# $Na^+$ Gradient-Dependent $Mg^{2+}$ Transport in Smooth Muscle Cells of Guinea Pig Tenia Cecum

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ABSTRACT Thin strips of guinea pig tenia cecum were loaded with the $Mg^{2+}$ indicator furaptra, and the indicator fluorescence signals measured in $Ca^{2+}$-free condition were converted to cytoplasmic-free $Mg^{2+}$ concentration ($[Mg^{2+}]_i$). Lowering the extracellular $Na^+$ concentration ($[Na^+]_o$) caused a reversible increase in $[Mg^{2+}]_i$, consistent with the inhibition of $Na^+$ gradient-dependent extrusion of cellular $Mg^{2+}$ ($Na^+$-$Mg^{2+}$ exchange). Curve-fitting analysis indicated that the relation between $[Na^+]_o$ and the rate of rise in $[Mg^{2+}]_i$ had a Hill coefficient of ~3, a $[Na^+]_o$ at the half-maximal rate of rise of ~30 mM, and a maximal rate of 0.16 ± 0.01 μM/s (mean ± SE, $n$ = 6). Depolarization with 56 mM $K^+$ shifted the curve slightly toward higher $[Na^+]_o$ without significantly changing the maximal rate, suggesting that the $Na^+$-$Mg^{2+}$ exchange was inhibited by depolarization. The maximal rate would correspond to a flux of 0.15–0.4 pmol/cm²/s, if cytoplasmic $Mg^{2+}$ buffering power (defined as the ratio of the changes in total $Mg^{2+}$ and free $Mg^{2+}$ concentrations) is assumed to be 2–5. Ouabain (1–5 μM) increased the intracellular $Na^+$ concentration, as assessed with fluorescence of SBFI (sodium-binding benzofuran isophthalate, a $Na^+$ indicator), and elevated $[Mg^{2+}]_i$. In ouabain-treated preparations, removal of extracellular $Na^+$ rapidly increased $[Mg^{2+}]_i$, with an initial rate of rise roughly proportional to the degree of the $Mg^{2+}$ load, and, probably, to the $Na^+$ load caused by ouabain. The enhanced rate of rise in $[Mg^{2+}]_i$ (up to ~1 μM/s) could be attributed to the $Mg^{2+}$ influx as a result of the reversed $Na^+$-$Mg^{2+}$ exchange. Our results support the presence of a reversible and possibly electrogenic $Na^+$-$Mg^{2+}$ exchange in the smooth muscle cells of tenia cecum.

## INTRODUCTION

Many studies have reported that the basal cytoplasmic free magnesium concentration ($[Mg^{2+}]_i$) is much lower than that expected from passive distribution of the ion across the cell membrane. Thus, $Mg^{2+}$ must be actively extruded from the cytoplasm to the extracellular space against the electrochemical gradient (for review, see Flatman, 1984; Romani and Scarpa, 1992). One candidate for such an $Mg^{2+}$ extrusion mechanism is the $Na^+$-$Mg^{2+}$ exchange, which uses the energy of passive $Na^+$ influx. Most evidence for the $Na^+$-$Mg^{2+}$ exchange has been obtained from squid giant axons (Baker and Crawford, 1972; De Weer, 1976; Mullins et al., 1977; Caldwell-Violich and Requena, 1979; Dipolo and Beauge, 1988; Gonzalez-Serratos and Rasgado-Flores, 1990), and erythrocytes (Gunther et al., 1984; Feray and Garay, 1986; Frenkel et al., 1989; Flatman and Smith, 1990; Xu and Willis, 1994). Three possibilities have been suggested for the stoichiometry of the exchange: $1Na^+$:$1Mg^{2+}$ (Baker and Crawford, 1972; Frenkel et al., 1989; Flatman and Smith, 1990; Gonzales-Serratos and Rasgado-Flores, 1990), $2Na^+$:$1Mg^{2+}$ (Mullins et al., 1977; Gunther et al., 1984; Dipolo and Beauge, 1988; Xu and Willis, 1994), and $3Na^+$:$1Mg^{2+}$ (Feray and Garay, 1988).

The $Na^+$-$Mg^{2+}$ exchange has been also postulated in other vertebrate cells, including neurons (Brocard et al., 1993; Stout et al., 1996; Gunzel and Schlue, 1996), thymocytes (Gunther and Vormann, 1992), sublingual acini (Zhang and Melvin, 1995), and hepatocytes (Luca et al., 1997). The transport might be either electroneutral ($2Na^+$:$1Mg^{2+}$; Zhang and Melvin, 1995) or electrogenic ($1Na^+$:$1Mg^{2+}$; Gunzel and Schlue, 1996). Evidence for the $Na^+$-$Mg^{2+}$ exchange is controversial in cardiac myocytes (for review, see Murphy et al., 1991; also see Handy et al., 1996).

In smooth muscles, the presence of the $Na^+$-$Mg^{2+}$ exchange has been also suggested by studies using various methods: atomic absorption spectroscopy (Palaty, 1974; Ford and Driska, 1986), electron probe x-ray microanalysis (Wasselman et al., 1986), $^{28}Mg$ flux measurement (Shetty and Weiss, 1988), and $^{31}P$-NMR (Nakayama and Tomita, 1991; Nakayama et al., 1994). However, the detailed properties of the exchange remain unclear.

To further characterize the $Na^+$-$Mg^{2+}$ exchange in smooth muscle cells, we used the $Mg^{2+}$ indicator furaptra to measure $[Mg^{2+}]_i$ in small strips of tenia cecum. The fluorescence signals of furaptra can be reliably detected from small preparations for many hours with an acceptable signal-to-noise ratio and time resolution. We converted the fluorescence signal to $[Mg^{2+}]_i$ with the intracellular calibration of furaptra as described in our companion article, because the intracellular calibration appears to become more important as $[Mg^{2+}]_i$ is elevated above the basal level (Tashiro and Konishi, 1997). In the present study, we examined the dependence of $[Mg^{2+}]_i$ on extracellular concentrations of $Na^+$ or $K^+$ or both, and estimated the stoichiometry of the $Na^+$-$Mg^{2+}$ exchange. In addition, we investigated the possibility that the exchange also occurs in

*Received for publication 2 June 1997 and in final form 3 September 1997.*
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0006-3495/97/12/3371/14 $2.00

the reverse direction, particularly when the intracellular $Na^+$ concentration is elevated.

A preliminary account of some of the results has appeared in abstract form (Tashiro et al., 1996; 1997a, b).

## METHODS

The preparation, furaptra loading, measurement of furaptra fluorescence signals, and analysis were described in our companion article (Tashiro and Konishi, 1997). Briefly, thin strips of longitudinal smooth muscle (~200 μm wide, ~100 μm thick, and ~10 mm long) were dissected from tenia cecum of guinea pig. After furaptra loading, the background-subtracted fluorescence intensity at 500 ± 20 nm was measured with excitation at 350 nm [F(350)] and 382 nm [F(382)], and the ratio signal [$R$ = F(382)/F(350)] was converted to $[Mg^{2+}]_i$ with a standard equation (Eq. 1 of Tashiro and Konishi, 1997). Calibration parameters obtained with an intracellular calibration method were used: $R_{min}$ = 0.986, $R_{max}$ = 0.199, and $K_D$ = 5.43 mM, where $R_{min}$ and $R_{max}$ are the values of $R$ at 0 $[Mg^{2+}]_i$ and saturating $[Mg^{2+}]_i$, respectively (Tashiro and Konishi, 1997). We found no significant change in the background fluorescence under the experimental conditions of the present study.

### Solutions and chemicals

Normal Tyrode's solution contained (mM): 137.9 NaCl, 5.9 $KHCO_3$, 2.4 $CaCl_2$, 1.2 $MgCl_2$, 11.8 glucose, and 5 HEPES (with pH adjusted to 7.4 at 25°C by ~2 mM NaOH). The $Ca^{2+}$-free solution contained 0.1 mM EGTA instead of $CaCl_2$. The high-$Mg^{2+}$ solution contained 10 mM $MgCl_2$ and a reduced NaCl concentration, 127.9 mM. High-$K^+$ (25.9 mM or 55.9 mM) solution of constant $[K^+] \times [Cl^-]$ product was made by substituting NaCl with potassium-methanesulfonate and sodium-methanesulfonate on an equimolar basis. The low-$K^+$ (2.0 mM) solution was prepared by replacing 3.9 mM $KHCO_3$ with 3.9 mM $NaHCO_3$. For the experiments in which the preparations were exposed to low $Na^+$ concentrations, $Na^+$ was substituted by equimolar *N*-methyl-D-glucamine (NMDG) or, in some experiments, by tetramethylammonium (TMA) to keep the solution osmolality at 280–290 mOsm/kg $H_2O$ (Osmotron-20 osmometer, Orion Research, Inc., MA). The preparation was continuously perfused with one of the above solutions at 25°C during the course of experiments, except for the period of indicator loading. The perfusate was usually bubbled with 100% $O_2$, but nearly identical results were obtained in experiments without bubbling. Furaptra (mag-fura-2, tetra potassium salt, lot 2211), furaptra-AM (mag-fura-2-AM, lot 2951), and SBFI-AM (lot 2632) were purchased from Molecular Probes, Inc. (Eugene, OR). Ouabain was obtained from Sigma Chemical Co. (St. Louis, MO). Atropine sulfate was purchased from Tanabe Seiyaku Co., Ltd. (Osaka, Japan). KB-R7943 (2-[2-[4-(4-nitrobenzyloxy) phenyl] ethyl]isothiourea methanesulfonate), was a kind gift of Kanebo, Ltd. (Osaka, Japan). All other chemicals were reagent grade.

### In vitro measurements of furaptra fluorescence

Effects of $[Na^+]$ on furaptra fluorescence signals were examined with a spectrofluorometer (FP-770, JASCO, Tokyo) by measuring F(382)/F(350) of 1 μM furaptra in 1-cm quartz cells (Fig. 1). In the absence of $Mg^{2+}$, a large change in $[Na^+]$ from 0 to 40 mM caused a slight decrease in the $R$ (−0.040). This $Na^+$-dependent change in $R$ appeared to be smaller in the presence of $Mg^{2+}$; at an $[Mg^{2+}]$ of 1 mM (near the basal cytoplasmic level) and 3.9 mM, the change in $R$ was −0.018 and −0.016, respectively, in a $[Na^+]$ range of 0 to 40 mM. This would cause an ~0.1-mM difference in the estimate of $[Mg^{2+}]_i$. The error in the estimate of $[Mg^{2+}]$ caused by the change in $[Na^+]_i$ around the basal level (probably 10–20 mM) is, therefore, expected to be smaller than 0.1 mM. Addition of 2 μM ouabain did not significantly effect the $R$ values (*crosses* in Fig. 1).

Neither the fluorescence nor the $Mg^{2+}$ ($Ca^{2+}$)-binding properties of furaptra is significantly affected by changes in pH between 6.5 and 7.2 (Konishi et al., 1993), findings that are consistent with the apparent pK of ~5 reported by Raju et al. (1989).

