Mechanisms of intracellular Mg^{2+} regulation affected by amiloride and ouabain in the guinea-pig taenia caeci

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1. The effects of amiloride and ouabain on the regulation of the intracellular, free Mg^{2+} concentration ($[Mg^{2+}]_i$) were investigated in the taenia isolated from the guinea-pig caecum, using nuclear magnetic resonance (NMR) techniques.

2. $[Mg^{2+}]_i$ were mainly estimated from the separation of the α- and β-ATP peaks observed in ^{31}P NMR spectra. In normal (physiological) and nominally Ca^{2+}-free solutions, $[Mg^{2+}]_i$ was approximately 0·3–0·4 mM. Application of either amiloride or ouabain in Ca^{2+}-free solutions significantly increased $[Mg^{2+}]_i$, with only a small change in ATP content. Wash-out of the drugs reversed the changes in $[Mg^{2+}]_i$.

3. Changes in pH_i were estimated from: (1) the chemical shift of phosphoethanolamine, and (2) solving two relational equations of pH_i and $[Mg^{2+}]_i$ obtained from the β- and γ-ATP peaks. Both estimations revealed some intracellular alkalosis during application of these two drugs. After correction for pH_i, a significant increase in $[Mg^{2+}]_i$ was still obtained 150 min after application of either drug.

4. In the presence of amiloride, simultaneous removal of extracellular Mg^{2+} and Ca^{2+} significantly depleted intracellular Mg^{2+}. This result suggests the presence of an amiloride-insensitive (or less sensitive) pathway which passively transports Mg^{2+} across the plasma membrane.

5. The intracellular Rb^+ concentration was monitored as an index of Na^+–K^+ pump activity, using ^{87}Rb NMR. In Ca^{2+}-free solutions containing 5 mM Rb^+, the intracellular Rb^+ concentration was hardly changed by amiloride, but was depleted by additional applications of ouabain. Wash-out of ouabain restored the intracellular Rb^+ in the presence of amiloride.

6. These results are consistent with the presence of Na^+–Mg^{2+} exchange as an effective Mg^{2+}-extruding mechanism in smooth muscle. Although many other factors may cause changes in $[Mg^{2+}]_i$, it seems likely that amiloride directly inhibits the Na^+–Mg^{2+} exchanger, whilst ouabain does so indirectly through reduction of the Na^+ gradient across the plasma membrane.

Na^+ transporters are known to co-operate in regulating the intracellular ionic composition. In smooth muscle, there are several established mechanisms for transmembrane Na^+ movement (e.g. Na^+–K^+ pump: Widdicombe, 1981; Na^+–Ca^{2+} exchange: Blaustein, 1989; Na^+–H^+ exchange and Na^+–HCO_3^- cotransport: Aickin, 1988 and Wray, 1988; Na^+–K^+–Cl^- cotransport: Aickin & Brading, 1990), including Na^+ channels (Sturek & Hermsmeyer, 1986).

Amiloride and its derivatives inhibit various Na^+ transporters. Some of the derivatives specifically block certain transporters (e.g. dimethylbenzamil for Na^+–Ca^{2+} exchange). However, amiloride itself affects numerous Na^+ transporters in a similar concentration range (Benos, 1988). For instance, in the isolated rat heart it has been reported that 0·1 mM amiloride prevents contractile dysfunction after reoxygenation, maybe as a result of combined inhibition of Na^+–H^+ and Na^+–Ca^{2+} exchange (Weiss, Lakatta & Gerstenblith, 1990). In the smooth muscle of the guinea-pig taenia caeci, we have recently shown the presence of Na^+–Mg^{2+} exchange which may be able to maintain a low internal magnesium concentration ($[Mg^{2+}]_i$) using the energy from the Na^+ gradient across the plasma membrane (Nakayama & Tomita, 1991). From the non-specific block by amiloride of Na^+ transporters, it is tempting to speculate that this drug may also inhibit Na^+–Mg^{2+} exchange and increase $[Mg^{2+}]_i$. Indeed, in red blood cells, amiloride has been reported to inhibit Na^+-dependent Mg^{2+} efflux (Lüdi & Schatzmann, 1987; Flatman & Smith, 1990). On the other hand, if Mg^{2+} is extruded by a Na^+–Mg^{2+} exchange system using the Na^+

gradient, Na^+–K^+ pump inhibitors would also increase $[Mg^{2+}]_i$. However, such changes in $[Mg^{2+}]_i$ have not yet been demonstrated.

