$^+$ in the presence of Mg$^{2+}$ gradually increased [Mg$^{2+}$]i in an extracellular Mg$^{2+}$-dependent manner. 2-aminoethoxydiphenyl borate (2-APB) attenuated both [Mg$^{2+}$]i load and depletion caused under Na$^+$- and Ca$^{2+}$-free conditions. Neither [ATP]i nor pH$_i$ correlated with changes in [Mg$^{2+}$]i. RT-PCR detected transcripts of both TRPM6 and TRPM7, although TRPM7 was predominant. In conclusion, the results suggest the presence of Mg$^{2+}$-permeable channels of TRPM family that contribute to Mg$^{2+}$ homeostasis in vascular smooth muscle cells. The low, basal [Mg$^{2+}$]i level in vascular smooth muscle cells is attributable to the relatively low activity of this Mg$^{2+}$ entry pathway.

Keywords: magnesium • vascular smooth muscle • 2-aminoethoxydiphenyl borate • ATP • NMR
Mg\textsuperscript{2+} is believed to be regulated by two transmembrane Mg\textsuperscript{2+} pathways: the Na\textsuperscript{+}–Mg\textsuperscript{2+} exchange driven by the Na\textsuperscript{+}-gradient, and the Na\textsuperscript{+}-independent ‘passive’ Mg\textsuperscript{2+} transport via Mg\textsuperscript{2+}-permeable channels [5–8]. Since the molecular identification of the latter Mg\textsuperscript{2+} pathway, such as melastatin-type transient receptor potential (TRPM) homologue channels, there has been an accumulating body of evidence for the crucial role that this pathway plays in Mg\textsuperscript{2+} homeostasis [9–11]. Also, TRPM homologue channels are bifunctional proteins, which contain a kinase domain in the C-terminus.

Mg\textsuperscript{2+} is known to change slowly, and thereby act as a chronic regulator. In addition, changes in the intracellular milieu, such as the intracellular pH (pHi) and [ATP] can affect [Mg\textsuperscript{2+}]i regulation. However, the importance of TRPM homologues in [Mg\textsuperscript{2+}]i regulation during relatively short durations has been assessed using fluorescent Mg\textsuperscript{2+} indicators. In the present study, we thus utilized \textsuperscript{31}P-NMR to estimate slow changes in [Mg\textsuperscript{2+}]i over several hours in carotid arteries, which are now frequently used as a model to evaluate arteriosclerotic changes, and assessed the contribution of TRPM-like Mg\textsuperscript{2+}-permeable channels.

Materials and methods

Preparation

Porcine carotid arteries were collected at an abattoir. The arteries were stripped of fat and connective tissue, and cut into segments of approximately 30 mm in length. The lumen was exposed by cutting the artery segments into two strips along the longitudinal direction. The endothelium was removed by scratching with a cotton-tipped stick. The resultant pig carotid artery strips (~2 g wet weight) were mounted in a sample tube of 10 mm in diameter. This study was approved by the institutional committee of animal experiments.

\textsuperscript{31}P-NMR

The methods employed for the \textsuperscript{31}P-NMR measurements were essentially the same as those previously described [12]. NMR spectrometers (GSX270W: JEOL, Tokyo, Japan; UNITY-500plus: Varian, Tokyo, Japan) were operated at 109.4 and 202.3 MHz, respectively. The temperature of the sample was maintained at 32°C. Radio frequency pulses corresponding to a flip angle of 30 were applied every 0.6 sec. \textsuperscript{31}P-NMR spectra were obtained by accumulating 2500 signals (free induction decays) over 25 min. Before Fourier transformation, a broadening factor of 20 Hz was applied to enhance the signal-to-noise ratio. Spectral peak resonances (frequencies) were measured relative to that of phosphocreatine (PCr) in p.p.m.

Control spectra were acquired in the absence of Ca\textsuperscript{2+}. Then, experiments were carried out in the absence of extracellular Na\textsuperscript{+} to rule out the contribution of Na\textsuperscript{+}-coupled Mg\textsuperscript{2+} transport, that is, Na\textsuperscript{+}–Mg\textsuperscript{2+} exchange. Six major peaks were observed (Fig. 1): phosphomonoesters (PME), inorganic phosphate (Pi), PCr and the γ-, α- and β-phosphorus atoms of ATP (γ-, α- and β-ATP). Concentrations of phosphorus compounds were estimated by integrating the peak areas (Scion image; Scion Corp., Fredrick, MA, U.S.A.) and by correcting with their saturation factors (Pi, 1.60; PCr, 1.36; β-ATP, 1.07).