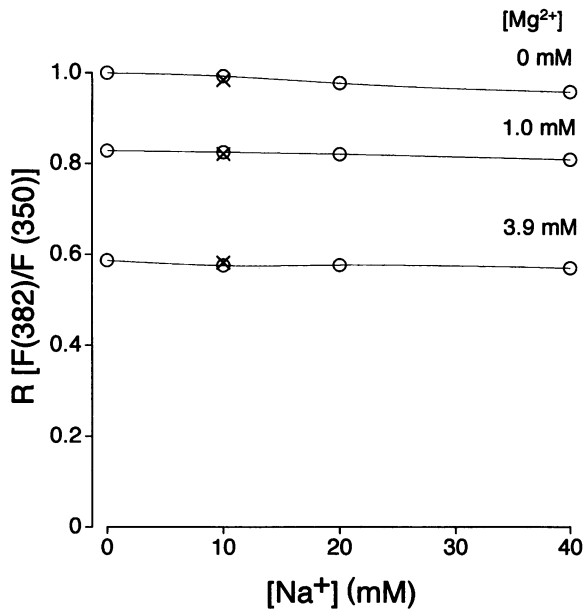


FIGURE 1 The effect of $[Na^+]$ and ouabain on the fluorescence ratio $R$ of furaptra (25°C). The emission wavelength was 510 ± 5 nm. The $R$ measured in the solutions with various $[Na^+]$ (○; 0–40 mM KCl of the basic solution was substituted with equimolar NaCl) and in the presence of 2 μM ouabain (×). All $R$ values were normalized to that obtained in the absence of $Mg^{2+}$ and $Na^+$. The basic solution contained (mM) 150 KCl, 0.5 EGTA, 0.001 furaptra (K salt), and 10 PIPES, at pH 7.1. Various amounts of $MgCl_2$ were added to yield the $[Mg^{2+}]$ indicated near each symbol; the apparent dissociation constant for the $Mg^{2+}$-EGTA reaction was assumed to be 16.9 mM (Martell and Smith, 1974).

### Measurement of SBFI fluorescence

In some preparations, relative changes in the cytoplasmic $Na^+$ concentration ($[Na^+]_i$) were monitored with the fluorescent $Na^+$ indicator SBFI (sodium-binding benzofuran isophthalate; Harootunian et al., 1989). The preparation was incubated with 5 μM SBFI-AM, 0.0125% pluronic, and 0.0625% dimethyl sulfoxide (as a solvent) for 12 h (23–25°C). The resultant SBFI fluorescence intensities were recorded at two excitation wavelengths [340 nm and 380 nm; background-subtracted fluorescence intensities denoted F(340) and F(380), respectively] with the emission band set at 500 ± 20 nm. No attempt was made to calibrate SBFI fluorescence in terms of $[Na^+]_i$; rather, a change in the ratio F(340)/F(380) was used as a qualitative indication of a change in $[Na^+]_i$. Because of similar excitation/emission bands appropriate for both furaptra and SBFI, fluorescence measurements of the two indicators in the same preparation were not possible.

### Data analysis

Nonlinear least-squares fitting was carried out with the program Origin (Microcal Software, Inc., Northampton, MA). Statistical values are shown as means ± SE. Differences between two sets of results were assessed with Student's two-tailed $t$-test and considered to be significant if $P < 0.05$.

## RESULTS

Fluorescence signals of furaptra were measured from strips of tenia cecum and were converted to $[Mg^{2+}]_i$. In the

present study, fluorescence was measured under $Ca^{2+}$-free conditions in the presence of 0.1 mM EGTA. However, preliminary experiments were performed in the presence of 2.4 mM $Ca^{2+}$. Fig. 2 shows an example of an experiment in which furaptra fluorescence was measured while extracellular $Na^+$ was removed in the presence of 2.4 mM $[Ca^{2+}]_o$. The fluorescence signal was calibrated in terms of $[Mg^{2+}]_i$ under the assumption that any change in the fluorescence signal was due solely to the change in $[Mg^{2+}]_i$. $Na^+$-free (plus high-$Mg^{2+}$) perfusion increased $[Mg^{2+}]_i$ (34–94 min in Fig. 2). The recovery of $[Mg^{2+}]_i$ after restoration of extracellular $Na^+$ was slow; the $[Mg^{2+}]_i$ level was still higher by ~0.1 mM than the initial baseline level even after 160-min incubation in normal Tyrode's solution. Surprisingly, with the second application of $Na^+$-free perfusion, now in the absence of $Mg^{2+}$, $[Mg^{2+}]_i$ was increased (256–316 min in Fig. 2), and did not decrease for 90 min after extracellular $Na^+$ was reintroduced. Qualitatively similar results were obtained in three other preparations. Because Nakayama et al. (1994) did not observe any increase in $[Mg^{2+}]_i$ (estimated with $^{31}$P-NMR) after perfusion with $Na^+$-free and $Mg^{2+}$-free, this apparent change in $[Mg^{2+}]_i$ in the absence of extracellular $Mg^{2+}$ does not likely reflect a true change in $[Mg^{2+}]_i$. Instead, the apparent rise in $[Mg^{2+}]_i$ might reflect an increase in $[Ca^{2+}]_i$ by inhibition of the $Na^+$-$Ca^{2+}$ exchange. The elevation in $[Ca^{2+}]_i$ could cause $Ca^{2+}$-related fluorescence change of furaptra ($K_D$ for $Ca^{2+}$ ~50 $\mu$M; Raju et al., 1989), which is nearly indistinguishable from the $Mg^{2+}$-related signal. The $Ca^{2+}$ loading in the stores as a result of the raised $[Ca^{2+}]_i$ could also affect furaptra fluorescence, because ~11% of furaptra molecules appear to be in the intracellular compartments (companion article). Alternatively, a continuous elevation of $[Ca^{2+}]_i$ might cause irreversible, or very slowly reversible, changes in the intracellular milieu, which influence the indicator signals in an unidentified way.

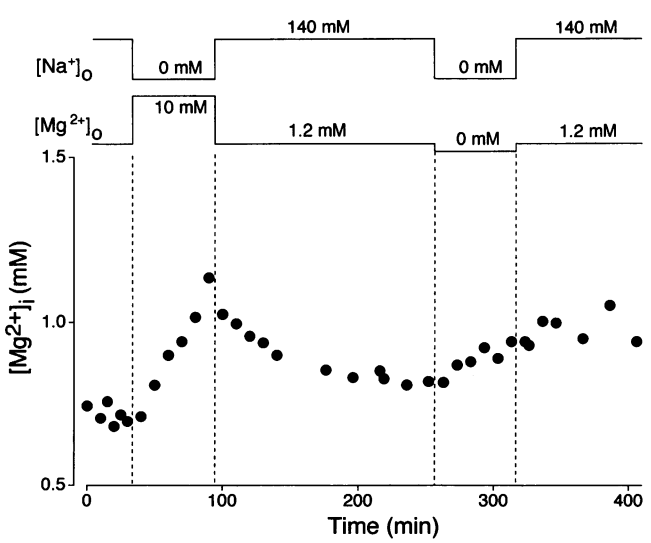


FIGURE 2 Long-term measurement of $[Mg^{2+}]_i$ from a single preparation in the presence of $[Ca^{2+}]_o$. Extracellular $Na^+$ and $Mg^{2+}$ concentrations were changed, as indicated above, with $Ca^{2+}$ concentration kept at 2.4 mM throughout. For 1-h periods between dashed lines, $Na^+$-free (NMDG-substituted) solution containing either 10 mM $[Mg^{2+}]$ (*left*) or 0 mM $[Mg^{2+}]$ (*right*) was superfused.

Another important point seen in Fig. 2 is that $Na^+$-free perfusion increased $[Mg^{2+}]_i$ much less in the presence of $Ca^{2+}$ than in the absence of $Ca^{2+}$(Fig. 3 *A*). In Fig. 2, the (true) rate of rise in $[Mg^{2+}]_i$ in the presence of 10 mM $Mg^{2+}$ may be estimated by subtraction of the apparent rate in the absence of $Mg^{2+}$ (0.059 $\mu$M/s) from that with 10 mM $Mg^{2+}$ (0.11 $\mu$M/s). The rate of rise thus estimated, 0.051 $\mu$M/s, is much smaller than 0.34 $\mu$M/s, the average value obtained in the absence of $Ca^{2+}$ (Fig. 3 *B*). The smaller change in $[Mg^{2+}]_i$ induced by $Na^+$-free perfusion in the presence of $Ca^{2+}$ was also found by Nakayama et al. (1994) and could be attributed to the reduced membrane permeability to $Mg^{2+}$ by $Ca^{2+}$. Extracellular $Ca^{2+}$-free would also help to inhibit the $Ca^{2+}$-activated $Mg^{2+}$ efflux, if present in the tenia, which has been proposed in hepatocyte membranes (Luca et al., 1997). We thus carried out experiments under $Ca^{2+}$-free conditions to demonstrate clearly and to characterize accurately the change in $[Mg^{2+}]_i$.

From 71 preparations used in the present study, the estimated $[Mg^{2+}]_i$ in $Ca^{2+}$-free Tyrode's solution was 0.99 ± 0.02 mM. This value was similar to that (0.98 ± 0.05 mM) obtained with normal Tyrode's solution in our companion article (Tashiro and Konishi, 1997).

## Removal of extracellular $Na^+$ causes an elevation in $[Mg^{2+}]_i$

Fig. 3 summarizes average results of two kinds of experiments in which $[Mg^{2+}]_i$ was continuously measured from the same preparations ($n$ = 2–3). When a preparation was superfused with $Ca^{2+}$-free Tyrode's solution containing normal $Na^+$ and $Mg^{2+}$, $[Mg^{2+}]_i$ was stable at ~1 mM for at least 4 h (*filled circles* in Fig. 3 *A*). Complete removal of extracellular $Na^+$ (by substitution with NMDG) caused a slow and approximately linear increase in $[Mg^{2+}]_i$, which reached 1.96 ± 0.09 mM ($n$ = 4) at the end of 2-h $Na^+$-free perfusion. After reintroduction of extracellular $Na^+$, $[Mg^{2+}]_i$ returned almost to the basal level (*open circles* in Fig. 3 *A*).

This reversible increase of $[Mg^{2+}]_i$ in the absence of $Na^+$ was enhanced in the presence of 10 mM $Mg^{2+}$, as expected from the presumed extracellular origin of the increased $[Mg^{2+}]_i$ (Fig. 3 *B*, *open circles*); the average $[Mg^{2+}]_i$ was 3.07 ± 0.19 mM ($n$ = 3) after 2-h perfusion with the 0-$Na^+$, high-$Mg^{2+}$ solution. When extracellular $Na^+$ and $Mg^{2+}$ concentrations were restored to control levels (140 mM and 1.2 mM, respectively), the $[Mg^{2+}]_i$ decreased toward the basal level, but had not fully recovered after 2 h. Even at nearly constant $[Na^+]_o$ (130 mM), high $[Mg^{2+}]_o$ alone caused some increase in $[Mg^{2+}]_i$ to 1.59 ± 0.27 mM ($n$ = 4) in 2 h (*filled circles* in Fig. 3 *B*), suggesting substantial passive influx of $Mg^{2+}$.