In the present study, we examined the effects of amiloride and ouabain – representative inhibitory drugs for Na^+ transporters and Na^+–K^+ pumps, respectively – on the regulation of $[Mg^{2+}]_i$ in the guinea-pig taenia caeci. $[Mg^{2+}]_i$ was estimated from the chemical shifts of the ATP peaks in ^{31}P NMR spectra. The effects of pH_i on $[Mg^{2+}]_i$ were also assessed. The intracellular Rb^+ concentration (^{87}Rb NMR) was monitored as an index of Na^+ pump activity.

METHODS

Preparation

Guinea-pigs (300–400 g) of either sex were stunned and bled, and the taeniae were dissected from the caecum. The taeniae (0·4–0·6 g), obtained from four guinea-pigs, were isometrically mounted in a sample tube of 10 mm diameter, and initially superfused with normal solution (for composition see below) at a constant flow rate of 12 ml min^{-1}. The temperature in the sample tube was kept at 32 °C. The perfusing set-up was 3 m apart from the main magnet. After inserting the sample into the bore of the main magnet (6·4 T), the proton signal from water (270 MHz) was used to improve the homogeneity of the magnetic field.

^{31}P NMR

The methods employed for ^{31}P NMR measurements were essentially the same as those used previously (Nakayama & Tomita, 1990, 1991). An NMR spectrometer (GSX270W, JEOL, Tokyo, Japan) was operated at 109·4 MHz for measurements of phosphorous compounds. Radio frequency pulses corresponding to a flip angle of 30 deg were applied every 0·6 s. ^{31}P NMR spectra obtained by the accumulation of 2500 signals (free induction decays: FIDs) over 25 min. Before Fourier transformation, a broadening factor of 15 Hz was applied to enhance the signal-to-noise ratio. Spectral peak resonances (frequencies) were measured relative to that of PCr (phosphocreatine) in parts per million (p.p.m.). Digital resolution of the spectrum was set to be approximately 0·005 p.p.m. by zero-filling.

Experiments were started after equilibrating preparations in normal solution for at least 100 min. In normal solution, six major peaks were observed: phosphomonoesters (PME), inorganic phosphate (P_i), PCr and the γ-, α- and β-peaks of ATP. The PME consisted of two peaks resonating at around 6·8 and 6·3 p.p.m. and they were assigned as PME 1 and PME 2. Concentrations of the phosphorous compounds were estimated by integrating the spectral peaks and by correcting with their saturation factors (Nakayama, Seo, Takai, Tomita & Watari, 1988).

Intracellular pH and Mg^{2+}

Since changes in intracellular pH (pH_i) are known to affect the estimation of $[Mg^{2+}]_i$ (especially when pH_i is less than 7; Fig. 1 in Nakayama, Nomura & Tomita, 1994), pH_i was monitored using the chemical shifts of the P_i (−log of dissociation constant (pK_a)), 6·70; chemical shifts of $H_2PO_4^-$ and HPO_4^{2-}, 3·15 and 5·72 p.p.m., respectively; Nakayama & Tomita, 1990) and PME 1 peaks (phosphorylethanolamine: pK_a, 5·70; chemical shifts of protonated and deprotonated forms, 3·27 and 6·95 p.p.m., respectively; Nakayama & Tomita, 1991).

Free Mg^{2+} concentration can be estimated from the ratio of metal-free and Mg^{2+}-binding forms of ATP, assuming the apparent dissociation constant of MgATP ($K_D^{\text{'MgATP'}}$). The chemical shifts of the ATP peaks change in proportion to the ratio of Mg^{2+} binding. In the present study, $[Mg^{2+}]_i$ was normally estimated from the observed chemical shift difference between the α- and β-ATP peaks ($\delta_{o(\alpha-\beta)}$), as previously described (Nakayama & Tomita, 1990, 1991):

$$[Mg^{2+}]_i = K_D^{\text{'MgATP'}}(\delta_{o(\alpha-\beta)} - \delta_{f(\alpha-\beta)}) / (\delta_{b(\alpha-\beta)} - \delta_{o(\alpha-\beta)}). \quad (1)$$

The values used for $\delta_{b(\alpha-\beta)}$ and $\delta_{f(\alpha-\beta)}$ (under Mg^{2+}-binding and metal-free conditions, respectively) were 8·35 and 10·85 p.p.m., respectively, and $K_D^{\text{'MgATP'}}$ was assumed to be 41 μM (at 32 °C, pH 7·2; Nakayama & Tomita, 1990).

