Intracellular-free Magnesium in the Smooth Muscle of Guinea Pig Taenia Caeci: A Concomitant Analysis for Magnesium and pH upon Sodium Removal

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ABSTRACT This study is concerned with the regulation of intracellular-free Mg^{2+} concentration ($[Mg^{2+}]_i$) in the smooth muscle of guinea pig taenia caeci. To assess an interaction of Ca^{2+} on the Na^+-dependent Mg^{2+}-extrusion mechanism (Na^+-Mg^{2+} exchange), effects of Na^+ removal (*N*-methyl-D-glucamine substitution) were examined in Ca^{2+}-containing solutions. As changes in pH_i in Na^+-free solutions perturb estimation of $[Mg^{2+}]_i$ using the single chemical shift only of the β-ATP peak in ^{31}P NMR (nuclear magnetic resonance) spectra, $[Mg^{2+}]_i$ and pH_i were concomitantly estimated from the chemical shifts of the γ- and β-peaks. When extracellular Na^+ was substituted with *N*-methyl-D-glucamine, $[Mg^{2+}]_i$ was reversibly increased. This increase in $[Mg^{2+}]_i$ was eliminated in Mg^{2+}-free solutions and enhanced in excess Mg^{2+} solutions. ATP content fluctuated little during removal and readmission of Na^+, indicating that $[Mg^{2+}]_i$ changes were not induced by Mg^{2+} release from ATP, and that Mg^{2+}-extruding system would not be inhibited by fuel restriction. A slow acidification in Na^+-free solutions and transient alkalosis by a readmission of Na^+ were observed regardless of the extracellular Mg^{2+} concentration. When the extracellular Ca^{2+} concentration was increased from normal (2.4 mM) to 12 mM, only a marginal increase in $[Mg^{2+}]_i$ was caused by Na^+ removal, whereas a similar slow acidosis was observed, indicating that extracellular Ca^{2+} inhibits Mg^{2+} entry, and that the increase in $[Mg^{2+}]_i$ is negligible through competition between Mg^{2+} and Ca^{2+} in intracellular sites. These results imply that Na^+-Mg^{2+} exchange is the main mechanism to maintain low $[Mg^{2+}]_i$ even under physiological conditions.

INTRODUCTION

Magnesium (Mg^{2+}) is a necessary cofactor for many intracellular enzyme reactions and other important processes. Since Mg^{2+} is the second or third most abundant cation in cells, intracellular free-Mg^{2+} concentration ($[Mg^{2+}]_i$) was thought to be in the order of millimolar (3–10 mM), at which concentration Mg^{2+}-dependent systems would be saturated (White and Hartzell, 1989). Therefore, the effects of intracellular

J. GEN. PHYSIOL. © The Rockefeller University Press · 0022-1295/94/05/0833/19 $2.00
Volume 103 May 1994 833–851

Ca^{2+} and other second messengers have usually been investigated assuming that the Mg^{2+} concentration was stable.

Recently, using improved techniques, evidence that the $[Mg^{2+}]_i$ is kept submillimolar and is thus not passively distributed has been accumulated in various tissues (reviewed by White and Hartzell, 1989; Flatman, 1991; Murphy, Freudenrich, and Lieberman, 1991). In smooth muscle, $[Mg^{2+}]_i$ has been reported to be 0.2–0.5 mM from the measurements of ^{31}P nuclear magnetic resonance (NMR) (summarized by McGuigan, Blatter, and Buri, 1991). The activities of several enzymes are significantly affected by a small change of Mg^{2+} concentration in submillimolar range: in smooth muscle, acto-myosin ATPase activity strongly depends on the free-Mg^{2+} concentration (Chacko and Rosenfeld, 1982). We can thus expect an important influence on smooth muscle contraction by changing $[Mg^{2+}]_i$. Indeed, it has been reported that contractions induced by adrenaline in vascular smooth muscle (Ford and Driska, 1986) and by carbachol in intestinal smooth muscle (Nakayama and Tomita, 1990) were attenuated by depletion of intracellular Mg^{2+}.

Na^+-Mg^{2+} exchange is accepted as a regulatory mechanism of $[Mg^{2+}]_i$ (Flatman, 1991). In smooth muscle, from measurements of Mg^{2+} content, Palatý (1974) predicted the presence of Na^+-Mg^{2+} exchange and the role of the exchange as an effective Mg^{2+}-extruding mechanism. It has recently been shown that $[Mg^{2+}]_i$ is altered by procedures which change the Na^+ concentration gradient across the plasma membrane (Nakayama and Tomita, 1991; Nakayama, Nomura, and Tomita, 1992). However, the experiments were performed mainly in the absence of extracellular Ca^{2+}.

In our preliminary measurements of ^{31}P NMR in the taenia isolated from guinea pig caecum, prolonged removal of extracellular Na^+ in the presence of Ca^{2+} was required to obtain clear shifts of the ATP peaks, corresponding to an increase in $[Mg^{2+}]_i$. During this procedure, the P_i (inorganic phosphate) peak became smaller and it was thus more difficult to estimate pH_i, but in some spectra the chemical shift indicated a considerable acidification with a pH_i below 7. Similar effects of Na^+ removal on pH_i have been reported in other smooth muscles using different techniques, and have been explained by a block of both proton extruding mechanisms through Na^+-H^+ exchange and Na^+-HCO_3^- co-transport (Aickin, 1988). The intracellular acidosis upon Na^+ removal may perturb estimation of $[Mg^{2+}]_i$ (London, 1991). Experiments involving removal of Na^+ in Ca^{2+}-containing solutions are, however, necessary to evaluate the physiological importance of Na^+-Mg^{2+} exchange, otherwise, one may propose that Mg^{2+} efflux in the absence of Ca^{2+} is due to a Na^+-Ca^{2+} exchanger which mainly transports Ca^{2+} under normal conditions. Furthermore, as ATP content tends to continuously decrease in the absence of Ca^{2+}, there is some difficulty in addressing the question: Are changes in $[Mg^{2+}]_i$ induced through inhibition of ATP-dependent Mg^{2+}-extrusion or through Mg^{2+} release from ATP upon its breakdown?

In the present study, we have thus applied an analytical technique to estimate $[Mg^{2+}]_i$ and pH_i from the chemical shifts of the γ- and β-ATP peaks. Most NMR measurements have been performed in the presence of extracellular Ca^{2+}.

MATERIALS AND METHODS

Guinea pigs (300–450 g) of either sex were stunned and exsanguinated, and the taeniae were dissected from the caecum. The taeniae (0.4–0.6 g), obtained from four guinea pigs, were isometrically mounted in an NMR tube (10-mm diam), and were superfused with normal solution bubbled with 100% O_2, at a flow rate of 12 ml min^{-1}. An NMR spectrometer (JEOL GSX270W) was operated at 109.4 MHz for measurements of phosphorous compounds. Radiofrequency pulses of 8.5 μs, corresponding to the 30° flip angle, were repeated at 0.6 s interval. Spectra were usually obtained by accumulating 2,500 signals over 25 min, and a line broadening factor of 15 Hz was applied. Peak positions were measured relative to that of phosphocreatine (PCr) in parts per million (p.p.m.). Digital resolution of the spectrum was set to be ~0.005 p.p.m. by zero-filling to free induction decay. Concentrations of intracellular phosphorous compounds were estimated by integrating the spectral peaks and by correcting with their saturation factors (Nakayama, Seo, Takai, Tomita, and Watari, 1988).