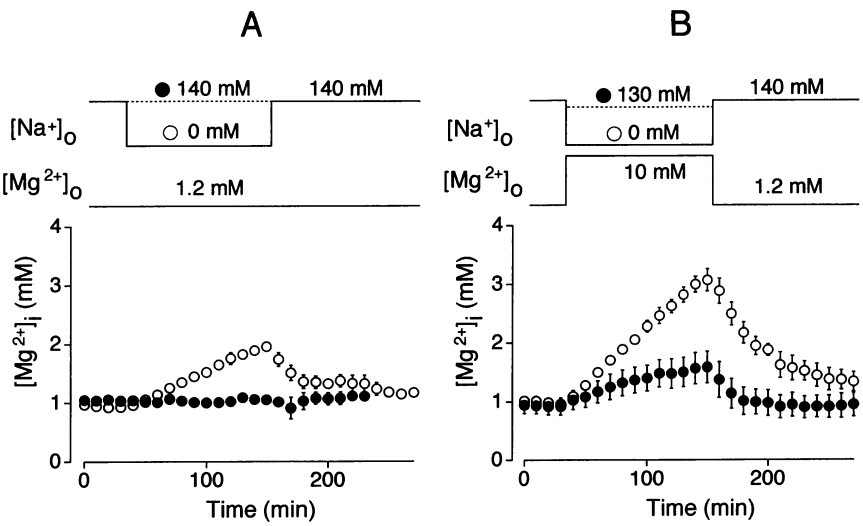


FIGURE 3 Long-term measurement of $[Mg^{2+}]_i$ with extracellular perfusion of the $Na^+$-free (NMDG-substituted) solution containing normal $[Mg^{2+}]$ (*A*, 1.2 mM) or high $[Mg^{2+}]$ (*B*, 10 mM) for 2 h. In *A* and *B*, the results obtained at 0 mM $[Na^+]_o$ (○) and 130–140 mM $[Na^+]_o$ (●) are shown, as indicated above. Each symbol represents a mean (±SE) value obtained from three preparations, except for filled circles in *A* ($n$ = 2).

In two preparations, we measured fluorescence excitation spectra of furaptra (330–420 nm), rather than fluorescence intensities at only two wavelengths (Fig. 4 *A*). In these experiments, the excitation spectrum of intracellular furaptra was first measured in the $Ca^{2+}$-free Tyrode's solution, after which the spectrum was remeasured after 100-min exposure to the $Na^+$-free solution containing 10 mM $Mg^{2+}$. The spectral shape of the fluorescence change of intracellular furaptra closely followed the $Mg^{2+}$ difference spectrum obtained in the in vitro solutions (Fig. 4 *B*). Thus, changes in furaptra fluorescence were consistent with the changes in $[Mg^{2+}]_i$, at least under this experimental condition.

Fig. 5 *A* shows examples of experiments in which NaCl was substituted by TMA Cl, so that the solution contained only ~2 mM $Na^+$. When NaCl was replaced with TMA Cl, $[Mg^{2+}]_i$ increased at an approximately constant rate for the first 40–50 min, as was observed after NMDG replacement (Fig. 3 *A*). In the later phase, however, $[Mg^{2+}]_i$ decreased toward the basal level even with continuous exposure to the low-$Na^+$ (TMA) solution (*filled circles* in Fig. 5 *A*).

Because TMA has been reported to stimulate muscarinic receptors (Kennedy et al., 1995), the experiment was repeated in the presence of 1 μM atropine (*open circles*). In this experiment and another experiment (not shown), the spontaneous decay of $[Mg^{2+}]_i$ was partially inhibited by 1 μM atropine; the $[Mg^{2+}]_i$ reached a plateau after increasing for 40–50 min. These results imply that a secondary mechanism related to muscarinic receptor stimulation, but not necessarily related to $[Na^+]_o$, might be involved in the regulation of $[Mg^{2+}]_i$ at later times. On the other hand, the rise in $[Mg^{2+}]_i$ for the initial 40 min was not significantly different when either NMDG or TMA replaced $Na^+$; the rate of rise in $[Mg^{2+}]_i$, defined as the slope of the least-squares-fitted line through the data points between 6 and 39

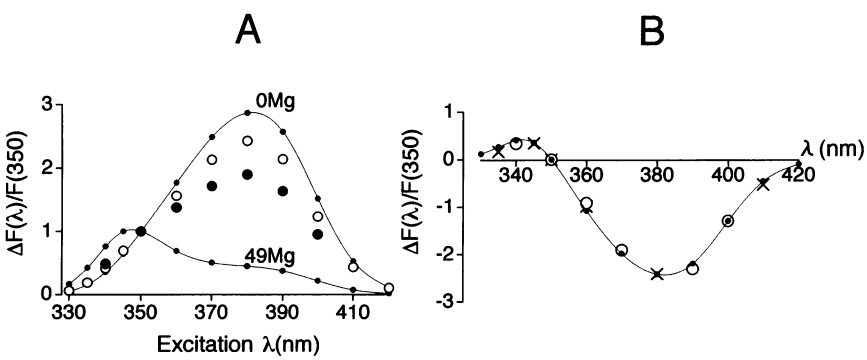


FIGURE 4 Comparison of fluorescence excitation spectra of furaptra measured from the preparations and in solutions. (*A*) Open and filled circles were two spectra recorded sequentially from a preparation before (○) and 100 min after (●) superfusion with a $Na^+$-free (NMDG-substitution) 10 mM-$Mg^{2+}$ solution (see Fig. 3 *B* for the solution protocol). In vitro spectra of 50 μM furaptra (*small filled circles*) were measured in quartz capillaries that contained the calibration solutions of 0 mM (0 Mg) and 49 mM $[Mg^{2+}]$ (49 Mg) with composition given in the preceding article (see Methods in Tashiro and Konishi, 1997). The solid lines through data points were drawn by spline interpolation. Each spectrum has been normalized to F(350) to correct for the decrease in the indicator concentration. (*B*) Open circles: the difference spectrum calculated at each wavelength by subtraction of ○ from ● in *A*. Crosses were obtained from a similar experiment to that shown in *A* in another preparation (not shown). The in vitro $Mg^{2+}$ difference spectrum calculated by subtraction of the spectrum at 0 mM $[Mg^{2+}]$ from that at 49 mM $[Mg^{2+}]$ is shown in *B* (*small filled circles*). Each difference spectrum obtained in the preparations has been scaled to fit the in vitro spectrum.

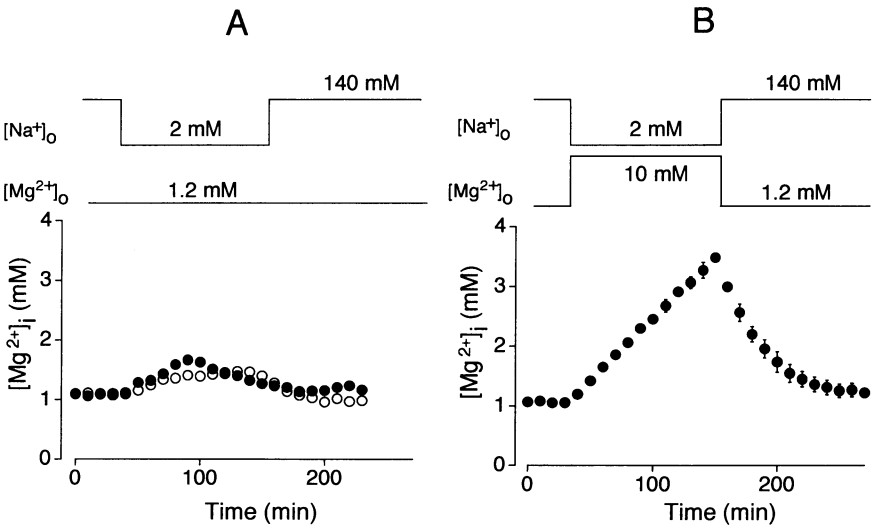


FIGURE 5 Long-term measurement of $[Mg^{2+}]_i$ with extracellular perfusion of low-$Na^+$ (TMA-substituted) solution containing normal $[Mg^{2+}]$ (*A*, 1.2 mM) or high $[Mg^{2+}]$ (*B*, 10 mM) for 2 h. The solution protocol was similar to that shown in Fig. 3, except for the use of TMA Cl to replace NaCl. (*A*) Values represented by open and filled circles were obtained from two different preparations. In one preparation (○), 1 μM atropine was included in all solutions. (*B*) Each symbol represents mean (±SE) value obtained from three preparations.

min after $Na^+$ removal (see below), was 0.16 ± 0.01 μM/s ($n$ = 6) and 0.14 ± 0.02 μM/s ($n$ = 3) with NMDG and TMA substitution, respectively. In the following sections, the rate of rise in $[Mg^{2+}]_i$ was, therefore, primarily analyzed as a $Na^+$-dependent change in $[Mg^{2+}]_i$, and NMDG was used to replace $Na^+$. Interestingly, in the presence of 10 mM $[Mg^{2+}]_o$, the spontaneous decay in the low-$Na^+$ (TMA) solution appeared to be masked by the large increase in $[Mg^{2+}]_i$ (Fig. 5 *B*); only the approximately linear increase in $[Mg^{2+}]_i$ was seen during low-$Na^+$ (and high-$Mg^{2+}$) perfusion, as was observed with NMDG substitution. We did not further characterize the spontaneous decay of $[Mg^{2+}]_i$ in the TMA-substituted low-$Na^+$ (and 1.2-mM $Mg^{2+}$) solution. Overall, the experiments in Figs. 3–5 support the presence of a $Na^+$-$Mg^{2+}$ exchange mechanism in smooth muscle cells of tenia cecum, which has been suggested from experiments with other methods (Palaty, 1974; Nakayama and Tomita, 1991; Nakayama et al., 1994). The results also suggest that furaptra fluorescence can be used for quantitative study of the $Na^+$-$Mg^{2+}$ exchange.