Estimation of [Mg\textsuperscript{2+}]i and pHi

Intracellular pH (pHi) was estimated from the chemical shift observed for the Pi peak ([δo(\Pi)]) using the following equation [13,14]:

\[
\text{pHi} = \text{pK}_a + \log_{10}\left[\frac{\delta_o(\Pi) - \delta_o(pHi)}{\delta_o(pHi) - \delta_o(\Pi)}\right], \quad \text{Eq}(1)
\]

where pK\textsubscript{a} is the negative logarithm of the dissociation constant of Pi (= 6.70), and δ\textsubscript{o}(\Pi) and δ\textsubscript{o}(pHi) are the chemical shifts for H\textsubscript{2}PO\textsubscript{4}\textsuperscript{2–} (= 3.15 p.p.m.) and HPO\textsubscript{4}\textsuperscript{2–} (= 5.72 p.p.m.), respectively. The pH\textsubscript{i} value was used to correct the [Mg\textsuperscript{2+}]i estimation.

Mg\textsuperscript{2+} usually binds to ATP as a 1 to 1 complex. [Mg\textsuperscript{2+}]i was thus estimated from the chemical shift observed for the β-ATP peak (δ\textsubscript{o}β) using the following equation [13,14]:

\[
[Mg^{2+}]_i = K_D^{-\beta-ATP} (\delta_{o\beta} - \delta_{f\beta}) / (\delta_{b\beta} - \delta_{o\beta}), \quad \text{Eq}(2)
\]

where δ\textsubscript{f\beta} and δ\textsubscript{b\beta} are the chemical shifts of metal-free and Mg\textsuperscript{2+}-bound forms of β-ATP, respectively. We have previously shown that K\textsubscript{D}\textsuperscript{-β-ATP} can be described as functions of pH [15] (See, Supplementary Material S1: Details of estimation procedures). K\textsubscript{D}\textsuperscript{-β-ATP(pHi)} was derived from quadratic pH-functions for K\textsubscript{D}\textsuperscript{-β-ATP at 25 and 37°C [16], using the van’t Hoff iso-chores. The pH–functions of δ\textsubscript{f\beta} and δ\textsubscript{b\beta} were constructed by fitting the data points of model solutions with sigmoid curves [17]. Thus, Eq(2) can be rewritten as:

\[
[Mg^{2+}]_i = K_D^{-\beta-ATP(pHi)} (\delta_{o\beta} - \delta_{f\beta(pHi)}) / (\delta_{b\beta(pHi)} - \delta_{o\beta}), \quad \text{Eq}(3)
\]

In Table 2, [Mg\textsuperscript{2+}]i and pH\textsubscript{i} values were also estimated from the chemical shifts of β- and γ-ATP [12,17]. For the
chemical shift of $\gamma$-ATP, an equation analogous to Eq(3) can be written:

$$[\text{Mg}^{2+}]_i = K_0 \text{MgATP}^\circ (p\text{H}_i) (\delta_{\gamma} - \delta_{\gamma}(p\text{H}_i))/ (\delta_{\beta}(p\text{H}_i) - \delta_{\gamma}^0)$$

where $\delta_{\gamma}$ and $\delta_{\beta}$ are the chemical shifts of metal-free and Mg$^{2+}$-bound forms of $\gamma$-ATP, respectively, and these parameters are also expressed as pH-functions. $[\text{Mg}^{2+}]_i$ and $p\text{H}_i$ were estimated by solving the Eq(3) and Eq(4) simultaneously.

Similar to the effect of Mg$^{2+}$, Ca$^{2+}$-binding to ATP changes the chemical shifts of the ATP peaks. However, basal [Ca$^{2+}]_i$ is maintained at ~100 nM in smooth muscle cells under physiological conditions. Furthermore, experiments in the present study were carried out mainly under Ca$^{2+}$-free conditions. Therefore, the effect of Ca$^{2+}$-binding to ATP is considered negligible.