$[Mg^{2+}]_i$ can also be estimated from the observed chemical shift difference between PCr and β-ATP ($\delta_{o\beta}$), based on the same theory described above:

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

where $\delta_{b\beta}$ and $\delta_{f\beta}$ are the chemical shifts of the β-ATP peak under Mg^{2+}-binding and metal-free conditions respectively. $K_D^{\text{'MgATP'}}$ (Bock, Wenz & Gupta, 1985) and $\delta_{f\beta}$ and $\delta_{b\beta}$ (Nakayama *et al.* 1994) can be expressed as pH functions. Equation (2) is rewritten as a relational function of $[Mg^{2+}]_i$, pH_i and $\delta_{o\beta}$:

$$[Mg^{2+}]_i = K_D^{\text{'MgATP'}}(pH_i)\frac{\delta_{o\beta} - \delta_{f\beta}(pH_i)}{\delta_{b\beta}(pH_i) - \delta_{o\beta}} = F_\beta(\delta_{o\beta}, pH_i). \quad (3)$$

Thus, estimation of $[Mg^{2+}]_i$ from the chemical shift of β-ATP can be corrected by pH_i. Since the P_i was often undetectable in Ca^{2+}-free solutions (Fig. 2), pH_i was mainly estimated from the chemical shift of PME 1 (phosphoethanolamine).

A similar relational function of $[Mg^{2+}]_i$ and pH_i is obtained for γ-ATP:

$$[Mg^{2+}]_i = F_\gamma(\delta_{o\gamma}, pH_i), \quad (4)$$

where $\delta_{o\gamma}$ is the observed chemical shift of the γ-ATP peak (observed chemical shift difference between PCr and γ-ATP). Two relational functions for $[Mg^{2+}]_i$ and pH_i are obtained by substituting the observed chemical shifts of β- and γ-ATP into eqns (3) and (4), respectively. Solving these simultaneous equations gives a value for $[Mg^{2+}]_i$ and pH_i (Nakayama *et al.* 1994).

In this study, we estimated $[Mg^{2+}]_i$ using eqn (1). However, in the main results (effects of amiloride and ouabain in the absence of Ca^{2+}), we modified eqn (2) to take changes in pH_i into consideration. To do this we used either eqn (3) with pH_i (estimated from PME 1) or the solutions of eqns (3) and (4) (as described above). The mathematical meaning of the estimation of $[Mg^{2+}]_i$ using eqn (1) is identical to eqn (3) assuming a pH_i of 7·2. This method contains an experimental error from the chemical shift of the α-ATP peak, the right hump of which contains nicotineamide adenine dinucleotide (NAD–NADH). We compared the estimated $[Mg^{2+}]_i$ and pH_i values using these three methods (Tables 1 and 2). The pH_i estimated using eqns (3) and (4) were rather varied compared with that from PME 1. This is probably because when pH_i is very alkaline, the changes in the chemical shifts of free- and Mg^{2+}-binding forms of both the β- and γ-ATP peaks are very small (Nakayama *et al.* 1994). During the continuous absence of extracellular Ca^{2+}, the chemical shift of PME 1 may be relatively more useful for monitoring pH_i.

^{87}Rb NMR

For measurements of ^{87}Rb NMR the spectrometer was operated at 88·4 MHz. Radio frequency pulses corresponding to a flip angle of 90 deg were repeated at 0·15 s intervals. The interval would allow full relaxation of the magnetization (we used a much longer interval than the reported values of the longitudinal relaxation time, e.g. Allis, Dixon, Till & Radda, 1989). Each ^{87}Rb spectrum was obtained by accumulation of 2000 signals over 5 min. The sample preparations and superfusions for ^{87}Rb NMR measurements were the same as for ^{31}P NMR measurements.