## Effect of extracellular $Mg^{2+}$

The increase in $[Mg^{2+}]_i$ upon extracellular $Na^+$ removal was clearly enhanced in the presence of 10 mM $[Mg^{2+}]_o$. However, the high $[Mg^{2+}]_o$ caused some increase in $[Mg^{2+}]_i$ even in the presence of normal levels of $[Na^+]_o$ (Fig. 3 *B*). The extracellular $Na^+$-dependent component of the $[Mg^{2+}]_i$ change was estimated by subtracting the change in $[Mg^{2+}]_i$ measured in the $Na^+$-containing solution from that measured in the $Na^+$-free solution (Fig. 6). The rate of $Na^+$-dependent rise in $[Mg^{2+}]_i$ at 10 mM $[Mg^{2+}]_o$ was 0.22 ± 0.04 μM/s ($n$ = 3), which was ~1.4-fold larger than the increase (0.16 ± 0.02 μM/s, $n$ = 6) obtained at 1.2 mM $[Mg^{2+}]_o$. The amplitude of $Na^+$-dependent $[Mg^{2+}]_i$ change in 2 h was also significantly different: 1.45 ± 0.26 mM vs. 0.90 ± 0.12 mM (10 mM $[Mg^{2+}]_o$ vs. 1.2 mM $[Mg^{2+}]_o$, respectively).

## Effect of extracellular $Na^+$ and $K^+$

Extracellular $Na^+$-dependence of $[Mg^{2+}]_i$ was further studied by monitoring the rate of rise in $[Mg^{2+}]_i$ on changing $[Na^+]_o$ from 140 mM to various levels between 0 and 88 mM. In this series of experiments, each preparation was exposed to lowered $[Na^+]_o$ only once, and the rates of rise in $[Mg^{2+}]_i$ during the initial 39 min were accumulated from a total of 45 preparations. This was done because exposure to low $[Na^+]_o$ might cause a secondary change in the intracellular environment, which might be restored only in the slow time course after reintroduction of normal $[Na^+]_o$.

The change in $[Mg^{2+}]_i$ was approximately linear between 6 and 39 min after the onset of low $[Na^+]_o$ perfusion, and the rate of rise (Fig. 7, *solid lines*) was defined as the slope

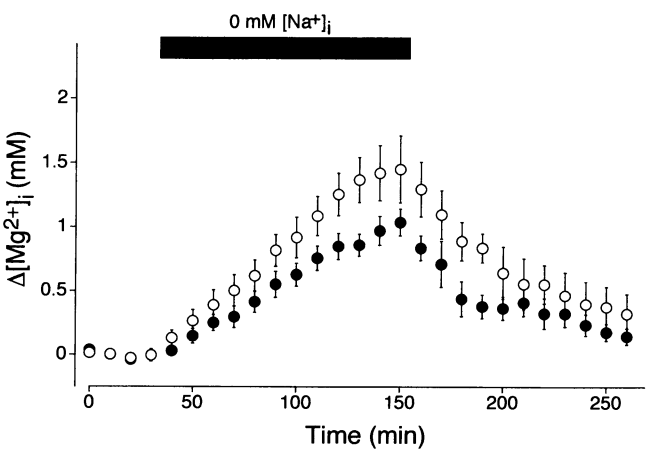


FIGURE 6 $[Na^+]_o$-dependent change in $[Mg^{2+}]_i$ (*ordinate*) in the presence of 1.2 mM (●) and 10 mM (○) $[Mg^{2+}]_o$. Extracellular $Na^+$ was replaced by NMDG for the period indicated by the horizontal bar. Values indicated by filled circles were calculated by subtracting the $[Mg^{2+}]_i$ in $Ca^{2+}$-free Tyrode's solution (● in Fig. 3 *A*) from that measured in $Na^+$-free solution (○ in Fig. 3 *A*). Values indicated by open circles were similarly calculated by subtracting the $[Mg^{2+}]_i$ in $Na^+$-containing solution (● in Fig. 3 *B*) from that in $Na^+$-free solution (○ in Fig. 3 *B*).

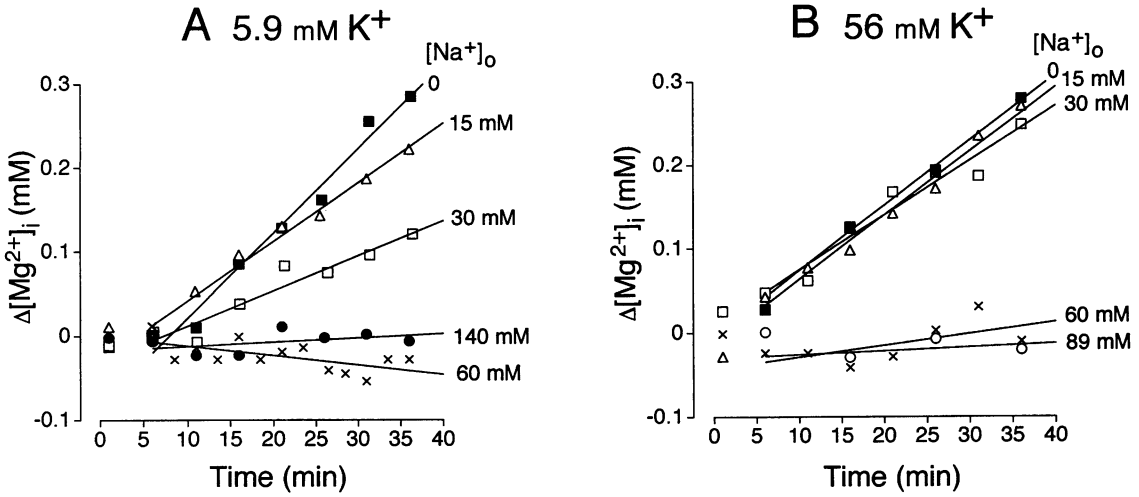


FIGURE 7 Examples of the early change in $[Mg^{2+}]_i$ induced by low $[Na^+]_o$ at either normal $[K^+]_o$ (*A*, 5.9 mM) or high $[K^+]_o$ (*B*, 56 mM). Each symbol in *A* and *B* represents data obtained from different preparations. The preparations were initially perfused with the $Ca^{2+}$-free Tyrode's solution, and one of the solutions with various $[Na^+]_o$ (marked on the right) was introduced at time 0 on the abscissa. $Na^+$ of the $Ca^{2+}$-free Tyrode's solution was replaced with NMDG in *A*. In *B*, 50 mM $Na^+$ of the $Ca^{2+}$-free Tyrode's solution was replaced with $K^+$, and the rest was replaced with NMDG. For each data set, the average $[Mg^{2+}]_i$ value in $Ca^{2+}$-free Tyrode's solution was taken as 0 $\Delta[Mg^{2+}]_i$, and the regression line for data points between 6 and 36 min is shown as a solid line.

of the best-fitted line. To clarify whether the $Na^+$-dependent change in $[Mg^{2+}]_i$ was influenced by cell membrane depolarization or hyperpolarization, the experiments were carried out at high $[K^+]_o$ (26 mM, not shown; 56 mM, Fig. 7 *B*) or low $[K^+]_o$ (2.0 mM, not shown), as well as at normal $[K^+]_o$ (5.9 mM, Fig. 7 *A*).

In the presence of relatively high $[Na^+]_o$ ($\geq$88 mM), the increase in $[Mg^{2+}]_i$ was minimal (on average, <0.01 $\mu$M/s) independent of $[K^+]_o$ (Fig. 8 *A*). The average rate of rise in $[Mg^{2+}]_i$ after a large reduction in $[Na^+]_o$ (to the levels <than 4 mM) was 0.16 ($\pm$0.01, $n$ = 6) $\mu$M/s at normal $[K^+]_o$, and was slightly less both at high $[K^+]_o$ and low $[K^+]_o$ (Fig. 8 *A*). However, the difference was not statistically significant [0.15 $\pm$ 0.01 $\mu$M/s ($n$ = 3), 0.14 $\pm$ 0.01 $\mu$M/s ($n$ = 3), and 0.12 $\pm$ 0.03 $\mu$M/s ($n$ = 3) at 26 mM, 56 mM, and 2.0 mM $[K^+]_o$, respectively]. At 30 mM $[Na^+]_o$, the rate of rise in $[Mg^{2+}]_i$ was, on average, lower at 2.0 mM $[K^+]_o$, and higher at 56 mM $[K^+]_o$ than at normal $[K^+]_o$ (Fig. 8 *A*); the difference was statistically significant between low $[K^+]_o$ and normal $[K^+]_o$. Although different levels of $[K^+]_o$ (between 2.0 and 56 mM) did not always cause a statistically significant change in the rate of rise in $[Mg^{2+}]_i$ at each $[Na^+]_o$, $[K^+]_o$ might have caused some alteration in the $[Na^+]_o$-dependence of the rate of rise in $[Mg^{2+}]_i$.

Fig. 8 *B* shows a more complete analysis of the relation between $[Na^+]_o$ and the rate of rise in $[Mg^{2+}]_i$ at normal (5.9 mM) and high (56 mM) $[K^+]_o$ where the rate of rise in $[Mg^{2+}]_i$ was estimated at five to six different $[Na^+]_o$ (between 0 and 140 mM). Data at low (2.0 mM) $[K^+]_o$ and three different $[Na^+]_o$ are also shown. In the presence of normal $[K^+]_o$, the rate of rise in $[Mg^{2+}]_i$ was significantly higher at $[Na^+]_o$ of 30 mM or lower. At $[Na^+]_o$ of 60 mM and 88 mM, the average rates of increase were slightly negative [$-0.021 \pm 0.010$ $\mu$M/s ($n$ = 5) at 60 mM and $-0.018 \pm 0.011$ $\mu$M/s ($n$ = 3) at 88 mM] but not significantly different from zero. Implication of these possible negative slopes (decrease of $[Mg^{2+}]_i$) will be discussed later (see Discussion). At $[Na^+]_o$ of 30 mM, 60 mM, and 88 mM, the rate of rise in $[Mg^{2+}]_i$ was, on average, larger at 56 mM $[K^+]_o$ than 5.9 mM $[K^+]_o$; the difference was statistically significant at 60 mM $[Na^+]_o$. At low $[K^+]_o$, the rates of rise in $[Mg^{2+}]_i$ at $[Na^+]_o$ of 15 mM and 30 mM were significantly less than corresponding values at normal $[K^+]_o$.