Solutions and chemicals

The extracellular solution used for the 'normal' solution had the following composition in mM: NaCl, 137.9; KHCO$_3$ 5.9; CaCl$_2$ 2.4; MgCl$_2$ 1.2; glucose 11.8; HEPES 5 (pH adjusted...
The ionic composition was modified iso-osmotically. Also, divalent cation-free solutions contained 1 mM EDTA. The solutions used for 31P-NMR measurements were normally aerated with 95% O2/5%CO2. 2-aminoethoxydiphenyl borate (2-APB) was purchased from Calbiochem (San Diego, CA, USA).

**RT-PCR**

The procedures for RT-PCR were essentially the same as previously described [18]. Total RNA was extracted from porcine carotid arteries. After treatment with RNase-free DNase (Promega, Madison, WI, USA), the total RNA was subjected to an RT (reverse-transcription) reaction. RT was performed using a random hexamer (12 pmol/reaction) and Moloney murine leukemia virus (MMLV) reverse transcriptase (100 U/reaction) according to the manufacturer’s instructions (Gibco-BRL, Rockville, MD, U.S.A.). The cycling condition was 3 min of initial denaturation at 95°C followed by 35 cycles of 95°C for 30 sec, 54°C for 30 sec and 72°C for 35 sec. The RT sample was then used as a template for the PCR reaction. The amplicons (5 μl) were run on a 2.5% agarose gel and stained with ethidium bromide. Because the cDNA sequences for porcine TRP (transient receptor potential) homologue cation channels have not been published, the PCR primers were designed by using the conserved sequences in humans and mice. The primers used are listed in Table 1.

**Statistics**

Numerical data are expressed as the mean (S.D). Differences between groups with different experimental protocols were evaluated by use of ANOVA for repeated measures. When a significant difference was identified between the groups (P<0.05), individual comparisons at the same time point were performed using an unpaired t-test.

**Results**

**Depletion of [Mg2+] via Na+-independent Mg2+ pathways**

31P-NMR was used to continuously measure phosphorus compounds in porcine carotid artery smooth muscle (Fig. 1). In this study, we mainly estimated [Mg2+]: from the chemical shift of the β-ATP peak, and correction was made by pH, estimated from the chemical shift of the Pi peak.

During exposure to a divalent cation-free solution (i.e. 0 Ca2+, 0 Mg2+), [Mg2+] decreased from 0.74±0.11 to 0.49±0.08 mM (n = 7; Fig. 2A, □) after 125 min, while pH did not change significantly (Fig. 2B, ●). Essentially the same decrease in [Mg2+] (from 0.75±0.09 to 0.46±0.05 mM; n = 7; Fig. 2A, □) was observed in the absence of Na+, suggesting that Mg2+-permeable channels make a major contribution to the changes in [Mg2+] under divalent cation-free conditions. On the other hand, pH decreased from 7.09±0.05 to 6.92±0.05 (n = 7) after 125 min in the absence of Na+ (Fig. 2B, ○), presumably due to the inhibition of Na+-coupled pH regulatory mechanisms, such as Na+/H+ exchange and Na+-HCO3− co-transport.

| Clones   | Primer Sequence (+): Sense, (-): Antisense | Primer site (in human clones) | Accession No (human) (mouse) |
|----------|-------------------------------------------|-------------------------------|-----------------------------|
| TRPM2    | (+): 5’TTC CAG GAG ATG TTT GAG AC-3’      | 1604-1938                     | NM_003307                   |
|          | (-): 5’TCA GGC TTG TTG GAG ATG AG-3’      |                               | NM_138301                   |
| TRPM4    | (+): 5’GTG GGA GGG ACT GGA ATT GA-3’      | 863-1263                      | NM_017636                   |
|          | (-): 5’AGC TCA TCC AGG TAG GCT GA-3’      |                               | NM_175130                   |
| TRPM6    | (+): 5’TGT TGG TGG AGA TGC AGC C-3’      | 2862-3174                     | NM_017662                   |
|          | (-): 5’CCT GCA TGT TGA TTC ACA GC-3’      |                               | NM_153417                   |
| TRPM7    | (+): 5’GAT GCC CTC AAA GAA CAT GC-3’      | 794-1269                      | NM_017672                   |
|          | (-): 5’GGC TCT GCT GCA TCA GGA AG-3’      |                               | NM_021450                   |