When the intracellular Rb^+ was measured, 5 mM RbCl ($^{87}Rb^+$, 27·85% and $^{85}Rb^+$, 72·15%) was added in Ca^{2+}-free solution. Assuming that the intracellular accumulation of Rb^+ was negligible in the first 5 min after addition of RbCl (the NMR signal was considered to be obtained only from extracellular solution), the intracellular Rb^+ was estimated by subtracting the NMR signals accumulated in the first 5 min from each signal obtained subsequently. A different spectral subtraction has enabled intra- and extracellular Rb^+ in rat salivary glands to be successfully distinguished (Steward, Seo, Murakami & Watari, 1991). We monitored intracellular Rb^+ as an index of Na^+–K^+ pump activity in the guinea-pig taenia caeci. In this tissue, using radioisotopes, it has been shown that the Rb^+ concentration for half-maximal Na^+–K^+ pump activity is nearly equal to that of K^+ (Widdicombe, 1981). Also, the sensitivity of ^{87}Rb NMR is 19 times higher than that of ^{39}K (Allis *et al.* 1989).

Since ^{87}Rb has a quadrupolar nucleus, a multiple-quantum filter can be used to detect intracellular Rb^+ (Allis & Radda, 1989; Steward *et al.* 1991). However, the time resolution of this method is much less than that of single-pulse detection, and in salivary glands it has been shown that double-quantum filtration requires 3–36 h accumulation of FIDs (Steward *et al.* 1991). Also, shift reagents can be applied to shift the extracellular Rb^+ peak. Since the spectral width of the Rb^+ peak is much broader than those of other ions (due to its shorter transverse relaxation time; Allis *et al.* 1989), it is necessary to use higher than normal concentrations of the shift reagents. However, high concentrations of the shift reagents may be toxic in the guinea-pig taenia cacei, and may confuse the interpretation of the results. Thus, we judged that spectral subtraction is the best experimental method for this tissue, although a large proportion of the ^{87}Rb NMR signal (single-pulse detection) may be lost during the pulse–receiver delay period because of its fast relaxation.

Solutions and chemicals

The normal solution had the following composition (mM): NaCl, 137·9; $KHCO_3$, 5·9; $CaCl_2$, 2·4; $MgCl_2$, 1·2; glucose, 11·8; Hepes, 5 (pH adjusted to 7·4–7·5 at 32 °C). When the ionic composition was modified, Na^+ was isosmotically substituted. Ca^{2+}-free solution contained 0·1 mM EGTA. Mg^{2+}- and Ca^{2+}-free solutions contained 1 mM EDTA. These solutions were bubbled with 100% O_2 before the preparation was superfused. Amiloride was kindly provided by Merck-Banyu Pharmaceutical Co. (Tokyo, Japan). Ouabain was purchased from Merck (Germany).

Statistics

Numerical data are expressed as means ± standard deviation. Differences between means were evaluated by Student's paired t test, and a test value of less than 0·05 was taken as a statistically significant difference.

RESULTS

The effects of amiloride, which is known to inhibit numerous Na^+ transporters (Benos, 1988), were examined with respect to Mg^{2+} regulation, because it has previously been suggested that $[Mg^{2+}]_i$ is kept much lower than its electrochemical equilibrium value by Na^+–Mg^{2+} exchange. $[Mg^{2+}]_i$ was mainly estimated from the separation between the α- and β-ATP peaks (eqn (1)), following a protocol previously used in this tissue (Nakayama & Tomita, 1990, 1991). Since the chemical shift of the α-ATP peak is nearly constant under various conditions, the changes in $[Mg^{2+}]_i$ correspond mainly to shifts of the β-ATP peak (an increase in $[Mg^{2+}]_i$ represented by a shift to a higher frequency). Thus, β-ATP peaks are shown expanded (Figs 1 and 2). The mean value of $[Mg^{2+}]_i$ in the preparations obtained in this study was 0·34 ± 0·04 mM ($n = 18$).