The data points at any given $[K^+]_o$ were fitted with a Hill-type equation:

$$\Delta[Mg^{2+}]_i/\Delta t = (\Delta[Mg^{2+}]_i/\Delta t)_{max}\left[1 - \frac{[Na^+]_o^N}{(K^N + [Na^+]_o^N)}\right] \quad (1)$$

where $\Delta[Mg^{2+}]_i/\Delta t$ and $(\Delta[Mg^{2+}]_i/\Delta t)_{max}$ denote the rate of rise in $[Mg^{2+}]_i$ at $[Na^+]_o \geq 0$ and $[Na^+]_o = 0$, respectively. $N$ is the Hill coefficient, and $K$ is $[Na^+]_o$ that gives the half-maximal rate of rise in $[Mg^{2+}]_i$. For curve fitting at 5.9 mM $[K^+]_o$, we did not use data points at 60 mM $[Na^+]_o$ (*open circle* in Fig. 8 *B*), because inclusion of these points made the fitting unreliable. The least-squares-fitted curve thus obtained had $K$ = 27 mM, $N$ = 2.8, and $(\Delta[Mg^{2+}]_i/\Delta t)_{max}$ = 0.16 $\mu$M/s (*solid line* in Fig. 8 *B*). The fitting for data points obtained at 56 mM $[K^+]_o$ yielded a curve with $K$ = 40 mM, $N$ = 3.6, and $(\Delta[Mg^{2+}]_i/\Delta t)_{max}$ = 0.14 $\mu$M/s (*dashed line* in Fig. 8 *B*). A similar analysis was applied to the data obtained at 2.0 mM $[K^+]_o$. For the fitting, however, the value of $(\Delta[Mg^{2+}]_i/\Delta t)_{max}$ was fixed at 0.123 $\mu$M/s, the average value estimated at 3.9 mM $[Na^+]_o$, because the rate

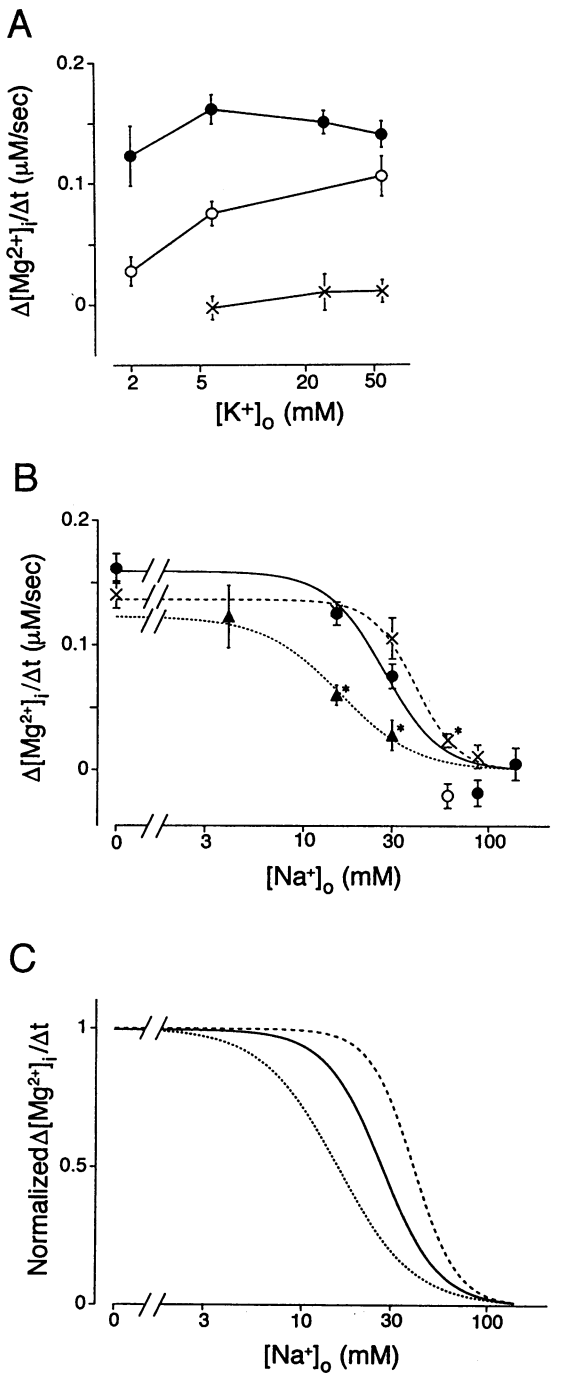


FIGURE 8 The effect of $[K^+]_o$ and $[Na^+]_o$ on the rate of rise in $[Mg^{2+}]_i$ ($\Delta[Mg^{2+}]_i/\Delta t$), estimated as shown in Fig. 7. (*A*) $[K^+]_o$-dependence of $\Delta[Mg^{2+}]_i/\Delta t$ measured at $[Na^+]_o$ of ≤4 mM (●), 30 mM (○), and ≥88 mM (×). (*B*) $[Na^+]_o$-dependence of $\Delta[Mg^{2+}]_i/\Delta t$ measured at $[K^+]_o$ of 2 mM $[K^+]_o$ (▲), 5.9 mM $[K^+]_o$ (●, ○) and 56 mM (×). The solid line indicates the least-squares fit of ● by the Hill-type curve. The dashed line indicates the least-squares fit of ×, and the dotted line indicates the least-squares fit of ▲ by the curve. (*C*) The normalized least-squares-fitted curves. Each fitted curve in *B* was scaled to match the maximal value. In *A* and *B*, each data point represents mean ± SE of three to six preparations (*$P < 0.05$ versus data at normal $[K^+]_o$), except for one data point at 56 mM $[K^+]_o$ and 15 mM $[Na^+]_o$ in *B* ($n = 1$). The total number of data points was nine (from nine preparations) at 2.0 mM $[K^+]_o$, 26 (from 22 preparations) at 5.9 mM $[K^+]_o$, six (from six preparations) at 26 mM $[K^+]_o$, and 14 (from 14 preparations) at 56 mM $[K^+]_o$.

of rise in $[Mg^{2+}]_i$ was estimated only within the limited range of $[Na^+]_o$ (4–30 mM). Data points at 2.0 mM $[K^+]_o$ yielded the least-squares-fitted curve with $K = 16$ mM and $N = 2.1$ (*dotted line* in Fig. 8 *B*). Fig. 8 *C* displays the $Na^+$-dependence of the rate of rise in $[Mg^{2+}]_i$ at $[K^+]_o$ of 2.0 mM, 5.9 mM, and 56 mM after normalization to the maximal rate.

Overall, relative to normal $[K^+]_o$, high $[K^+]_o$ (depolarization) appeared to cause a shift of the curve toward higher $[Na^+]_o$ without a marked change in the slope or the maximal rate of rise, whereas low $[K^+]_o$ (hyperpolarization) caused a shift of the curve toward lower $[Na^+]_o$ with some reduction of slope and possibly a slight decrease in the maximal rate of rise.

## Effect of intracellular $Na^+$

In the previous section, $[Na^+]_o$ was lowered to study the effect of $Na^+$ gradient across the cell membrane on $[Mg^{2+}]_i$. However, the reduction of $[Na^+]_o$ would cause a decrease in $[Na^+]_i$ (e.g., Fig. 11), which might also influence $Mg^{2+}$ efflux. It was thus important to carry out experiments in which $[Na^+]_i$ was altered while $[Na^+]_o$ was kept constant.

Nakayama and Nomura (1995) applied 10 μM ouabain, a well-known inhibitor of the $Na^+$-$K^+$ pump, to preparations of guinea pig tenia cecum and reported an increase in $[Mg^{2+}]_i$ using $^{31}$P-NMR (32°C). Since this concentration of ouabain completely blocks $Na^+$-$K^+$ pump-related oxygen consumption in smooth muscle of the tenia cecum (Nakayama and Tomita, 1990), a large increase in $[Na^+]_i$ is expected. We used a similar approach in the present study, but used relatively low concentrations of ouabain (1–5 μM) to produce a slow and moderate rise in $[Na^+]_i$.

Fig. 9 shows examples of two experiments (*A* and *B*), in which preparations were sequentially treated with 1 and 2 μM ouabain. In the preparation shown in Fig. 9 *A*, the application of 1 μM ouabain caused a slow increase in $[Mg^{2+}]_i$, which appeared to be further accelerated by 2 μM ouabain. The effect of ouabain was slowly reversed with a significant delay after washout of ouabain, probably due to slow dissociation of the drug from the binding sites.

In another preparation (Fig. 9 *B*), $[Mg^{2+}]_i$ was rapidly increased by ouabain, with no clear concentration-dependence between 1 and 2 μM, and reached a quasi-steady-state level after washout of ouabain. Removal of extracellular $Na^+$ (NMDG substitution) in the $Mg^{2+}$-loaded (and probably also $Na^+$-loaded) preparation caused a rapid rise in $[Mg^{2+}]_i$ (Fig. 9 *B*). The initial rate of rise, defined as the slope best-fitting the data points during the initial 10 min after $Na^+$ removal, was 1.17 μM/s in this preparation; this value was 7.3 times larger than the average rate of rise (0.16 μM/s) obtained in preparations not treated with ouabain. The initial rate of rise (slope between 0 and 10 min), rather than the rate of rise at a later time (slope between 6 and 39 min), was analyzed because, in some ouabain-treated prep-

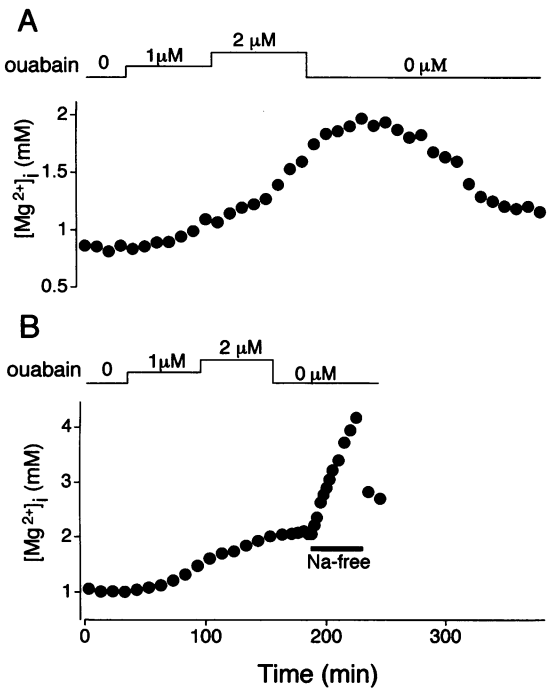


FIGURE 9 The effect of ouabain on $[Mg^{2+}]_i$. *A* and *B* were obtained from two different preparations. Ouabain was added to the $Ca^{2+}$-free Tyrode's solution as indicated at the top of each panel. In *B*, $Na^+$-free (NMDG-substituted) solution was perfused for the period indicated by a horizontal bar.

arations, the rise in $[Mg^{2+}]_i$ was linear for only the first 10–15 min and was clearly slowed for the rest of the period (not shown).

In one experiment, extracellular $Na^+$ was removed before and after treatment with 5 μM ouabain, and the effect of $Na^+$ removal on the observed elevation of $[Mg^{2+}]_i$ could thus be compared in the same preparation. The rise in $[Mg^{2+}]_i$ was clearly facilitated after treatment with ouabain, compared with the control without ouabain treatment (Fig. 10 *A*, *open* and *filled circles*).