Table 1 PCR primers. PCR primers for porcine sample were designed by using the conserved sequences in humans and mice.
Table 2 ([Mg^{2+}] and pHi values estimated using two methods: (1) from the chemical shifts of β- and γ-ATP or 2) from the chemical shifts of β-ATP and P_i. (See Materials and methods for details). [Mg^{2+}] and pHi values during [Mg^{2+}] depletion were compared in A and B. In C and D, [Mg^{2+}] and pHi values were during the elevation of [Mg^{2+}]. Single (*) and double asterisks (**) indicate P<0.05 and P<0.01 versus control, respectively.

(A) Exposure to a divalent cation- and Na^+-free solution (n = 7).

|                  | 1) from β- and γ-ATP | 2) from β-ATP and P_i |
|------------------|-----------------------|-----------------------|
|                  | [Mg^{2+}] (mM) | pHi | [Mg^{2+}] (mM) | pHi |
| Control (0 Ca^{2+}) | 0.70±0.13 | 7.20±0.11 | 0.75±0.09 | 7.09±0.05 |
| 0 Ca^{2+}, 0 Mg^{2+}, 0 Na^+ (K^+) 25–50 min | 0.61±0.07** | 7.09±0.09** | 0.62±0.04** | 7.06±0.04** |
| 0 Ca^{2+}, 0 Mg^{2+}, 0 Na^+ (K^+) 100–125 min | 0.43±0.04** | 7.01±0.08** | 0.46±0.05** | 6.92±0.05** |

(B) Exposure to a divalent cation- and Na^+-free solution containing 2-APB (n = 7).

|                  | 1) from β- and γ-ATP | 2) from β-ATP and P_i |
|------------------|-----------------------|-----------------------|
|                  | [Mg^{2+}] (mM) | pHi | [Mg^{2+}] (mM) | pHi |
| Control (0 Ca^{2+}) | 0.68±0.04 | 7.21±0.05 | 0.74±0.05 | 7.09±0.04 |
| 0 Ca^{2+}, 0 Mg^{2+}, 0 Na^+ (K^+) +150μM 2-APB 25–50 min | 0.65±0.08* | 7.09±0.08** | 0.68±0.07* | 7.03±0.04** |
| 0 Ca^{2+}, 0 Mg^{2+}, 0 Na^+ (K^+) +150μM 2-APB 100–125 min | 0.60±0.12** | 7.00±0.11** | 0.62±0.08** | 6.94±0.05** |

(C) Exposure to a Ca^{2+}- and Na^+-free, high Mg^{2+} (6.0 mM) solutions (n = 7).

|                  | 1) from β- and γ-ATP | 2) from β-ATP and P_i |
|------------------|-----------------------|-----------------------|
|                  | [Mg^{2+}] (mM) | pHi | [Mg^{2+}] (mM) | pHi |
| Control (0 Ca^{2+}) | 0.72±0.10 | 7.20±0.09 | 0.78±0.08 | 7.08±0.12 |
| 0 Ca^{2+}, 6.0 Mg^{2+}, 0 Na^+ (K^+) 25–50 min | 1.11±0.28** | 7.12±0.10** | 1.18±0.28** | 7.03±0.05** |
| 0 Ca^{2+}, 6.0 Mg^{2+}, 0 Na^+ (K^+) 100–125 min | 1.63±0.23** | 6.99±0.11** | 1.79±0.18** | 6.92±0.04** |

(D) Exposure to a Ca^{2+}- and Na^+-free, high Mg^{2+} (6.0 mM) solution containing 2-APB (n = 6).