The composition of normal solution was as follows (in millimolar): NaCl, 137.9; $KHCO_3$, 5.9; $CaCl_2$, 2.4; $MgCl_2$, 1.2; glucose 11.8; HEPES (*N*-2-hydroxyethlpiperazine-*N*-2-ethanesulphonic acid), 5; pH adjusted to 7.4–7.5 at 32°C. In Na^+-free solution, Na^+ was replaced by *N*-methyl-D-glucamine (NMDG) on an equimolar basis. Mg^{2+}-free solution and Ca^{2+}-free solution contained 0.1 mM EDTA (ethylenediaminetetraacetic acid) and 0.1 mM EGTA (ethyleneglycol-bis-(β-aminoethylether) *N,N,N',N'*-tetraacetic acid), respectively. NMR measurements were started after equilibrating preparations in normal solution for at least 100 min. After inserting the sample into the magnet, the ^1H signal from water was used to improve the homogeneity of the magnetic field.

Intracellular free-Mg^{2+} concentration ($[Mg^{2+}]_i$) and pH (pH_i) were concomitantly estimated from the chemical shifts of the β- and γ-ATP peaks. This estimation theoretically depends on the fact that the ATP peaks carry information for both $[Mg^{2+}]_i$ and pH_i. Since it is possible to calculate two functional relationships between $[Mg^{2+}]_i$ and pH_i from the γ- and β-ATP peaks, a solution can be found for $[Mg^{2+}]_i$ and pH_i.

Since Mg^{2+} usually binds to ATP as a 1 to 1 complex, $[Mg^{2+}]_i$ is expressed by using the apparent dissociation constant of MgATP ($K_D^{\text{"MgATP"}}$):

$$[Mg^{2+}] = K_D^{\text{"MgATP"}}(\Phi^{-1} - 1), \tag{1}$$

where Φ is the free to total ATP ratio (Gupta and Moore, 1980; Gupta, Gupta, and Moore, 1984). The chemical shift of the observed ATP peak (δ_o) depends on the ratio of Mg^{2+}-bound to free ATP. This gives:

$$\Phi = (\delta_o - \delta_b)/(\delta_f - \delta_b), \tag{2}$$

where δ_f and δ_b are the chemical shifts of metal-free and Mg^{2+}-binding forms of ATP, respectively (Fig. 1*A*). These values can be obtained from model solutions. $[Mg^{2+}]_i$ is thus estimated from the observed chemical shifts of ATP (Gupta and Moore, 1980; Gupta et al., 1984):

$$[Mg^{2+}] = K_D^{\text{"MgATP"}}(\delta_o - \delta_f)/(\delta_b - \delta_o). \tag{3}$$

Chemical shift difference between the α- and β-ATP peaks (Gupta et al., 1984; Nakayama and Tomita, 1990, 1991) or the PCr and β-ATP peaks (Kushmerick, Dillon, Meyer, Brown, Krisanda, and Sweeney, 1986) is normally used as δ in Eq. 3.

Intracellular pH (pH_i) is known to be affected by treatments such as Na^+ removal. As protons bind to ATP, the $K_D^{\text{"MgATP"}}$ in Eq. 3 can be expressed as a function of pH (London, 1991), based on the definition of "MgATP" (Mg^{2+}-binding ATP) and "ATP" (metal-free ATP) described by

Garfinkel and Garfinkel (1984):

$$K_D^{"MgATP"}(pH) = \frac{K_D^{MgATP}(1 + 10^{pK-pH})}{1 + (K_D^{MgATP}/K_D^{MgHATP})10^{pK-pH}}, \quad (4)$$

where K_D^{MgATP} and K_D^{MgHATP} are dissociation constants between Mg^{2+} and free ATP, and between Mg^{2+} and HATP, respectively, and pK is the negative logarithm of K_D^{HATP}. When $K_D^{MgHATP} \gg K_D^{MgATP}$, Eq. 4 can be rewritten, using an apparent dissociation constant of MgATP ($K_{Da}^{"MgATP"}$) measured at a specific pH (pH_a, Bock, Wenz, and Gupta, 1985):

$$K_D^{"MgATP"}(pH) = K_{Da}^{"MgATP"} \frac{1 + 10^{pK-pH}}{1 + 10^{pK-pHa}}. \quad (5)$$

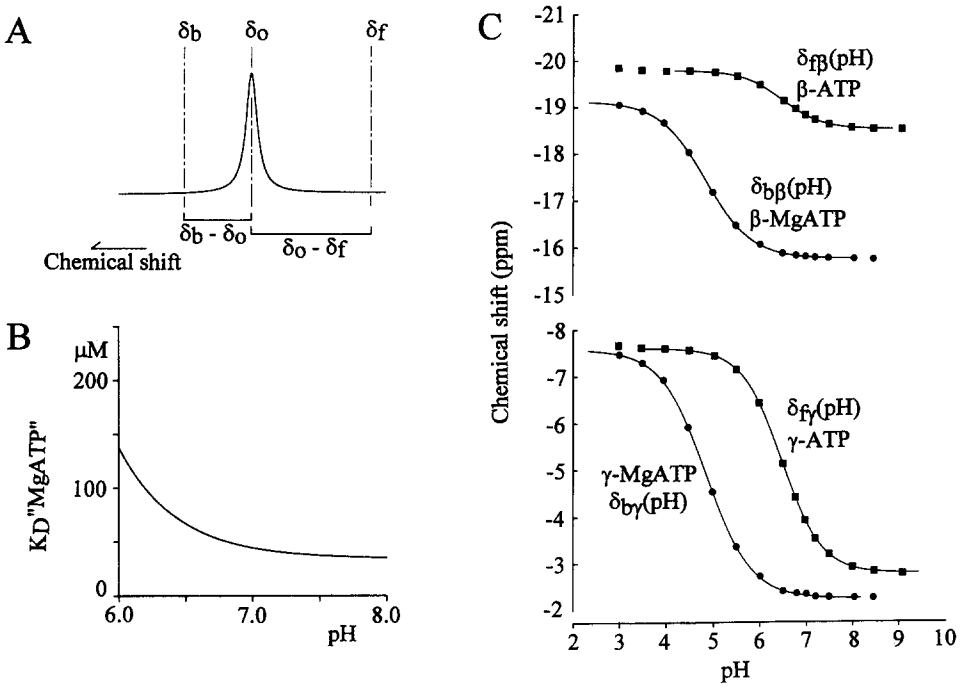

FIGURE 1. (*A*) The ATP ^{31}P NMR peak and terms for estimation of $[Mg^{2+}]_i$; (*B*) Effect of pH on the apparent dissociation constant of MgATP; (*C*) chemical shifts of metal-free (δ_f) and Mg^{2+}-binding ATP peaks (δ_b). The chemical shift of the ATP peak depends on the free to total ATP ratio (Φ) as: $\Phi = (\delta_o - \delta_b)/(\delta_f - \delta_b)$. The terms shown in *A* are for both analyses in the β- and γ-ATP peaks. In *B*, the solid curve is drawn based on Eq. 5. In *C*, the titration data for metal-free and Mg^{2+}-binding ATP are fitted by sigmoid curves (Eq. 6, e.g., $\delta_{f\beta}[pH]$). The fitting parameters are shown in Table I (abbreviations in text).

Eq. 5 was used in the present estimation of $[Mg^{2+}]_i$. The $K_{Da}^{"MgATP"}$ at 32°C (41 μM at pH 7.2) was estimated from the values at different temperatures, using the van't Hoff isochore (Nakayama and Tomita, 1990). In Fig. 1 *B*, the solid line was drawn based on Eq. 5 using the calculated $K_{Da}^{"MgATP"}$.