During the course of the study, we found considerable variation among preparations in the $Mg^{2+}$ load by ouabain treatment. We used this natural variation to analyze the correlation between the ouabain-induced $Mg^{2+}$ load, which was presumed to reflect $Na^+$ load, and the initial rate of rise in $[Mg^{2+}]_i$ upon $Na^+$ removal. The experimental protocol was similar to that shown in Fig. 9 *B*; after treatment with 2–5 μM ouabain for 60–125 min and washout of ouabain, extracellular $Na^+$ was replaced with NMDG. Fig. 10 *B* plots the initial rate of rise in $[Mg^{2+}]_i$ as a function of the degree of $Mg^{2+}$ load by ouabain; pooled data from seven preparations indicate the dependence of the initial rate on the $Mg^{2+}$ load by ouabain in a roughly proportional manner. In one experiment (⊠), 5 μM KB-R7943 was applied during ouabain washout (30 min) and $Na^+$ removal. This drug has been reported to inhibit the reverse mode of $Na^+$-$Ca^{2+}$ exchange with the half inhibition by 0.3–3 μM in cardiac and smooth muscles (Watano et al., 1996; Iwamoto et al., 1996). KB-R7943 did not exhibit a clear effect on the initial rate of rise in $[Mg^{2+}]_i$ in this preparation. One preparation was loaded with $Mg^{2+}$ by incubation in 20 mM $[Mg^{2+}]_o$ (10 mM $MgCl_2$ added to the high-$Mg^{2+}$ solution). Even though the basal $[Mg^{2+}]_i$ was elevated by 0.81 mM, no substantial increase in the initial rate of rise in $[Mg^{2+}]_i$ was observed. It is thus unlikely that the $Mg^{2+}$ load of ~0.8 mM above the basal level, by itself, significantly modifies the initial rate of rise in $[Mg^{2+}]_i$ (e.g., via the change in cytoplasmic $Mg^{2+}$ buffering).

## Alteration of $[Na^+]_i$ monitored with SBFI fluorescence

Relative changes in $[Na^+]_i$ by perfusion with low $[Na^+]_o$ or ouabain treatment or both were monitored with the fluorescence signals of SBFI. During the initial phase of the experimental run with an SBFI-loaded preparation, both F(340) and F(380) of SBFI slowly decreased with time, probably reflecting slow leak of the indicator (Fig. 11). Low $[Na^+]_o$ or the application of ouabain (2 μM) caused a clear increase or decrease, respectively, in the F(380), whereas the F(340) was essentially unchanged. This observation suggests that the isosbestic wavelength of SBFI in the intracellular environment of the tenia preparation is close to 340 nm, which is ~20 nm blue-shifted compared with the isosbestic wavelength in salt solutions (~360 nm). The blue-shift of the SBFI isosbestic wavelength, in close agreement with earlier reports in cardiac myocytes (Donoso et al., 1992), probably results from the binding of SBFI to intracellular constituents. Despite the evidence of altered properties of the intracellular indicator, we assumed that the fluorescence intensity ratio of SBFI [F(340)/F(380)] showed a relative change in $[Na^+]_i$.

When $[Na^+]_o$ was decreased to various levels (0–90 mM), the fluorescence ratio decreased rapidly, as expected from the decrease in $[Na^+]_i$, and reached new steady levels within 10–12 min (Fig. 11, *bottom*). The ratio returned to near the control level with a similar time course when extracellular $Na^+$ was restored. Thus, the level of $[Na^+]_i$ is influenced by $[Na^+]_o$, and even a small reduction in $[Na^+]_o$ appears to cause some decrease in $[Na^+]_i$ (Fig. 12 *A*; also see *e* in Fig. 12 *B*). The rapid change in $[Na^+]_i$ suggests that, in most of the time range during which the rate of rise in $[Mg^{2+}]_i$ was assessed (6–39 min after solution exchange), $[Na^+]_i$ was nearly steady.

The effect of ouabain on $[Na^+]_i$ was also investigated (Fig. 11), and the results obtained from this and two other preparations of a very similar protocol are summarized in Fig. 12 *B*. The fluorescence ratio increased in the presence of 2 μM ouabain (*h*) and stayed high after ouabain washout (*i*), probably reflecting a sustained elevation of $[Na^+]_i$ by the inhibition of $Na^+$-$K^+$ ATPase; recovery from $Na^+$ load, as well as $Mg^{2+}$ load (see above), was extremely slow. Removal of extracellular $Na^+$ from the ouabain-treated preparation caused a fall in the ratio (*j*). However, the ratio

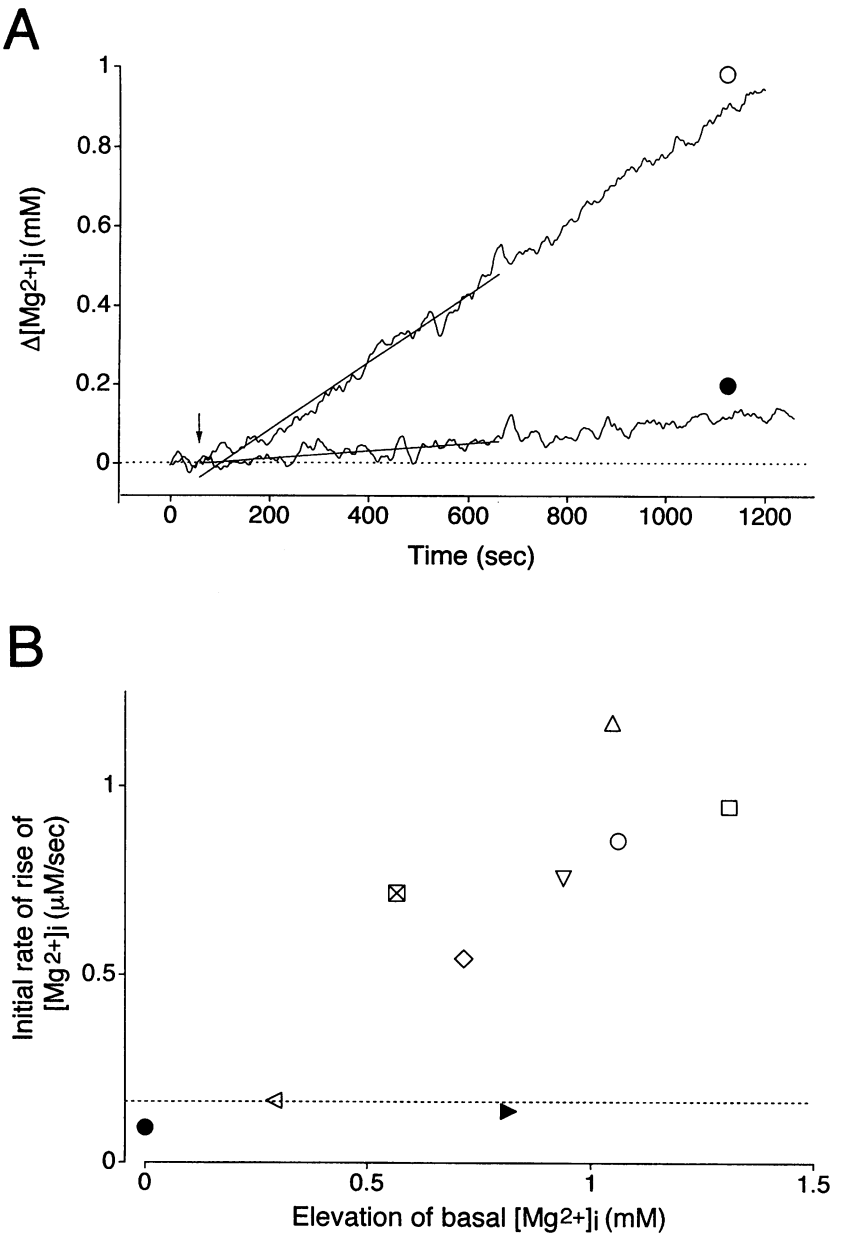


FIGURE 10 (*A*) Rise in $[Mg^{2+}]_i$ upon removal of extracellular $Na^+$ continuously recorded from a preparation. Two noisy traces are records obtained before (●) and after (○) ouabain treatment. The trace marked with the filled circle was recorded first in the absence of ouabain, and reintroduction of $Na^+$ caused full recovery of $[Mg^{2+}]_i$ (not shown). Then, the trace marked with the open circle was recorded after treatment with 5 μM ouabain and washout. Extracellular $Na^+$ was replaced with NMDG at the time indicated by an arrow, and the ordinate shows the change in $[Mg^{2+}]_i$ from the baseline obtained just before $Na^+$ removal. For each record, the least-squares-fitted lines for the initial 10 min after $Na^+$ removal is also shown by a solid line. (*B*) Relationship between the $Mg^{2+}$ load, defined as the change in the basal $[Mg^{2+}]_i$ in the presence of $Na^+$ (*abscissa*), and the initial rate of rise in $[Mg^{2+}]_i$ upon $Na^+$ removal (*ordinate*). Each symbol type represents values obtained from a single experiment. The open symbols and ⊠ represent measurements in ouabain-treated preparations. A filled circle was obtained from a preparation without ouabain treatment; ○ and ● in *B* correspond to the slope of the solid lines shown in *A*. In a preparation (▶), $Mg^{2+}$ was loaded with high $[Mg^{2+}]_o$ (20 mM) without ouabain treatment. Dashed line indicates the mean level for the rate of rise in $[Mg^{2+}]_i$, estimated as shown in Fig. 7 without the $Mg^{2+}$ load ($n$ = 6).

after 20-min perfusion in the $Na^+$-free solution (*j* in Fig. 12 *B*) was still about the same level as those obtained at normal $[Na^+]_o$ without ouabain treatment (*b* and *g* in Fig. 12 *B*).

## DISCUSSION

When extracellular $Na^+$ was removed by substitution with NMDG, $[Mg^{2+}]_i$ slowly increased in guinea pig tenia cecum. This elevation in $[Mg^{2+}]_i$ was mostly reversed by the reintroduction of $Na^+$. These results are consistent with earlier observations with $^{31}$P-NMR (Nakayama and Tomita, 1991; Nakayama et al., 1994), and support the hypothesis of $Na^+$-$Mg^{2+}$ exchange proposed in this and other cell types (see Introduction for references). Interestingly, even though basal $[Mg^{2+}]_i$ levels estimated in the same tissue with $^{31}$P-NMR (Nakayama et al., 1994) and furaptra (companion article) were quite different, similar increases in $[Mg^{2+}]_i$ after $Na^+$-free perfusion were observed in both studies. Nakayama et al. (1994) reported that extracellular $Na^+$-free (and $Ca^{2+}$-free) perfusion for 100 min increased $[Mg^{2+}]_i$ by 0.84 mM (average of two measurements), compared with 0.93 ± 0.06 mM ($n$ = 4) in the present study.