|                  | 1) from β- and γ-ATP | 2) from β-ATP and P_i |
|------------------|-----------------------|-----------------------|
|                  | [Mg^{2+}] (mM) | pHi | [Mg^{2+}] (mM) | pHi |
| Control (0 Ca^{2+}) | 0.70±0.09 | 7.20±0.11 | 0.75±0.04 | 7.10±0.03 |
| 0 Ca^{2+}, 6.0 Mg^{2+}, 0 Na^+ (K^+) +150μM 2-APB 25–50 min | 0.91±0.10** | 7.06±0.07** | 0.93±0.06** | 7.02±0.02** |
| 0 Ca^{2+}, 6.0 Mg^{2+}, 0 Na^+ (K^+) +150μM 2-APB 100–125 min | 1.10±0.08** | 6.99±0.04** | 1.14±0.08** | 6.95±0.03** |
Effects of 2-APB in Na⁺-independent depletion of [Mg²⁺]:

2-APB is known to block TRPM7 [11]. To substantiate the involvement of analogous Mg²⁺-permeable channels in vascular muscle cells, the effect of 2-APB was examined (Fig. 1C and D). As shown in Figure 3, application of 150 µM 2-APB to the divalent cation- and Na⁺-free solution significantly attenuated the depletion of [Mg²⁺] (from 0.74 ± 0.05 to 0.62 ± 0.08 mM after 125 min; Fig. 3A, ■). This inhibitory effect of 2-APB was concentration-dependent (Fig. 3C). The decrease in [Mg²⁺] after 125 min was –0.27 ± 0.11 mM at 15 µM (n = 5), (0.22 ± 0.07 mM at 50 µM (n = 5) and –0.12±0.08 mM at 150 µM (n = 7). On the other hand, this drug had little effect on the changes in pHi (unpaired t-test, P >0.05, n = 7; Fig. 3B, ●).

Increase in [Mg²⁺]: via Na⁺-independent Mg²⁺ pathways

Next, to demonstrate the increase in [Mg²⁺], via transmembrane Mg²⁺-permeable channels, the effect of Na⁺ removal was examined in the presence of Mg²⁺ (Supplementary Fig. S1, A). Extracellular Ca²⁺ was again removed to potentiate Mg²⁺ transport, i.e. to reduce competition between divalent cations at the channel pore.

Changes in [Mg²⁺] and pHi during exposure to Ca²⁺- and Na⁺-free solutions are shown in Figures 4A and B, respectively. In the presence of 1.2 mM Mg²⁺ (the ‘normal’ Mg²⁺ concentration in extracellular medium), [Mg²⁺] increased from 0.73±0.07 to 1.01±0.09 mM after 125 min (●; n = 5; P<0.01). When the concentration of extracellular Mg²⁺ was increased to 6.0 mM, [Mg²⁺] increased from 0.78±0.08 to 1.79 ± 0.18 mM after 125 min (■; n = 7; P<0.01). The [Mg²⁺] rise was clearly enhanced by raising the extracellular Mg²⁺ concentration (unpaired t-test, P<0.01).

Effects of 2-APB on the increase in [Mg²⁺]:

To assess whether the same Mg²⁺-permeable channels contributed to the inward and outward transport of Mg²⁺ under Na⁺-free conditions, we examined the effect of 2-APB in the presence of Mg²⁺. 2-APB (150 µM) was applied to a Ca²⁺- and Na⁺-free solution.
solution containing 6.0 mM Mg$^{2+}$ (Supplementary Fig. S1, B). After 125 min, [Mg$^{2+}$]$_i$ increased from 0.75±0.04 to 1.14±0.08 mM (Fig. 5A, ■; n = 7; P<0.01), but this increase in [Mg$^{2+}$]$_i$ was significantly smaller than without 2-APB (unpaired t-test, P<0.01). On the other hand, pHi with and without 2-APB, was comparable throughout experiments (not shown).

A gradual, and slow increase in [Mg$^{2+}$]$_i$ was caused by substituting extracellular Na$^+$ with N-methyl-D-glucamime (NMDG), even in the presence of Ca$^{2+}$ (Fig. 5B, □; n = 4). Application of 150 µM 2-APB again attenuated the increase in [Mg$^{2+}$]$_i$ significantly (Fig. 5B, ■; n = 4; P<0.01), suggesting that TRPM7-like Mg$^{2+}$-permeable channels sensitive to 2-APB play a crucial role in Mg$^{2+}$ regulation (i.e. uptake) under physiological conditions, in which Ca$^{2+}$ is present. Lower concentrations (15 and 50 µM) of 2-APB only attenuated the increase in [Mg$^{2+}$]$_i$ slightly (P>0.05; Fig. 5C).