When 1 mM amiloride was added to normal solution (containing 1·2 mM Mg^{2+}), there was little change in $[Mg^{2+}]_i$ over 150 min (an increase of 0·06 ± 0·02 mM after 150 min, $n = 3$). During the application of amiloride, only a small alkalinization was observed (mean change was 0·05 pH units, estimated from the chemical shift of P_i). In two out of the three preparations, the subsequent removal of extracellular Ca^{2+} caused an increase in $[Mg^{2+}]_i$ of 0·13 mM (mean value, $n = 2$) after 50 min. This is probably because passive Mg^{2+} flux is small in the presence of Ca^{2+} (Nakayama & Tomita, 1990). The other preparation was subsequently exposed to high-Mg^{2+} solution (12 mM Mg^{2+}, 2·4 mM Ca^{2+}) in the presence of amiloride. The $[Mg^{2+}]_i$ increased by 0·06 mM.

In another preparation, 1 mM amiloride was applied in the presence of high Mg^{2+} (12 mM Mg^{2+}, 2·4 mM Ca^{2+}). After 150 min, the β-ATP peak was shifted by 0·04 p.p.m. towards a higher frequency (Fig. 1). This shift corresponds to an increase in $[Mg^{2+}]_i$ of only 0·08 mM. Subsequent wash-out of amiloride fully restored $[Mg^{2+}]_i$. The P_i peak was very small, as shown in Fig. 1. There was little change in the pH_i estimated from PME 1 (7·19–7·21). In order to clearly demonstrate drug-induced effects on $[Mg^{2+}]_i$, subsequent experiments were thus performed in Ca^{2+}-free solutions.

Effects of amiloride and ouabain in the absence of Ca^{2+}

Reversible increase in $[Mg^{2+}]_i$

Figure 2 shows the effect of prolonged application of amiloride on the ^{31}P NMR spectrum. After smooth muscle preparations were exposed to Ca^{2+}-free solution for 50 min, 1 mM amiloride was added to the perfusate. The β-ATP peak shifted by 0·13 ± 0·05 p.p.m. ($n = 4$) towards a higher frequency 150 min after application of amiloride (Fig. 2, trace *b*), indicating that $[Mg^{2+}]_i$ increased from 0·38 ± 0·03 to 0·86 ± 0·28 mM (Fig. 3, ●). The subsequent

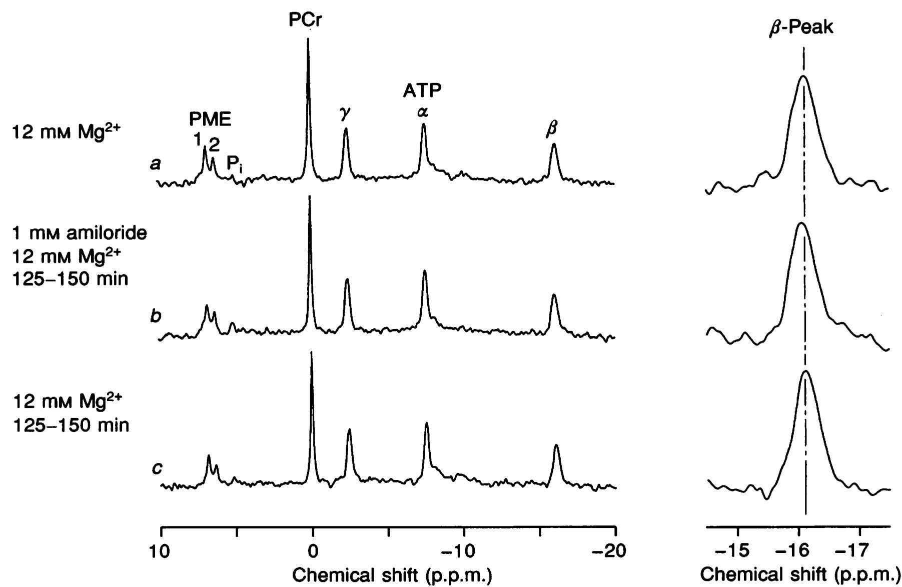

Figure 1. Effects of amiloride on ^{31}P NMR spectra in smooth muscle of the guinea-pig taenia caeci

Each spectrum was obtained by 2500 FIDs accumulated over 25 min. The preparation was initially superfused with normal solution for more than 100 min. After acquiring the spectrum in 12 mM Mg^{2+}-containing solution (2·4 mM Ca^{2+}; trace *a*), 1 mM amiloride was applied for 150 min (*b*). Subsequently amiloride was washed out with high-Mg^{2+} solution for 150 min (*c*). The β-ATP peak is shown expanded to the right of the spectrum. The vertical line corresponds to the initial chemical shift of the β-ATP peak.