One advantage of the present method over $^{31}$P-NMR is that the indicator fluorescence can be measured with an acceptable signal-to-noise ratio and time resolution from the small preparations. For example, Nakayama et al. (1994) accumulated the NMR spectra from 0.4–0.6 g of tenia cecum for 25 min. In the present experiment, we could obtain signals from small strips (<1 mg) with acceptable stability by signal averaging for 2 s. Furthermore, furaptra

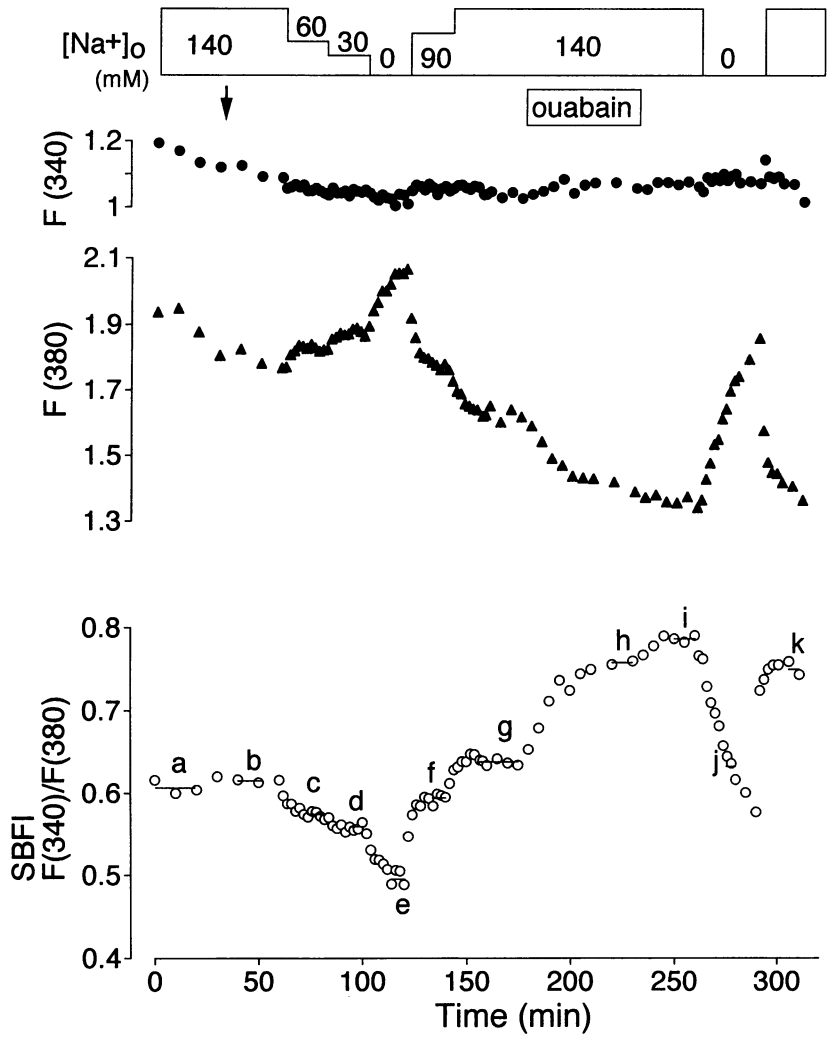


FIGURE 11 The fluorescence signals of SBFI in a preparation superfused with various $[Na^+]_o$ and 2 $\mu$M ouabain, as described above. SBFI F(340) (●, *top panel*; arbitrary unit), F(380) (▲, *middle panel*; arbitrary unit) and the ratio F(340)/F(380) (○, *bottom panel*) are plotted as functions of time. For superfusion, normal Tyrode's solution was substituted with $Ca^{2+}$-free Tyrode's solution at the time indicated by an arrow; the rest of the experiment was carried out under $Ca^{2+}$-free (0.1 mM EGTA) conditions. The average ratio values of two to six measurements in each experimental condition (*a–k*) are indicated by horizontal lines in the bottom panel.

fluorescence exhibits large changes in response to changes in $[Mg^{2+}]_i$, because the fluorescence signal is far from saturation near the basal level of $[Mg^{2+}]_i$.

A disadvantage of furaptra is its sensitivity to $Ca^{2+}$, and possibly to other intracellular substances, by which intracellular indicators might be influenced. Because $Na^+$ is related to many types of ion transport across the cell membrane, the alteration of $[Na^+]_o$ should change the concentrations of ions other than $Na^+$ and $Mg^{2+}$. When $[Na^+]_o$ is lowered in the presence of normal $[Ca^{2+}]_o$ (2.4 mM), the cytoplasmic free-$Ca^{2+}$ concentration ($[Ca^{2+}]_i$) can be expected to increase because of the inhibition of $Ca^{2+}$ efflux or the facilitation of $Ca^{2+}$ influx through a $Na^+$-$Ca^{2+}$ exchanger (e.g., Brading, 1978; Tomita et al., 1993) or both. The results illustrated in Fig. 2 suggest that the use of the present method may be limited in the experimental condition of $Ca^{2+}$ overload.

Removal of extracellular $Na^+$ is expected to cause a decrease in intracellular pH ($pH_i$) by inhibition of $Na^+$-$H^+$ exchange. By measuring $^{31}$P-NMR, Nakayama et al. (1994) reported gradual intracellular acidification during $Na^+$-free perfusion in guinea pig tenia cecum under solution conditions similar to those in the present study; $pH_i$ decreased by 0.2–0.3 in 150 min (see Fig. 5 of Nakayama et al., 1994). The degree of $Na^+$-free-induced acidification appears to be similar in the absence of extracellular $Ca^{2+}$ (S. Nakayama, personal communication). Despite a difference in the experimental temperature (32°C in Nakayama et al. and 25°C in the present study), the decrease in $pH_i$ is expected to be rather small, 0.1 pH unit or less, during the first 40 min after $Na^+$ removal (note that we primarily analyzed the rate of rise in $[Mg^{2+}]_i$ estimated between 6 and 39 min of $Na^+$-free perfusion).

To test the effect of acidosis on $[Mg^{2+}]_i$, two preparations were exposed to acid load by switching bubbling gas from 100% $O_2$ to 5% $CO_2$ and 95% $O_2$, which probably produced intracellular acidification of more than 0.1 pH unit. The estimated $[Mg^{2+}]_i$ during the 20-min acid load was, on average, only slightly greater than the bracketed control by 0.036 mM (average of −0.015 mM and 0.087 mM from the two preparations). Thus, the effect, if any, of change in $pH_i$ by $Na^+$ removal on $[Mg^{2+}]_i$ measurements appears to be

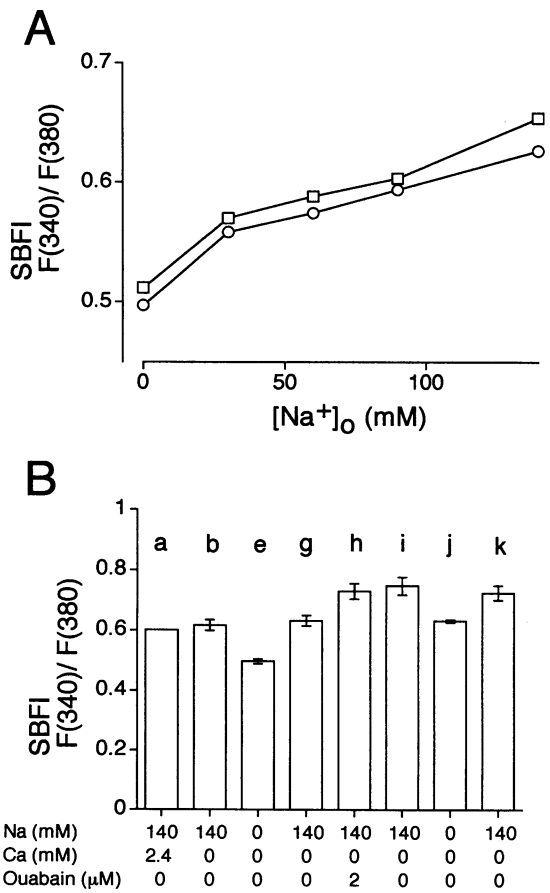


FIGURE 12 (*A*) Steady-state values of the fluorescence intensity ratio (*ordinate*) measured in two preparations (○ and □) at various $[Na^+]_o$ (*abscissa*). The data at the highest $[Na^+]_o$ are the average of two bracketed measurements (e.g., *b* and *g* of Fig. 11) in each preparation. (*B*) Mean fluorescence intensity ratio [F(340)/F(380)] of SBFI from three experiments with a protocol similar to that shown in Fig. 11. Columns *a* through *k* represent mean ± SE values under experimental conditions described below, which correspond to *a* through *k* in Fig. 11.

minor in the present study. Absence of the effects of change in pH on furaptra fluorescence signal has been also reported in cardiac myocytes (Hongo et al., 1994).

## Na$^+$ removal and elevation of $[Mg^{2+}]_i$

In the high-$Mg^{2+}$ solution containing 10 mM $Mg^{2+}$, $[Mg^{2+}]_i$ increased even in the continuous presence of high (130 mM) $Na^+$ (Fig. 3 *B*). This rise in $[Mg^{2+}]_i$ probably reflects the facilitated passive influx of $Mg^{2+}$ by a larger driving force. At normal $[Mg^{2+}]_o$ and $[Na^+]_o$, the steady, passive influx of $Mg^{2+}$ is thought to be counterbalanced by $Mg^{2+}$ extrusion in exchange with $Na^+$ (and possibly by other $Mg^{2+}$ transport processes) to maintain a stable $[Mg^{2+}]_i$ (Fig. 3 *A*). Removal of extracellular $Na^+$ should turn off the $Na^+$-dependent $Mg^{2+}$ extrusion, and $[Mg^{2+}]_i$ is elevated by the steady $Mg^{2+}$ leakage. Assuming that the rate of $[Mg^{2+}]_i$ elevation is, at least initially, equivalent to the rate of the $Mg^{2+}$ extrusion inhibited, we analyzed the relatively early rising phase of $[Mg^{2+}]_i$. We do not think that $Mg^{2+}$ influx through the reversed $Na^+$-$Mg^{2+}$ exchange contributes greatly to the increase in $[Mg^{2+}]_i$, because removal of extracellular $Na^+$ probably causes a rapid fall in $[Na^+]_i$ to a low level within 10–12 min (Borin et al., 1993; also see Fig. 11). The increase in $[Mg^{2+}]_o$ from 1.2 to 10 mM did not markedly enhance the $Na^+$-dependent rise in $[Mg^{2+}]_i$ (Fig. 6). Some increase in the $Na^+$-dependent rise in $[Mg^{2+}]_i$ at high $[Mg^{2+}]_o$ could reflect the larger leak influx of $Mg^{2+}$ and resultant stimulation of the $Na^+$-$Mg^{2+}$ exchange by the elevated $[Mg^{2+}]_i$. In the intermediate $[Na^+]_o$ (15–88 mM), however, part of the $Mg^{2+}$ influx may be coupled to the $Na^+$ efflux.