In the present experiments, Na$^+$ was frequently substituted with equimolar K$^+$. To rule out that Na$^+-$Mg$^{2+}$ exchangers coupled Mg$^{2+}$ transport with K$^+$ under Na$^+$-free conditions, we examined the effect of amiloride, which is known to inhibit a broad range of Na$^+$-coupled transporters [19], including Na$^+-$Mg$^{2+}$ exchange [12, 20]. Application of amiloride (1 mM) had little effect on the increase in [Mg$^{2+}$]$_i$ caused by exposure to a Ca$^{2+}$- and Na$^+$-free solution containing 6.0 mM Mg$^{2+}$ (unpaired t-test, P>0.05; see Supplementary Fig. S2).

**Estimation of [Mg$^{2+}$]$_i$ and pH$_i$ from the β and γ-ATP peaks**

To confirm the changes in [Mg$^{2+}$]$_i$ and pH$_i$ estimated above, we used a different procedure; specifically,
the chemical shifts of β- and γ-ATP were used to solve simultaneous equations for [Mg\(^{2+}\)]\(_i\) and pH\(_i\) (see, Material and methods). The changes in pH\(_i\), as well as [Mg\(^{2+}\)]\(_i\), were comparable between the two analyses (Table 2). [Mg\(^{2+}\)]\(_i\) estimated from β- and γ-ATP were slightly smaller because of the higher pH\(_i\) estimated; i.e. the apparent dissociation constant for MgATP is smaller in a higher pH.

**High-energy phosphates**

ATP is known to affect the activity of TRPM7 and to act as an important intracellular Mg\(^{2+}\) buffer. Correlation analysis revealed that [ATP]\(_i\) and [Mg\(^{2+}\)]\(_i\) are not correlated regardless of the presence of extracellular Mg\(^{2+}\) (Fig. 6). Also, [PCr]\(_i\) did not change significantly throughout (Supplementary Table S1).

**RT-PCR**

To confirm the transcription of genes for Mg\(^{2+}\)-permeable channels, RT-PCR was performed. Because the cDNA sequences for porcine TRP (transient receptor potential) homologue cation channels have not been published, the PCR primers were designed by using the conserved sequences from humans and mice. Of TRPM2, 4, 6 and 7, the TRPM7 was predominant. TRPM6 was also detectable under the same PCR condition (Fig. 7; see also Supplementary Fig. S3).

**Discussion**

The present 31P-NMR measurements revealed several features of [Mg\(^{2+}\)]\(_i\), modulation via transmembrane Mg\(^{2+}\)-permeable channels, including sensitivity to 2-APB. Simultaneous removal of extracellular Mg\(^{2+}\) and Ca\(^{2+}\) reduced [Mg\(^{2+}\)]\(_i\) to approximately 60% of the control after 125 min even in the absence of Na\(^+\) (Fig. 2), under which conditions Na\(^+\)-coupled Mg\(^{2+}\) transporters i.e. Na\(^+\)-Mg\(^{2+}\) exchange, do not operate. In addition, the removal of extracellular Na\(^+\) in the presence of extracellular Mg\(^{2+}\) increased [Mg\(^{2+}\)]\(_i\) in an extracellular Mg\(^{2+}\)-concentration-dependent manner (Fig. 4), and this effect was enhanced by the simultaneous removal of Ca\(^{2+}\). Altogether, these results suggested an important role of transmembrane Mg\(^{2+}\)-permeable channels in regulating [Mg\(^{2+}\)]\(_i\) in vascular smooth muscle cells.

As shown in Fig. 5B, the activity of Mg\(^{2+}\)-permeable channels are attenuated by extracellular Ca\(^{2+}\), but Mg\(^{2+}\) entry still occurred. In these experiments, extracellular Na\(^+\) was substituted with NMDG, and
this procedure maintained the negative membrane potential, unlike Na+ substitution with K+, which was used in other experiments. Furthermore, simultaneous removal of extracellular divalent cations, that is, Ca2+ and Mg2+, did not significantly decrease [Mg2+]i when extracellular Na+ was substituted with NMDG (data not shown). Therefore, it is considered that, although Mg2+ loss can be caused via Mg2+-permeable channels under extreme conditions, this mechanism mainly contributes to Mg2+ uptake under physiological conditions, in which the negative resting membrane potential is preserved (probably several tens of mV).