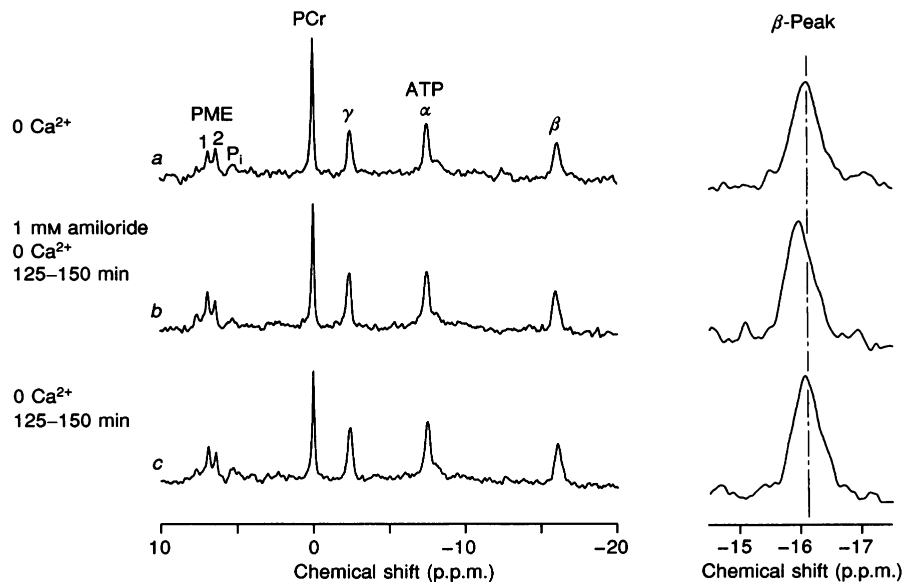

Figure 2. Effects of amiloride and ouabain on ^{31}P NMR spectra

Each spectrum has a common accumulation time of 25 min. After acquiring the spectrum in the absence of Ca^{2+} for 50 min (trace *a*), 1 mM amiloride was applied for 150 min (*b*). Recovery was observed after 150 min of washing out amiloride with a Ca^{2+}-free solution (*c*). The β-ATP peak is shown expanded to the right of the spectrum. The vertical line corresponds to the initial chemical shift of the β-ATP peak.

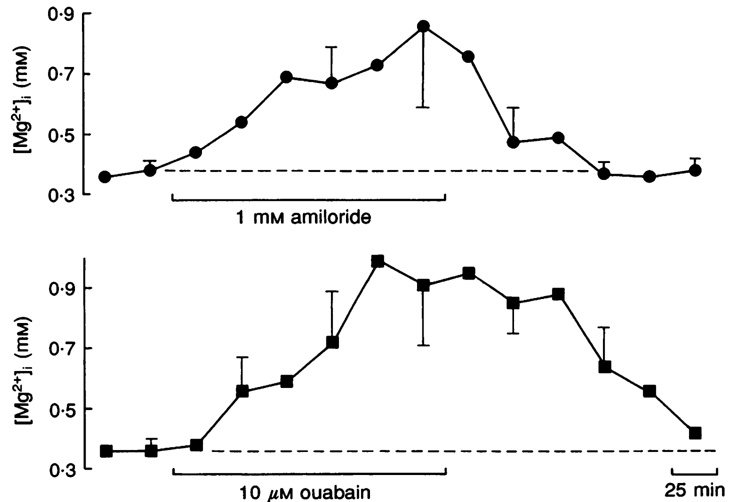

Figure 3. Time course of changes in $[Mg^{2+}]_i$ upon application of amiloride (upper) and ouabain (lower panel)

Each point was obtained from the accumulation of 2500 ^{31}P NMR FIDs over 25 min. Vertical bars represent S.D. values ($n = 4$).

wash-out of amiloride reversed the chemical shift of the β-ATP peak in 100 min. These results are in accord with the hypotheses that Na^+–Mg^{2+} exchange exists in smooth muscle and that amiloride increases $[Mg^{2+}]_i$ through inhibition of this mechanism.