As shown in Fig. 1 *C*, the chemical shifts of free (δ_f) and Mg^{2+}-bound ATP (δ_b in Eq. 2 and 3) are expressed as sigmoidal functions of pH:

$$\delta_f(pH) = \frac{\delta_{f,p} - \delta_{f,d}}{1 + 10^{[HL(pK-pH)]}} + \delta_{f,d}, \tag{6}$$

where $\delta_{f,d}$ and $\delta_{f,p}$ are the chemical shifts of deprotonated and protonated forms of metal-free ATP, respectively, HL is the Hill coefficient, and pK is the negative logarithm of the dissociation constant between H^+ and ATP. $\delta_b(pH)$ is similarly expressed. The titration data (Fig. 1 *C*) were iteratively fitted using a modified 'simplex' program. The fitted results are shown in Table I. The mean residual differences were less than 0.01 p.p.m. As the effect of pH on the chemical shift was very small (Gadian, Radda, Richard, and Seeley, 1979; Nakayama and Tomita, 1991), the α-ATP peak was not used for this analysis. The pK value for (metal)-free ATP in Table I was applied as the pK in Eqs. 4 and 5. The composition of the model solutions was as follows (mM): KCl, 150; HEPES, 10; ATP, 5; PCr, 1. 1 mM-EDTA and 10 mM $MgCl_2$ were added in free ATP and Mg^{2+}-binding ATP solutions, respectively. The pH was altered by titrating with KOH.

TABLE I

Fitting Parameters for Fig. 1

	δ_d	δ_p	HL	pK
β-Peak, free-ATP (δ_f)	−18.59	−19.79	−1.00	6.48
MgATP (δ_b)	−15.79	−19.12	−0.90	4.84
γ-Peak, free-ATP (δ_f)	−2.78	−7.61	−1.01	6.48
MgATP (δ_b)	−2.26	−7.57	−0.92	4.86

Abbreviations are described in text.

Using the pH functions for the β-ATP peak [$\delta_{f\beta}(pH)$, $\delta_{b\beta}(pH)$], Eq. 3 is rewritten:

$$[Mg^{2+}] = K_D^{\text{"MgATP"}}(pH)\frac{\delta_{o\beta} - \delta_{f\beta}(pH)}{\delta_{b\beta}(pH) - \delta_{o\beta}} \tag{7}$$

$$= F_\beta(\beta, pH). \tag{7}'$$

In Eqs. 7 and 7′, $[Mg^{2+}]$ is recognized as a function of pH and the observed chemical shift of the β-peak. ($\delta_{o\beta}$; (β) in Eq. 7′ represents $\delta_{o\beta}$). Similarly, for γ-ATP peak, Eq. 3 is rewritten as a function of γ ($\delta_{o\gamma}$) and pH:

$$[Mg^{2+}] = K_D^{\text{"MgATP"}}(pH)\frac{\delta_{o\gamma} - \delta_{f\gamma}(pH)}{\delta_{b\gamma}(pH) - \delta_{o\gamma}} \tag{8}$$

$$= F_\gamma(\gamma, pH). \tag{8}'$$

When certain observed chemical shifts of β- and γ-ATP peaks measured from an intact tissue are incorporated into 7′ and 8′, a pair of simultaneous equations in $[Mg^{2+}]_i$ and pH_i is obtained. Solving the two pH_i-functions for $[Mg^{2+}]_i$, the values are obtained concomitantly. In Fig. 2, the values of $[Mg^{2+}]_i$ and pH_i at the crossing point correspond to the solution of 7′ and 8′. As $K_D^{\text{"MgATP"}}$ (pH_i) is common to 7′ and 8′, solving these equations is equivalent to the estimation of the free to total ATP ratio and pH_i. The mathematical meaning of our previous $[Mg^{2+}]_i$ estimation (Nakayama and Tomita, 1990, 1991) is identical to a specific solution of Eq. 7, assuming a pH_i of 7.2. In *A*, the mean values of chemical shifts of the β- and γ-peaks in

normal solution were used, and in *B*, the chemical shifts during Na^+ removal (experiment shown in Fig. 4 *b*).

When pH_i is very alkaline, the reliability for pH_i estimation from the β- and γ-ATP peaks is reduced, because the changes in the chemical shifts of free-(δ_f) and Mg^{2+}-binding forms (δ_b) of both the β- and γ-ATP peaks are very small. Information from other phosphorus peaks is important to assess the reliability of the analysis described above. Intracellular pH (pH_i) is most accurately estimated from the chemical shift of inorganic phosphate (P_i), if the peak is observable. Phosphomonoester-1 (PME-1, phosphoethanolamine, in Fig. 4) can also be used:

$$pH_i = pK_{a(X)} + \log_{10}\{[\delta_{o(X)} - \delta_{p(X)}]/[\delta_{d(X)} - \delta_{o(X)}]\}, \quad (9)$$

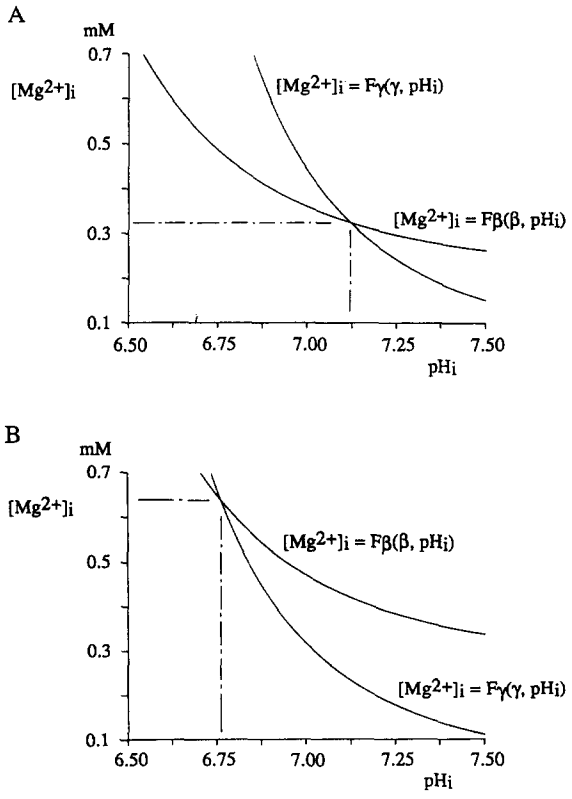

FIGURE 2. Estimation of $[Mg^{2+}]_i$ and pH_i. The two curves are based on pH_i functions for $[Mg^{2+}]_i$ obtained from the analyses for β- and γ-ATP peaks (Eq. 7′ and 8′, respectively). The crossing point corresponds to the solution of the functions. The dotted lines indicate the estimated values of $[Mg^{2+}]_i$ and pH_i. In *A*, the mean chemical shifts of β- (−16.16 p.p.m.) and γ-ATP peaks (−2.46 p.p.m.) in normal solution were used in the equations. In *B*, the chemical shifts observed in a Na^+-free solution (Fig. 4 *b*: β-, −16.09 p.p.m.; γ-peak, −2.51 p.p.m.) were used.

where $pK_{a(X)}$ is the negative logarithm of the dissociation constant in a phosphorous compound (X), and $\delta_{o(X)}$ is its observed chemical shift. The parameters for the titration curves previously described were used (P_i: $pK_a = 6.70$, $\delta_d = 5.72$, $\delta_p = 3.15$; PME-1: $pK_a = 5.70$, $\delta_d = 6.95$, $\delta_p = 3.27$; Nakayama and Tomita, 1990, 1991).