TRPM6 and TRPM7 are known Mg2+-permeable non-selective cation channels. It has been shown that TRPM6 is abundant in the kidney and small intestine, while TRPM7 is expressed ubiquitously in numerous tissues and organs [9, 21, 22]. It is thought that the former and latter are responsible for Mg2+ regulation at the organ and cellular levels, respectively. In line with this notion, RT-PCR examinations revealed that TRPM7 was a major component of TRPM homologues in porcine carotid arteries, but TRPM6 was also detectable (Fig. 7). Previous patch clamp studies have shown that the removal of extracellular divalent cations facilitates TRPM7-like current [9, 11, 23], a finding that is in good agreement with our [Mg2+]i measurements, that is, Figure 5.

2-APB is known to block store-operated Ca2+ release-activated Ca2+ (CRAC) channels as well as Mg2+-permeable channels [24]. However, Mg2+ transport via CRAC channels is considered negligible, because these channels have even greater Ca2+ selectivity than voltage-sensitive Ca2+ channels [25]. In the present study, Mg2+ transport via Mg2+-permeable channels demonstrated as Na+-independent [Mg2+]i changes, was not completely suppressed by 2-APB at <150 µM, which is three times greater than the IC50 (~50 µM) reported for TRPM7-like cation currents [11, 23]. This discrepancy may be explained as follows: (1) The IC50 of 2-APB in TRPM7 may be altered by the intracellular milieu, namely, [ATP]i is...
maintained in our $^{31}$P-NMR measurements, while no ATP was added in the intracellular solution of the previous patch clamp experiments in which the effect of 2-APB was examined [11, 23]; (2) Native TRP channels may act as multimeric proteins of complex compositions [26]; 3) some other, as yet unknown Mg$^{2+}$ transport mechanisms operate in parallel. Recently, it has been shown that the application of 2-APB does not block, but rather facilitates TRPM6 [27], which is contained as a minor component in porcine carotid arteries. In future studies, it would thus be interesting to examine the effects of drugs, such as 2-APB in TRPM6/7 knockdown cells. NMR measurements provide us with abundant information on [Mg$^{2+}$]; regulation, that is pH$_i$ and [ATP]; which affect intracellular Mg$^{2+}$-buffering, but require a considerable amount of vascular smooth muscle tissue to achieve reasonable time-resolution. For tissue level experiments, gene-targeting techniques, for example, RNA interference, remain to be improved.

The TRPM homologue channels consist of channel pore and kinase domains. Thus, these channels are referred to as ‘chanzymes’. It has been reported that Mg$^{2+}$ and MgATP regulate the activity of TRPM7 via the kinase domain [9–11, 28]. In addition to this mechanism, intracellular ATP itself acts as an important Mg$^{2+}$ buffer. With respect to the inhibitory effect of 2-APB on [Mg$^{2+}$]; rise in the absence of extracellular Ca$^{2+}$ and Na$^+$ (in the presence of 6 mM Mg$^{2+}$; Fig. 4), one may suspect that 2-APB interacts with the kinase domain to elevate its sensitivity to [Mg$^{2+}$]. If this mechanism operates, increase in [Mg$^{2+}$]; would be slowed at around the [Mg$^{2+}$]; which significantly reduces the open probability of the Mg$^{2+}$-permeable channels. The present $^{31}$P-NMR measurements, however, revealed that changes in [Mg$^{2+}$]; were suppressed throughout the application of 2-APB (Fig. 6; Supplementary Material IV, Supplementary Table S1). Therefore, the present results suggest that 2-APB directly blocks Mg$^{2+}$-permeable channels at the channel pore.