From the maximal rate of rise in $[Mg^{2+}]_i$ obtained at 0 $[Na^+]_o$ (0.16 μM/s), the rate of $Mg^{2+}$ efflux at physiological $[Mg^{2+}]_i$ (~1 mM) could be estimated. Cytoplasmic $Mg^{2+}$ buffering power (defined as the ratio of the total amount of $Mg^{2+}$ added to the cytoplasm and the change in $[Mg^{2+}]_i$) was assumed to be 2–5, similar to that reported in skeletal and cardiac muscle cells (Westerblad and Allen, 1992; Koss et al., 1993), and the surface-to-volume ratio of guinea pig tenia cecum was taken to be 1.5 $\mu m^{-1}$ (Gabella, 1976). We also assumed that cytoplasm occupies 70% of the cell volume as estimated in skeletal muscle (Baylor et al., 1986). With these values, the maximal rate of $Na^+$-dependent $Mg^{2+}$ efflux was calculated to be 0.15–0.4 pmol/cm$^2$/s at ~1 mM $[Mg^{2+}]_i$ and in the absence of extracellular $Ca^{2+}$.

## Dependence on $[Na^+]_o$ and membrane potential

The relation between $[Na^+]_o$ and the rate of rise in $[Mg^{2+}]_i$ can be described with a Hill-type curve that is shifted toward higher $[Na^+]_o$ by cell membrane depolarization with high (56 mM) $[K^+]_o$ (Fig. 8). According to Casteels and Kuriyama (1966), an increase in $[K^+]_o$ from 5.9 mM to 56 mM causes ~40 mV depolarization from ~−60 mV to −20 mV in guinea pig tenia cecum. Depolarization is probably less in the absence of extracellular $Ca^{2+}$ because membrane would already be depolarized at 5.9 mM $[K^+]_o$ (Bülbring and Tomita, 1970). The rightward shift of the curve (Fig. 8) could be attributed to the inhibition of $Mg^{2+}$ efflux through the $Na^+$-$Mg^{2+}$ exchange by cell membrane depolarization of ~40 mV or less.

We considered four other possibilities by which high $[K^+]_o$ or depolarization or both might influence the rise in $[Mg^{2+}]_i$: first, depolarization might effect the passive influx of $Mg^{2+}$ by changing the driving force. However, as passive leakage should be reduced by depolarization, one would expect to see a decrease in the rate of rise in $[Mg^{2+}]_i$, not the increase that was observed. A second possibility is that both high $[K^+]_o$ and depolarization stimulate the $Na^+$-$K^+$ pump, causing a decrease in $[Na^+]_i$. However, the decrease in $[Na^+]_i$ is expected to facilitate, rather than inhibit, $Mg^{2+}$ efflux through the $Na^+$-$Mg^{2+}$ exchange. The above two possibilities, if true, would mask the observed effect of depolarization. A third possibility is the presence of a "volt-

age-gated $Mg^{2+}$ channel," as yet unidentified, which when opened by depolarization, increases $Mg^{2+}$ influx. However, this hypothesis, in its simplest form, cannot explain the lack of change in the maximal rate of $[Mg^{2+}]_i$ increase with depolarization. Finally, high $[K^+]_o$ might directly inhibit $Mg^{2+}$ efflux (e.g., $K^+$ occupancy of an external site on the exchanger). Although we cannot rule out the possible contribution of other $Mg^{2+}$ influx/efflux transport processes and direct inhibition of the $Na^+$-$Mg^{2+}$ exchange by $K^+$, the most likely mechanism underlying the observation is the voltage (depolarization)-dependent inhibition of the $Na^+$-$Mg^{2+}$ exchange.

Hyperpolarization of membrane potential by low $[K^+]_o$ shifted the $[Na^+]_o$-dependence of the rate of rise in $[Mg^{2+}]_i$ toward lower $[Na^+]_o$ (Fig. 8). The direction of the change is opposite to that observed with high $[K^+]_o$ depolarization and is, therefore, consistent with the voltage sensitivity of the $Na^+$-$Mg^{2+}$ exchange (stimulation by hyperpolarization). However, the interpretation of the results with low $[K^+]_o$ is not straightforward quantitatively. The hyperpolarization caused by a threefold reduction in $[K^+]_o$ (from 5.9 mM to 2.0 mM) is probably much smaller than the depolarization caused by a ninefold increase in $[K^+]_o$ (from 5.9 mM to 56 mM). If the $Na^+$-$Mg^{2+}$ exchange is linearly modulated by membrane potential, the shift of the curve by 2.0 mM $[K^+]_o$ is expected to be smaller than that caused by 56 mM $[K^+]_o$. The rather large shift with shallower slope (and possibly with a reduced maximal rate) induced by low $[K^+]_o$ might result from a (direct or indirect) effect of $[K^+]_o$ in addition to the voltage effect on the $Na^+$-$Mg^{2+}$ exchange.

Thus, we assume that the $Na^+$-$Mg^{2+}$ exchange is voltage-sensitive and, therefore, electrogenic with a stoichiometric ratio of 3 (or more) $Na^+$ to 1 $Mg^{2+}$ in smooth muscle of tenia cecum. This stoichiometry is also consistent with the Hill coefficient of ~3 for describing the $[Na^+]_o$ dependence either at normal $[K^+]_o$ or at high $[K^+]_o$ (Fig. 8 *B*).

Although we cannot provide clear explanations for the small negative mean values of the rate of rise in $[Mg^{2+}]_i$ observed at 60–88 mM $[Na^+]_o$ and 5.9 mM $[K^+]_o$ (Fig. 8 *B*), one possible explanation is that the reduction in $[Na^+]_i$ (see Fig. 12 *A*), rather than $[Na^+]_o$, stimulates the $Mg^{2+}$ efflux and causes the decrease in $[Mg^{2+}]_i$. In fact, the increase in $[Na^+]_i$ from the basal level by treatment with ouabain gradually increased $[Mg^{2+}]_i$ (Fig. 9), a finding that suggests that the $Na^+$-$Mg^{2+}$ exchange is sensitive to $[Na^+]_i$ near the basal level. On the other hand, the $Na^+$-$Mg^{2+}$ exchange appears to be relatively insensitive to $[Na^+]_o \geq 60$ mM (Fig. 8 *B*). If this is the case, the rate of rise in $[Mg^{2+}]_i$ plotted versus $[Na^+]_o$ in Fig. 8 *B* is also a function of $[Na^+]_i$, which would complicate the interpretation of the results. Therefore, the $3Na^+$:$1Mg^{2+}$ stoichiometry proposed in the present study is still tentative. Further studies with proper control of $[Na^+]_i$ (and membrane potential) are required to conclusively determine the stoichiometry of the exchange and to understand whether the reported diversity of stoichiometries ($Na^+$:$Mg^{2+}$) between 1:1 and 3:1 reflects a true difference among cell types and animal species.

## $[Na^+]_i$ and reversal of the $Na^+$-$Mg^{2+}$ exchange

Sensitivity of the $Na^+$-$Mg^{2+}$ exchange to $[Na^+]_i$ suggests a reversal of transport (Gonzalez-Serratos and Rasgado-Flores, 1990) which was most clearly demonstrated by removal of extracellular $Na^+$ from the $Na^+$-loaded preparations (Figs. 9 and 10). In $Na^+$-free perfusion, the elevated $[Na^+]_i$ of the ouabain-treated preparations fell rapidly, probably because of efflux through several possible pathways, including the $Na^+$-$K^+$ pump (partially inhibited by ouabain but stimulated by high $[Na^+]_i$), the reversed $Na^+$-$Mg^{2+}$ exchange and, possibly, channels. The decrease in $[Na^+]_i$ should slow the rise in $[Mg^{2+}]_i$; the slowing of the rise was often clearly observed in the preparations moderately loaded with $Mg^{2+}$ (not shown). In three highly $Mg^{2+}$-loaded preparations (elevation of basal $[Mg^{2+}]_i > 1$ mM), however, the slowing was not obvious for at least 20 min after $Na^+$ removal (see Figs. 9 *B* and 10 *A*). In these preparations, in which the $Na^+$-$K^+$ pump was effectively inhibited by ouabain, the fall in $[Na^+]_i$ might be smaller than that seen in Fig. 12. Also, the decrease of $Mg^{2+}$ influx might be masked at very high $[Mg^{2+}]_i$, by the reduction of cytoplasmic buffering power. However, because we did not quantitatively estimate $[Na^+]_i$ and simultaneously measure $[Na^+]_i$ and $[Mg^{2+}]_i$, we were unable to quantitatively analyze the relation between $[Na^+]_i$ and $[Mg^{2+}]_i$.

In $Mg^{2+}$-loaded preparations with ouabain treatment, reintroduction of $Na^+$ after $Na^+$-free perfusion caused rapid fall in $[Mg^{2+}]_i$ (Fig. 9 *B*). The faster rate of $Mg^{2+}$ extrusion than that observed without ouabain treatment (Fig. 3 *A*) might be partly due to elevated levels of $[Mg^{2+}]_i$, although factors other than $Na^+$ and $Mg^{2+}$ might also contribute to this initial decrease of $[Mg^{2+}]_i$.

## Possible physiological significance of the $Na^+$-$Mg^{2+}$ exchange

In the presence of physiological extracellular $Ca^{2+}$, $Mg^{2+}$ efflux through the $Na^+$-$Mg^{2+}$ exchange was extremely slow. It is thus unlikely that the $Na^+$-$Mg^{2+}$ exchange plays a role in the rapid regulation of $[Mg^{2+}]_i$. Instead, the steady $Mg^{2+}$ efflux in exchange with $Na^+$ might be important to counterbalance the slow leak influx of $Mg^{2+}$. $[Na^+]_o$, at the physiological level, is probably not an important modulator of the exchange. However, the intracellular level of $Na^+$, as well as those of other possible regulating factors (Dipolo and Beauge, 1988; Flatman and Smith, 1990), might physiologically modulate the rate of $Mg^{2+}$ transport by the exchange mechanism and might consequently influence $[Mg^{2+}]_i$. We do not exclude possible existence of other $Mg^{2+}$ transport mechanisms (e.g., ATP-driven $Mg^{2+}$ pump).

The reversibility and the stoichiometry of the $Na^+$-$Mg^{2+}$ exchange tentatively proposed in the present study make one wonder if the $Na^+$-$Mg^{2+}$ exchange and the $Na^+$-$Ca^{2+}$ exchange might share the same transporter molecules; the $Na^+$-$Ca^{2+}$ exchange that has been demonstrated in the present tissue (e.g., Tomita et al., 1993) might slowly transport $Mg^{2+}$. Although $Mg^{2+}$ is generally believed to competitively inhibit the $Na^+$-$Ca^{2+}$ exchange without being transported (Lyu et al., 1991; Kimura, 1996), $Mg^{2+}$ (or $Na^+$) flux at an extremely low rate might not have been detected in earlier studies. At this point, we can neither exclude nor support this possibility; a single preliminary experiment with an inhibitor of the $Na^+$-$Ca^+$ exchange (KB-R7943) is too premature to draw any conclusion. Further electrophysiological, pharmacological, and molecular biological studies are required to elucidate this possibility.

We thank S. Kurihara and S. Nakayama for their helpful comments on the manuscript. We also thank M. Okazaki for his reading of the manuscript.

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan (to M.K.).

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