$[Mg^{2+}]_i$ can be also calculated from the chemical shift of β-ATP using Eq. 7 with the pH_i obtained from Eq. 9 (London, 1991). Since the chemical shift of P_i provides high resolution of pH_i, $[Mg^{2+}]_i$ could be more accurately estimated from this calculation. However, the P_i peak was often undetectable in Na^+-free solution. We thus used the concomitant estimation of $[Mg^{2+}]_i$ and pH_i from the β- and γ-ATP peaks in the present study. Also, if a part of the tissue (or organ) is not well perfused in the sample tube, the ischemic part, which may not have the same

pH_i as the rest of the tissue, would have a large contribution to the observed P_i peak. The present method could be used to check whether such a part exists in a preparation.

The numerical data were expressed as means ± standard deviation (SD). The significance of differences were evaluated by paired or unpaired *t* tests, and a probability of less than 0.05 was taken as statistically significant.

RESULTS

Concomitant Estimation of $[Mg^{2+}]_i$ and pH_i

^{31}P NMR spectra were obtained from muscle strips of taenia caecum. In the spectra observed after the sample was superfused with normal solution for 100 min, the proportion of peak integrals and the chemical shifts of the peaks (shown in Fig. 4A (a)) were essentially the same as those previously observed (Nakayama and Tomita, 1990, 1991). $[Mg^{2+}]_i$ can be estimated from the chemical shifts of ATP (Gupta et al., 1984). Since pH_i considerably decreases during prolonged exposures of Na^+-free

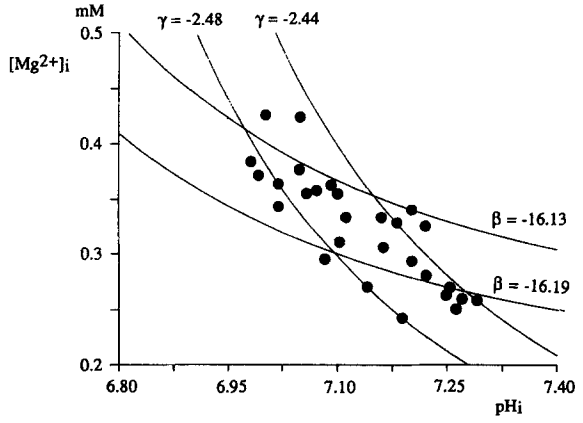

FIGURE 3. Distribution of $[Mg^{2+}]_i$ and pH_i in normal solution. Each filled circle corresponds to a pair of solutions obtained from measured chemical shifts of β- and γ-ATP peaks (n = 30). The curves are obtained using the means ± SD for the chemical shifts of β- and γ-peaks into Eqs. 7′ and 8′.

solutions, some correcting methods are necessary for accurate estimation of $[Mg^{2+}]_i$ (Bock et al., 1985; London, 1991). In the present study P_i peak, which is generally used to determine pH_i, was often unresolvable under these conditions. Thus, $[Mg^{2+}]_i$ and pH_i were concomitantly estimated from the chemical shifts of the β- and γ-ATP peaks (Eqs. 7 and 8, and Fig. 2).

In normal solution, the observed β- and γ-peaks were at −16.16 ± 0.03 and −2.46 ± 0.02 p.p.m., respectively (n = 30). The distribution of the estimated $[Mg^{2+}]_i$ and pH_i is shown in Fig. 3. The curves were drawn by inserting the means + or − the deviations (SD) for the β- and γ-ATP peaks into Eqs. 7 and 8. $[Mg^{2+}]_i$ values varied from 0.24 to 0.43 mM, while pH_is ranged from 6.98 to 7.29. The means were 0.33 ± 0.05 mM for $[Mg^{2+}]_i$ and 7.13 ± 0.09 for pH_i. The free ATP ratio was estimated to be 11.6 ± 1.3% (Eq. 1).

As the reliabilities on estimations of $[Mg^{2+}]_i$ and pH_i were dependent on the resolution of the β- and γ-ATP peaks in each sample, comparison with those estimated from other peaks was an important consideration. The observed chemical

shift of the α-ATP peak was −7.53 ± 0.02 p.p.m. in the spectra used for the above analysis. The $[Mg^{2+}]_i$ (0.33 ± 0.04 mM) calculated from the separation between α- and β-peaks ($\delta_{\alpha\beta}$), using the method applied previously (general method: Gupta et al., 1984) was consistent with that estimated from the chemical shifts of β- and γ-ATP peaks. In normal solution, the chemical shift of P_i was resolvable in the majority of the samples (n = 26 out of 30 samples, four peaks not resolvable). The pH_i estimated from the chemical shift (4.99 ± 0.06 p.p.m.) was 7.10 ± 0.05. This value also agreed well with that of the concomitant estimation. In the previous calculation of $[Mg^{2+}]_i$, pH_i was assumed to be 7.2 (Nakayama and Tomita, 1990, 1991). When the apparent dissociation constant of MgATP was corrected using the pH_i from the chemical shift of P_i, the $[Mg^{2+}]_i$ from $\delta_{\alpha\beta}$ increased by less than 0.02 mM.

Effects of Na^+ Removal and Readmission

A hypothesis that Na^+-Mg^{2+} exchange may regulate $[Mg^{2+}]_i$ under physiological conditions was examined in the following experiments. Fig. 4 shows an example of the effects of Na^+ removal and readmission on ^{31}P spectra. The phosphomonoesters (PME), P_i, γ- and β-ATP peaks are appropriately expanded in 4B. External Na^+ was completely replaced by NMDG, after observing the spectrum in normal solution (2.4 mM Ca^{2+}, 1.2 mM Mg^{2+}, [*a*]). A small shift to higher p.p.m. (leftward) of the β-ATP peak (−16.09 ± 0.01 p.p.m.) was observed after 150 min, whereas the γ-peak was shifted to lower p.p.m. (−2.50 ± 0.02 p.p.m., n = 5; [*b*]). $[Mg^{2+}]_i$ slowly increased while pH_i decreased, and reached 0.65 ± 0.06 mM and 6.77 ± 0.06 pH units, respectively, after 150 min (the concomitant estimation: Fig. 5). The free ATP ratio also decreased from 11.1 ± 0.8 to 7.4 ± 0.5% (Fig. 5 *D*).

Readmission of Na^+ resulted in full recovery of $[Mg^{2+}]_i$ (0.36 ± 0.03 mM) in 25 min, and $[Mg^{2+}]_i$ was stable for the rest of the observation over 75 min (Fig. 5). The recovery of the free ATP ratio was slower than $[Mg^{2+}]_i$. The free ATP ratios were 10.1 ± 0.5% after 25 min and 10.8 ± 0.8% after 100 min. On the other hand, pH_i was transiently increased to 7.23 ± 0.08 in the first spectrum after readmission of Na^+, and returned to the control level (7.11 ± 0.06) after 100 min. The transient intracellular alkalinization produced the small separation between recovery of $[Mg^{2+}]_i$ and the free ATP ratio. The changes in pH_i induced by removal and readmission of Na^+ (slow acidification and rapid alkalinization, respectively) agreed well with the measurements of pH_i using ion-sensitive microelectrodes in guinea pig ureter (Aickin, 1988).

During the exposure to Na^+-free solution, the intensity of the P_i peak decreased and pH_i was difficult to estimate from its chemical shift. In some spectra, a small peak was observed at lower p.p.m. values (rightward) of the initial chemical shift of P_i, indicating intracellular acidification. The chemical shift (4.67 p.p.m.) of the peak indicated by the arrow in Fig. 4 *B* (*b*) corresponded to a pH_i fall from 7.10 to 6.86, assuming that the peak arose from P_i. A small increase in the concentration of PCr ([PCr]) corresponding to the decrease in $[P_i]$ was observed (Table II). The PME-1 peak, which was considered to be mainly due to phosphoethanolamine (Kushmerick et al., 1986; Dawson and Wray, 1985; Nakayama and Tomita, 1991), shifted from 6.79 ± 0.01 to 6.70 ± 0.03 p.p.m. (n = 5), corresponding to a decrease in pH_i from 7.05 ± 0.03 to 6.85 ± 0.07. The PME-1 peak was transiently shifted to higher p.p.m.