Reported values for [Mg$^{2+}$]; in smooth muscle cells are lower than found in the other two muscle types, that is, skeletal and cardiac muscles. Furthermore, among smooth muscles distributed in numerous tissues and organs, we estimated [Mg$^{2+}$]; in vascular smooth muscle cells to be the lowest after correcting for the pH$_i$ and temperature [8]: (0.8–0.85 mM in taenia caeci; 0.7–0.9 mM in the uterus; 0.8 mM in the urinary bladder and 0.65–0.75 mM in the carotid
artery. Under divalent cation-free conditions, \([\text{Mg}^{2+}]_i\) decreased to \(-60\%\) of the control value after 125 min in porcine carotid arteries, however, the decrease in \([\text{Mg}^{2+}]_i\) was much slower than that previously observed in intestinal smooth muscle cells (a decrease to tens of \(\mu\text{M}\) after 100 min) [6, 17]. The difference in the rate of \([\text{Mg}^{2+}]_i\) depletion under Na+-free conditions suggests that the activity of \(\text{Mg}^{2+}\)-permeable channels is lower in vascular smooth muscle cells. This hypothesis may account for the fact that the lowest basal \([\text{Mg}^{2+}]_i\) level has been estimated in vascular smooth muscle cells and may contribute to our understanding of \(\text{Mg}^{2+}\)-dependent cellular mechanisms. For example, the physiological significance of \(\text{Ca}^{2+}\)-induced \(\text{Ca}^{2+}\) release from ryanodine receptors in vascular smooth muscle could be attributed to the low \([\text{Mg}^{2+}]_i\) [29]. Also, \(\text{Mg}^{2+}\)-permeable non-selective cation channels, such as TRPM7, have been recently shown to play a crucial role in generating spontaneous rhythmicity in pacemaker interstitial cells of intestinal smooth muscle tissues [30, 31]. Taken together, these facts warrant systematic investigation into cell- and tissue-specific roles of TRPM channels in numerous smooth muscle tissues containing pacemaker-like interstitial cells [32–35].

Functional mutations of ion channels are known to cause numerous diseases. Functional mutations of TRPM7 result in a significant effect on cellular \(\text{Mg}^{2+}\) homeostasis. It has been experimentally shown that mutations of the kinase domain in TRPM7 modulate the sensitivity to \([\text{Mg}^{2+}]_i\). Furthermore, knockout of TRPM7 causes intracellular \(\text{Mg}^{2+}\)-deficiency in cultured cells [36]. In clinical fields, it has long been recognized that \(\text{Mg}^{2+}\) is associated with several important diseases, e.g. diabetes mellitus, hypertension, and cardiovascular and cerebrovascular diseases [37, 38]. Subgroups of these diseases may involve functional mutations of \(\text{Mg}^{2+}\)-permeable channels contributing to passive \(\text{Mg}^{2+}\) transport via the electrochemical gradient. Similarly to the studies on TRPM6 [39, 40], future genetic analyses to clarify the contribution of TRPM7-like \(\text{Mg}^{2+}\)-permeable channels to cardiovascular diseases will be of great interest. Indeed, a TRPM7 variant has been recently reported for two Guamanian neurodegenerative disorders [41].

In conclusion, the present \(^{31}\text{P-NMR}\) data have suggested the existence of \(\text{Mg}^{2+}\)-permeable channels of TRPM homologues, contributing to \([\text{Mg}^{2+}]_i\) regulation in vascular smooth muscle cells. The fact that \([\text{Mg}^{2+}]_i\) decreases slowly under divalent cation-free conditions, presumably corresponding to the low expression level and/or activity of \(\text{Mg}^{2+}\)-permeable channels, may account for the low basal \([\text{Mg}^{2+}]_i\) in vascular smooth muscle cells. From the inhibitory effect of 2-APB and the results of RT-PCR, TRPM7 appears to play a predominant role in the passive \(\text{Mg}^{2+}\) transport.

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Supplementary material

The following supplementary material is available for this article:

Material S1 Details of estimation procedures.

Fig. S1 Example spectra for changes in the \(\text{H}^{25}\)-ATP peak during exposures to a \(\text{Ca}^{2+}\)- and Na+-free, high (6.0 mM) \(\text{Mg}^{2+}\) solution.

Fig. S2 The effect of amiloride on \([\text{Mg}^{2+}]_i\).

Fig. S3 Tests of primers.

Table S1 Changes in the concentration of high-energy phosphates.

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