If Na^+–Mg^{2+} exchange exists and the antiporter extrudes Mg^{2+} using the energy of Na^+ influx, an increase in $[Mg^{2+}]_i$ would be expected when the Na^+ gradient across the plasma membrane is reduced. Ouabain is a well-known inhibitor of the Na^+–K^+ pump and a concentration of 10 μM completely blocks the oxygen consumption which corresponds to the pump activity in smooth muscle of the taenia (Nakayama & Tomita, 1990). When ouabain (10 μM) was applied in the absence of external Ca^{2+}, the β-ATP peak shifted towards a higher frequency as seen during application of amiloride. The $[Mg^{2+}]_i$ increased from $0·36 \pm 0·04$ to $0·91 \pm 0·20$ mM ($n = 4$) after 150 min (Fig. 3, ■). Wash-out of ouabain reversibly decreased $[Mg^{2+}]_i$ close to the control value after 150 min.

Dose-dependent change in $[Mg^{2+}]_i$ was also tested in three preparations. The concentration of amiloride was cumulatively increased (0·1, 0·5 and 1 mM) in the absence of Ca^{2+}. Each concentration of amiloride was applied for 75 min. The $[Mg^{2+}]_i$ only slightly increased with 0·1 mM amiloride, but increased up to 1 mM with a higher concentration of amiloride (1 mM; Fig. 4).

In the four muscle preparations used in the amiloride experiments, the relative concentration of PCr ([PCr]) in Ca^{2+}-free solution was $2·22 \pm 0·12$ taking the [ATP] in the control spectrum as 1. The [PCr] gradually decreased throughout the experiments ($2·13 \pm 0·11$, 150 min after application of amiloride; $1·89 \pm 0·16$, 150 min after wash-out), irrespective of changes in $[Mg^{2+}]_i$. On the other hand, there was little change in [ATP] ($1·02 \pm 0·04$, 150 min after application of ouabain; $0·98 \pm 0·06$, 150 min after wash-out). Similar changes in the concentrations of high-energy phosphates were observed upon application and wash-out of ouabain. Throughout the experiments [PCr] gradually decreased by 12%, while [ATP] decreased by 5%. In both experiments for amiloride and ouabain, changes in the concentrations of high-energy phosphates did not correlate with those in $[Mg^{2+}]_i$.

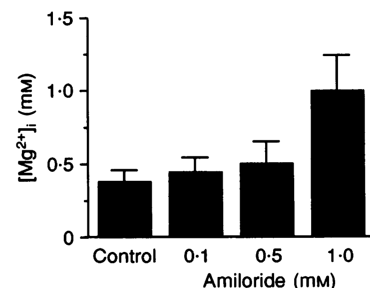

Figure 4. The effects of cumulative application of amiloride on $[Mg^{2+}]_i$

The $[Mg^{2+}]_i$ was estimated from the chemical shift difference between the α- and β-ATP peaks. Each concentration of amiloride was applied for 75 min. Changes in $[Mg^{2+}]_i$ were measured during the last 25 min.

Table 1. Effects of amiloride on the chemical shifts of the PME 1, and γ-, α- and β-ATP peaks

	Control	150 min amiloride	150 min wash-out
PME 1 (p.p.m.)	6·81 ± 0·01	6·85 ± 0·02	6·84 ± 0·02
γ-ATP (p.p.m.)	−2·44 ± 0·01	−2·36 ± 0·02	−2·42 ± 0·02
α-ATP (p.p.m.)	−7·53 ± 0·01	−7·52 ± 0·02	−7·53 ± 0·01
β-ATP (p.p.m.)	−16·12 ± 0·02	−15·99 ± 0·05	−16·13 ± 0·03
$[Mg^{2+}]_i$ from α- and β-ATP (mM)	0·38 ± 0·03	0·86 ± 0·28	0·38 ± 0·04
$[Mg^{2+}]_i$ from γ- and β-ATP (mM)	0·35 ± 0·02	0·71 ± 0·33	0·34 ± 0·06
pH_i from γ- and β-ATP	7·16 ± 0·04	7·25 ± 0·20	7·28 ± 0·15
$[Mg^{2+}]_i$ from β-ATP and PME 1 (mM)	0·36 ± 0·01	0·68 ± 0·25	0·34 ± 0·06
pH_i from PME 1	7·11 ± 0·05	7·26 ± 0·09	7·23 ± 0·09