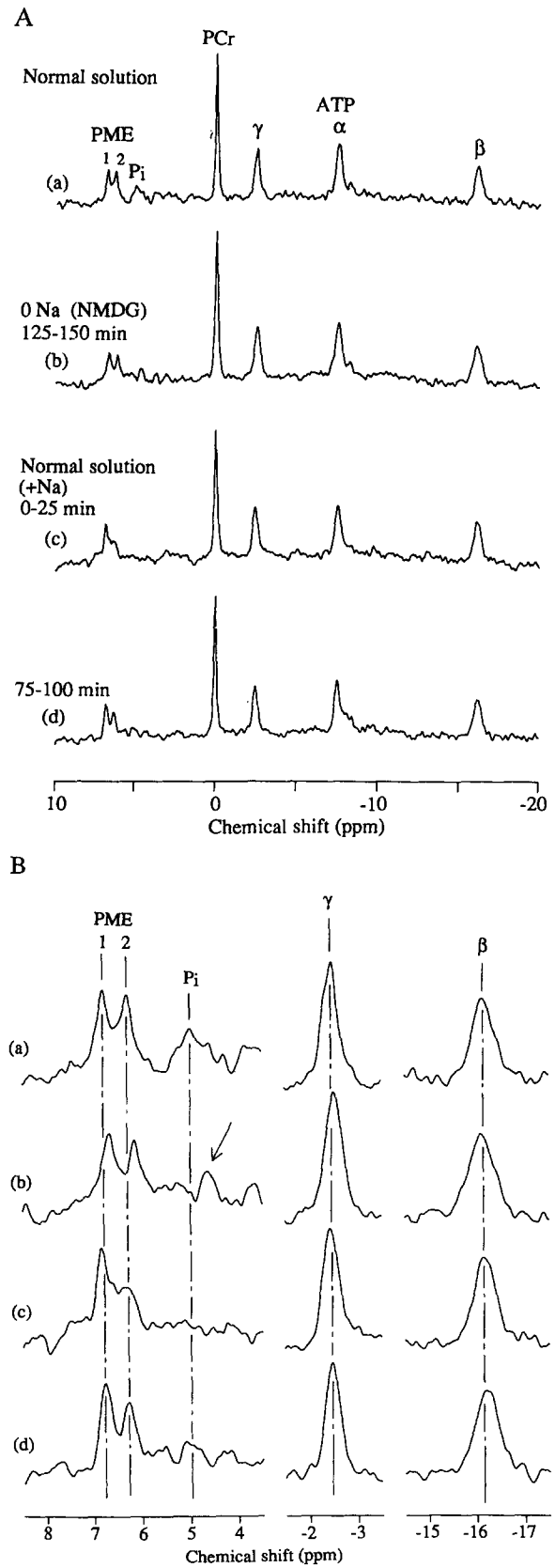

FIGURE 4. Example of effects of Na^+-removal on ^{31}P NMR spectra. Spectral peaks were assigned as PME, P_i, PCr and three ATP peaks. PME was divided into two peaks resonating around 6.8 (1) and 6.3 (2) p.p.m. (*A*) After acquiring NMR signals in normal solution (*a*), extracellular Na^+ was replaced by equimolar NMDG for 150 min (*b*). The spectra (*c* and *d*) were recorded 0–25 and 75–100 min after readmission of Na^+, respectively. Each spectrum was obtained by accumulation of 2,500 signals (25 min). In *B*, the PME, P_i, and γ- and β-ATP peaks are shown expanded. Vertical lines indicate the initial values of each peak (*a*) in this and the following figures. See text for explanation of the arrow (*B* [*b*]).

(leftward) by 0.15 U during readmission of Na^+ after 0–25 min (Fig. 4 *B*, [*c*]). The chemical shift implied a pH_i of 7.27 ± 0.04. The pH_i estimated from the PME-1 peak then slowly recovered to nearly the control value (7.08 ± 0.05). The time course of changes in pH_i during the removal and readmission of Na^+ was consistent with that estimated from the chemical shifts of the ATP peaks (Fig. 5). The PME-2 peak was also similarly shifted to lower p.p.m. during exposure to Na^+-free solution, and transiently to higher p.p.m. by readmission of Na^+. These estimations of pH_i supported the suitability of the estimation of $[Mg^{2+}]_i$ from the β- and γ-ATP peaks.

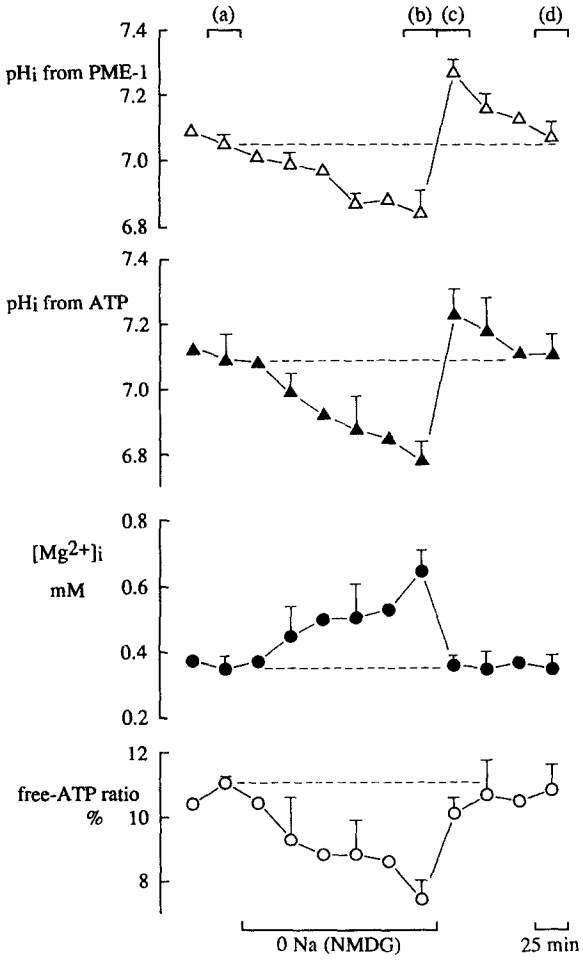

FIGURE 5. Time courses of changes in pH_i, $[Mg^{2+}]_i$ and free ATP ratio during removal and readmission of Na^+. In *A*, the pH_i (*open triangles*) was calculated from the chemical shift of PME-1. The pH_i (*B, filled triangles*) and $[Mg^{2+}]_i$ (*C, filled circles*) were estimated concomitantly from the chemical shifts of β- and γ-ATP peaks by solving Eqs. 7 and 8. The free-ATP ratio (*D, open circle*) was also estimated from $[Mg^{2+}]_i$ using Eq. 1. (*a–d*) correspond to those in Fig. 4. Vertical bars, SD (n = 5).

After Na^+ concentration was returned to normal, $[P_i]$ was further decreased and undetectable in the first spectrum Fig. 4 *c*, it then partially recovered after 100 min Fig. 4 (*d*). A spectrum obtained after 0–25 min readmission of Na^+ showed a small split peak ~5.2 p.p.m., agreeing with the transient intracellular alkalosis. On the other hand, [PCr] decreased slightly below the initial concentration during 100 min Na^+ readmission (Table II). [ATP] was practically stable throughout the removal and

readmission of Na^+, except for a 15% decrease observed in one experiment. The tendency for a decrease in the concentration of phosphorous compounds was smaller than previously observed in Na^+- (K substitution) or K^+-free solution in the absence of Ca^{2+} (Nakayama and Tomita, 1991). ATP is known as an important intracellular Mg^{2+}-buffer (Palatý, 1971). An increase in $[Mg^{2+}]_i$ accompanied by decrease in [ATP] has been reported in inotropically stimulated rat heart (Headrick and Willis, 1989). The effect of Mg^{2+} release from ATP on increase in $[Mg^{2+}]_i$, however, seemed to be negligible in the present experiments under Na^+-free conditions.

Effects of Changing the External Mg^{2+} Concentration

The extracellular Mg^{2+} concentration ($[Mg^{2+}]_o$) was changed to clarify the origin of the increase in $[Mg^{2+}]_i$ during exposure to Na^+-free solution. In four experiments, 12 mM Mg^{2+} was added to the perfusate 50 min before removal of Na^+ (substituted by NMDG). The $[Mg^{2+}]_i$ and pH_i concomitantly estimated from the γ- and β-ATP peaks were 0.35 ± 0.06 mM and 7.07 ± 0.06, respectively, during superfusion with normal solution, and 0.37 ± 0.05 mM and 7.11 ± 0.07 ($n = 4$), respectively, after increasing $[Mg^{2+}]_o$ for 50 min (open symbols in Fig. 6 [*a*]). The small intracellular

TABLE II

Relative Concentration of Phosphorous Compounds ($n = 5$)

	ATP	PCr	P_i
Normal solution (control)	1.00	1.66 ± 0.20	0.57 ± 0.16
0 Na^+ (NMDG) 125–150 min	1.05 ± 0.05	1.95 ± 0.18	—
Normal solution (+Na^+)			
0–25 min	1.03 ± 0.09	1.69 ± 0.20	—
75–100 min	0.95 ± 0.07	1.54 ± 0.11	0.44 ± 0.06

The concentrations are expressed relative to the unitary [ATP] value ascribed to the initial normal solution (Fig. 4*a*).

alkalinization was consistent with the changes in pH_i estimated from the PME-1 peak (7.03 ± 0.02 to 7.09 ± 0.02) and with the previous observation in excess Mg^{2+} solution (Nakayama and Tomita, 1991), but the deviations were larger in the present estimation. On the other hand, the estimation of $[Mg^{2+}]_i$ was qualitatively similar to the previous method (i.e., stable $[Mg^{2+}]_i$ in excess Mg^{2+} solution and similar SD values).

When Na^+ was removed in the presence of 12 mM Mg^{2+}, the $[Mg^{2+}]_i$ was increased to 1.19 ± 0.39 mM and the pH_i decreased to 6.69 ± 0.05 after 150 min (Fig. 6 [*b*]). The increase in $[Mg^{2+}]_i$ was significantly larger than in the presence of 1.2 mM Mg^{2+}, accompanied by a greater shift to higher p.p.m. of the β-ATP peak (−16.01 ± 0.04 p.p.m.). The pH_i increased to 7.18 ± 0.08 after 0–25 min after readmission of Na^+ (Fig. 6 [*c*]), then returned to the initial control level observed in the presence of excess Mg^{2+} (Fig. 6 [*d*]). The $[Mg^{2+}]_i$ was drastically decreased by readmission of Na^+, but did not fully recover (0.46 ± 0.08 mM) after 100 min.

The time course of pH_i shown in Fig. 6 (*open triangle*) was consistent with that estimated from the chemical shifts of the PME peaks (see also Fig. 8). The values of pH_i estimated from the PME-1 peak were 6.82 ± 0.03 125–150 min after Na^+

removal, and 7.18 ± 0.08 0–25 min after readmission of Na^+. In the presence of 12 mM Mg^{2+}, no significant increase in [PCr] was observed during the prolonged removal of Na^+. The [ATP] was stable with a maximum of 5% deviation from the mean throughout. In the presence of 1.2 mM Mg^{2+}, removal of external Na^+ increased [PCr] by 17% (Table II). This was probably due to suppression of the spontaneous tension development (tension recording performed separately is not shown). On the other hand, in high-Mg^{2+} solution, spontaneous contraction had ceased before Na^+ removal.

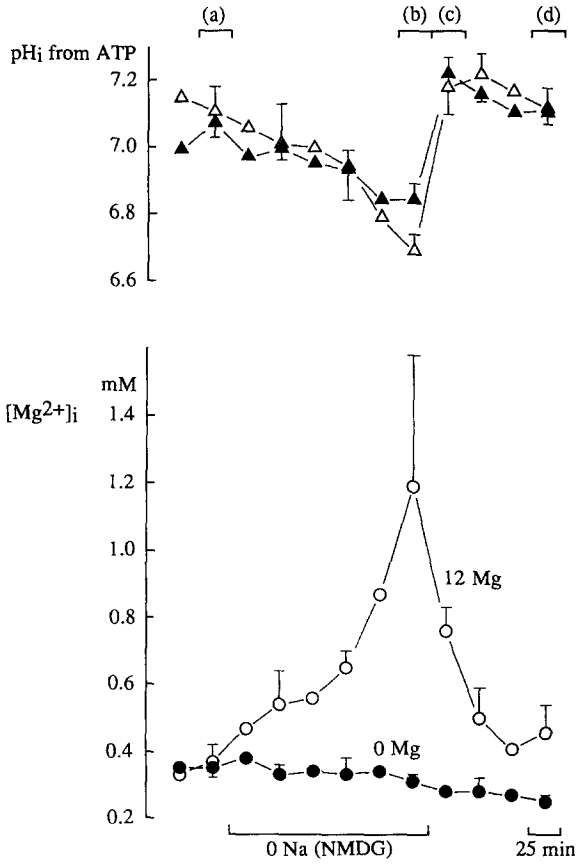

FIGURE 6. Changes in pH_i (*triangles*) and $[Mg^{2+}]_i$ (*circles*) during removal and readmission of Na^+ in excess Mg^{2+} (12 mM, *open*) and Mg^{2+}-free solutions (*filled*) (n = 4, respectively). (*a–d*) see text. Vertical bars represent SD values.

The effects of Na^+ removal and readmission were also examined in the absence of extracellular Mg^{2+} (with 0.1 mM EDTA) (Fig. 6, *filled data*). The estimated $[Mg^{2+}]_i$ from ATP peaks was stable (0.35 ± 0.03 mM, n = 4) 50 min after Mg^{2+}-free solution was applied, while the pH_i decreased by 0.07 U. The decrease in pH_i agreed with the previous measurements (Nakayama and Tomita, 1990), and presumably related to the corresponding increase in spontaneous activity. In contrast to the effects of Na^+ removal in the presence of extracellular Mg^{2+}, the $[Mg^{2+}]_i$ very slowly decreased throughout the experiments in Mg^{2+}-free solution, irrespective of modification of

external Na^+ concentration (0.31 ± 0.02 mM, 150 min after Na^+-removal; 0.25 ± 0.02 mM, 100 min after Na^+ readmission). The changes in pH_i due to the removal and readmission of Na^+ were similar to those in the presence of Mg^{2+}, a finding also supported by the changes in chemical shift of the PME-1 peak (see also Fig. 8).

In the same experiments performed in the absence of Mg^{2+}, the P_i peak was decreased by removal of Na^+. Conversely, [PCr] was significantly increased by ~30% after 150 min (from 1.50 ± 0.21 [*a*] to 1.92 ± 0.09 [*b*]). The average [ATP] varied within a range of 8% throughout (1.07 ± 0.03, 150 min after Na^+ removal [*b*]; 0.92 ± 0.06, 100 min after Na^+ readmission [*e*]).

Effects of Extracellular Ca^{2+} on $[Mg^{2+}]_i$

The extracellular Ca^{2+} concentration was changed in order to investigate the role of Ca^{2+} on the regulation of $[Mg^{2+}]_i$ during exposure to Na^+-free solution. In two experiments, 12 mM Ca^{2+} was concomitantly added in Na^+-free solution. Fig. 7

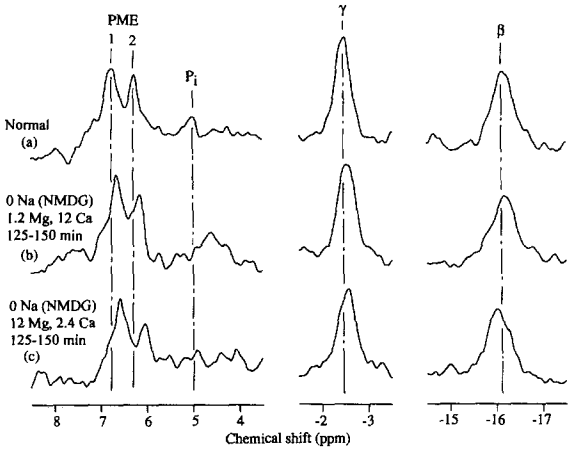

FIGURE 7. Effects of increasing extracellular Ca^{2+} concentration on the chemical shifts of phosphorous compounds (PMEs, P_i, and γ- and β-ATP). After observing the peaks in normal solution (*a*), Na^+ was completely replaced by NMDG and Ca^{2+} was simultaneously increased to 12 mM for 150 min (*b*). The Ca^{2+} concentration was then returned to normal (2.4 mM) and Mg^{2+} was simultaneously increased to 12 mM (*c*) with no Na^+.

shows an example of the effects of increasing the Ca^{2+} concentration on the spectral peaks. The chemical shifts of the γ- and β-ATP peaks were −2.53 and −16.16 p.p.m., respectively, 150 min after removal of Na^+ (Fig. 7 [*b*]), corresponding to an intracellular acidosis (pH_i 6.85) and marginal increase in $[Mg^{2+}]_i$ (0.43 mM). During the following exposure to Na^+-free solution, the extracellular Ca^{2+} concentration was returned to normal (2.4 mM) and $[Mg^{2+}]_o$ was increased to 12 mM. The β-ATP peak was shifted to higher p.p.m. (to −16.04 p.p.m.), corresponding to a large increase in $[Mg^{2+}]_i$ (1.48 mM). The pH_i estimated from the ATP peaks was further decreased to 6.50 by the modification of extracellular divalent cations. The chemical shifts of the PME peaks (Fig. 7 *b* and *c*) support continuous acidification in Na^+-free solution. In another experiment similar results were obtained.

The effects of removing Na^+ were also examined in the absence of extracellular Ca^{2+} (with 0.1 mM EGTA). The average $[Mg^{2+}]_i$ (n = 2) increased from 0.32 to 1.16 mM when Na^+ was removed from the Ca^{2+}-free solution for 100 min.

DISCUSSION

$[Mg^{2+}]_i$ can be calculated from the chemical shifts of ATP in ^{31}P NMR spectrum (Gupta et al., 1984). Since the pH_i, tentatively calculated from the chemical shift PME-1, sizeably decreased during the exposures to Na^+-free solution, analysis taking pH_i into consideration was necessary (Bock et al., 1985; London, 1991). If accurate pH_i were always estimated, $[Mg^{2+}]_i$ could have been calculated by correcting the effects of pH_i on the apparent dissociation constant of ATP (Bock et al., 1985), and the chemical shifts of free- and Mg^{2+}-binding ATP peaks (London, 1991). In the present study, however, the P_i peak which is generally used for pH_i estimation in ^{31}P NMR spectra (Moon and Richards, 1973) was often below the noise level in Na^+-free solution. Furthermore, in some smooth muscles lacking spontaneous activity, the P_i peak has been reported to be undetectable even in normal solution (Spurway and Wray, 1987; Hellstrand and Vogel, 1985). We thus used a method which concomitantly estimates $[Mg^{2+}]_i$ and pH_i using the difference of proton- and Mg^{2+}-binding effects on chemical shifts of β- and γ-ATP peaks. The analysis corresponds to extracting the information on pH_i as well as $[Mg^{2+}]_i$ involved in ATP peaks.

Under superfusion with normal solution, $[Mg^{2+}]_i$ and pH_i estimated from the chemical shifts of ATP peaks agreed well with the values previously obtained from the same tissue (Nakayama and Tomita, 1991) using conventional methods (pH_i: Moon and Richards, 1973; $[Mg^{2+}]_i$: Gupta et al., 1984), although the SD for pH_i estimated in the present study was larger. In the figure showing the distribution of $[Mg^{2+}]_i$ and pH_i (Fig. 3), a tendency for $[Mg^{2+}]_i$ to increase as pH_i decreased ($R = -0.84$) was seen. This might reflect the effect of protonation of intracellular Mg^{2+}-binding sites. However, the increase in $[Mg^{2+}]_i$ in Na^+-free solution was not fully explained by extrapolating the regression line based on this assumption. The correlation between $[Mg^{2+}]_i$ and the free ATP ratio, which are independent of $K_D^{\text{"MgATP"}}$ (pH), was weaker ($R = 0.75$).

When Na^+ was removed from the normal solution, $[Mg^{2+}]_i$ increased and intracellular acidosis occurred in the taenia. The increase in $[Mg^{2+}]_i$ supports the presence of Na^+-Mg^{2+} exchange, which has been suggested from experiments under Ca^{2+}-free conditions (Nakayama and Tomita, 1991). The acidosis can be attributed to the inhibition of Na^+-H^+ exchange and Na^+-HCO_3^- co-transport, both of which have been proposed in smooth muscle (Aickin, 1988). On the other hand, the transient alkalinization upon Na^+ readmission could be due to excess facilitation of these proton-extruding mechanisms.

In various kinds of smooth muscle, the presence of Na^+-Ca^{2+} exchange has been suggested (Blaustein, 1989). In the guinea pig ureter Na^+ extrusion accompanied by Ca^{2+} influx has been confirmed in the absence of a functional Na^+ pump (Aickin, 1987; Aickin, Brading, and Walmsley, 1987), and in the guinea-pig taenia caeci, some contributions of Na^+-Ca^{2+} exchange to Ca^{2+} movement have been suggested under limited conditions (Katase and Tomita, 1972; Brading, 1978). In cultured cardiac cells, (Murphy, Wheeler, LeFurgey, Jacob, Lobaugh, and Lieberman, 1986; Murphy, Freudenrich, Levy, London, and Lieberman, 1989) it has been reported that Na^+ removal increased intracellular Ca^{2+} through Na^+-Ca^{2+} exchange and that the $[Mg^{2+}]_i$ was secondarily increased by displacement by Ca^{2+} at intracellular, divalent

cation binding sites. We have thus designed the present experiments to clarify the source of Mg^{2+} which increases $[Mg^{2+}]_i$ during exposure of Na^+-free solutions in the guinea-pig taenia caeci.

When Na^+ was removed from a medium containing 12 mM Mg^{2+}, the increase in $[Mg^{2+}]_i$ was significantly enhanced, whereas, $[Mg^{2+}]_i$ decreased during Na^+ removal in the absence of extracellular Mg^{2+} despite similar intracellular acidification being observed (Fig. 6). These results suggest that the influx of Mg^{2+} from the external solution is the main source for the increase in $[Mg^{2+}]_i$ and that $[Mg^{2+}]_i$ is maintained by continuous operation of Na^+-Mg^{2+} exchange even in the presence of extracellular Ca^{2+}. However, a small increase in $[Mg^{2+}]_i$ was observed 25 min after the removal of Na^+ in the absence of extracellular Mg^{2+}. This might involve the competitive effects between Mg^{2+} and Ca^{2+} at the intracellular binding sites reported in cultured cardiac cells (Murphy et al., 1986, 1989).

Changes in chemical shifts of the ATP peaks by Ca^{2+} binding are known to be similar to the effects of Mg^{2+} binding (Nageswara Rao, 1984). Generally, the effects of Ca^{2+} binding to ATP are considered to be negligible in the estimation of $[Mg^{2+}]_i$ using ^{31}P NMR, because the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is much lower than $[Mg^{2+}]_i$ (Gupta and Moore, 1980). In the guinea pig taenia, however, neither the degree nor the time course of the increase in $[Ca^{2+}]_i$ are well known in Na^+-free solution, although an increase is expected through Na^+-Ca^{2+} exchange. We thus elevated the extracellular Ca^{2+} concentration to 12 mM in order to evaluate the effects of Ca^{2+} binding on the chemical shifts of ATP during Na^+ removal (Fig. 7). In the presence of excess Ca^{2+}, however, only a marginal increase in $[Mg^{2+}]_i$ was seen after 150 min in Na^+-free solution compared to that in the presence of normal Ca^{2+} concentration (2.4 mM), although a similar degree of intracellular acidosis was observed. Furthermore, when the solution was subsequently changed to excess Mg^{2+} and normal Ca^{2+} medium at the same time, the β-ATP peak was significantly shifted to higher p.p.m. (leftward). This confirmed the dominant binding effects of intracellular Mg^{2+} and the negligible effects of Ca^{2+}. The results obtained in high-Ca^{2+} solution also agreed well with the inhibitory effects of Ca^{2+} on Mg^{2+} transport reported by Palatý (1971, 1974).

At every concentration of extracellular Mg^{2+}, the pH_i was decreased by Na^+ removal, irrespective of changes in $[Mg^{2+}]_i$. Therefore, the increases in $[Mg^{2+}]_i$ estimated from the β- and γ-ATP peaks are not considered to be due to a systematic analytical error. The decreases in pH_i deduced from the chemical shifts of the PME-1 peak were, however, smaller than that from the ATP peaks in the presence of 1.2 and 12 mM Mg^{2+} (Fig. 8). In all data (shown in Figs. 5 and 6), the acidification estimated from PME-1 was ~25% smaller (Fig. 8, *solid line*). This discrepancy was less in the absence of extracellular Mg^{2+} (*dotted line*). In measurements of model solutions, the pK_a value of PME-1 (phosphoethanolamine) was decreased by addition of Mg^{2+} (S. Nakayama, unpublished observation). In the presence of Mg^{2+}, the $[Mg^{2+}]_i$ significantly increased in Na^+-free solution, whereas it only very slowly decreased in the absence of Mg^{2+}. This discrepancy between the fall in pH_i values estimated by these two methods can, in part, be explained by effects of increasing $[Mg^{2+}]_i$ on the pK_a value of PME-1.

When Na^+ was substituted by NMDG after superfusing with normal solution, spontaneous tension development was transiently increased but subsequently decreased, and disappeared on prolonged removal. Readmission of Na^+ restored tension development after a short delay (data not shown). Similar observations have been reported with other Na^+ substitutes (choline: Holman, 1957; Li^+: Axelsson, 1961; sucrose: Brading, Burnett, and Sneddon, 1980). On the other hand, in NMR measurements [P_i] decreased, accompanied by an increase in [PCr] in Na^+-free solution without large changes in [ATP] (Table II), suggesting that the tension

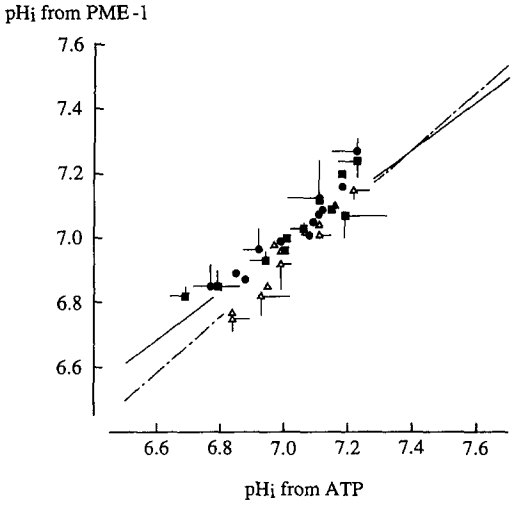

FIGURE 8. Correlation of estimated pH_is from ATP and from PME-1 peaks. Filled circles and squares correspond to the values in normal (2.4 mM) and excess Mg^{2+} (12 mM), respectively, and open triangles are for Mg^{2+}-free solution. Each point represents the mean pH_i values estimated from the series of spectra successively obtained by 25 min accumulation. The vertical and horizontal bars are standard deviations for the pH_i values estimated from the PME-1 and ATP peaks, respectively. The solid line is a regression line for all data (Y = 1.837 + 0.735X) and the dotted line for Mg^{2+}-free solution (Y = 0.824 + 0.873X). The correlation coefficients are 0.83 and 0.87, respectively. Regression line ($Y = b + aX$) and correlation coefficient (R) were obtained as follows:

$$a = \frac{\Sigma(X_i - X_{mean})(Y_i - Y_{mean})}{\Sigma(X_i - X_{mean})^2}$$

$$= R\, SD_Y/SD_X,$$

$$b = Y_{mean} - aX_{mean}$$

where X_{mean} and SD_X are the mean and standard deviation of X data, respectively.

development was not impaired by metabolic inhibition. Conversely, the changes in the concentrations of phosphorous compounds may be secondary to the reduction of tension development. The preservation of high energy phosphates and the reduction of the tension implies that intracellular Ca^{2+} can be kept low by mechanisms other than Na^+-Ca^{2+} exchange (Raemaekers, Wuytack, and Casteels, 1974) in the Na^+-free solution (NMDG substitution): presumably by the ATP-driven Ca^{2+} pump in the guinea pig taenia caeci (Casteels and van Breemen, 1975; Aaronson and van Breemen, 1981). Thus, it is suggested that under the Na^+-free conditions estimation of $[Mg^{2+}]_i$ was little perturbed by Ca^{2+}-binding to ATP.

In conclusion, the physiological importance of Na^+-Mg^{2+} exchange for Mg^{2+} extrusion is shown by the changes in $[Mg^{2+}]_i$ during removal and readmission of Na^+ even in the presence of Ca^{2+}. Since changes in $[Mg^{2+}]_i$ were not correlated with high energy phosphate levels, the energy for Mg^{2+} extrusion seems to be supplied mainly from Na^+ gradient, although it is likely that other regulating factors exist (DiPolo and Beaugé, 1988; Flatman and Smith, 1990).

We would like to thank Professor Sadayuki Sakuma and Dr. Kengo Itoh, Nagoya University, for their pertinent help and advice, and we are further grateful to Drs. Andrew J. Cook, Joseph F. Clark, Claire C. Aickin, Melissa Bridgewater, and Alison F. Brading, Oxford University, for their useful comments and critical reading of the manuscript.

Original version received 18 September 1992 and accepted version received 13 December 1993.

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