After observing a control spectrum in Ca^{2+}-free solution, 1 mM amiloride was added for 150 min. $[Mg^{2+}]_i$ was calculated by three methods: (1) from the difference between the chemical shifts of α- and β-ATP peaks (the values were not corrected by pH_i); (2) $[Mg^{2+}]_i$ and pH_i were calculated from the chemical shifts of γ- and β-ATP peaks; (3) $[Mg^{2+}]_i$ was calculated from the chemical shift of the β-ATP peak, and corrected by pH_i from PME 1. The values were expressed as means ± S.D.

pH_i analysis

Both amiloride and ouabain seem likely to affect Na^+-dependent pH regulatory mechanisms. The chemical shift of P_i changes most widely as pH changes, and therefore provides a best resolution for pH estimation. However, the P_i peak was often undetectable in Ca^{2+}-free solutions. The pH_i was thus estimated using two other methods: (a) simultaneous estimation of pH_i and $[Mg^{2+}]_i$ from the chemical shifts of β- and γ-ATP (by solving eqns (3) and (4)) (Nakayama *et al.* 1994); and (b) estimation of pH_i from the chemical shift of PME 1 (phosphoethanolamine; Nakayama & Tomita, 1991). Tables 1 and 2 summarize the chemical shifts of PME 1 and ATP peaks, and the resultant estimation of pH_i and $[Mg^{2+}]_i$. In the absence of Ca^{2+}, the pH_i estimated by either method was very slightly increased during application of amiloride (pH_i from β- and γ-ATP, from 7·16 ± 0·04 to 7·25 ± 0·20; pH_i from PME 1, from 7·11 ± 0·05 to 7·26 ± 0·09, $n = 4$). The increased pH_i was not reversed by subsequent wash-out of amiloride. In this tissue, slow and gradual alkalinization has been observed in Ca^{2+}-free solutions (e.g. Fig. 6 in Nakayama *et al.* 1988). It is likely that pH_i did not reach a stable level when NMR signals were accumulated for the control spectrum (25–50 min after removal of Ca^{2+}).

Changes in pH_i may affect regulation mechanisms for $[Mg^{2+}]_i$ and also perturb the estimation of $[Mg^{2+}]_i$ itself. $[Mg^{2+}]_i$, as well as pH_i, was estimated from the chemical shifts of β- and γ-ATP. $[Mg^{2+}]_i$ increased from 0·35 ± 0·02 to 0·71 ± 0·33 mM ($n = 4$) during application of amiloride (Table 1). $[Mg^{2+}]_i$, estimated from the chemical shift of β-ATP (eqn (3)) was also corrected by pH_i from PME 1. Using this technique for estimation, the mean value of $[Mg^{2+}]_i$ increased from 0·36 to 0·68 mM. In all three methods described here, $[Mg^{2+}]_i$ was significantly increased by application of amiloride, and restored by subsequent wash-out, irrespective of sustained alkalinization under Ca^{2+}-free conditions. The increase in $[Mg^{2+}]_i$, estimated from the chemical shifts of the α- and β-ATP peaks, was

Table 2. Effects of ouabain on the chemical shifts of the PME 1, and γ-, α- and β-ATP peaks

	Control	150 min ouabain	150 min wash-out
PME 1 (p.p.m.)	6·82 ± 0·01	6·86 ± 0·01	6·87 ± 0·01
γ-ATP (p.p.m.)	−2·43 ± 0·02	−2·36 ± 0·01	−2·38 ± 0·01
α-ATP (p.p.m.)	−7·53 ± 0·01	−7·52 ± 0·02	−7·54 ± 0·02
β-ATP (p.p.m.)	−16·14 ± 0·02	−15·99 ± 0·02	−16·11 ± 0·02
$[Mg^{2+}]_i$ from α- and β-ATP (mM)	0·36 ± 0·04	0·91 ± 0·20	0·42 ± 0·02
$[Mg^{2+}]_i$ from γ- and β-ATP (mM)	0·32 ± 0·05	0·66 ± 0·17	0·32 ± 0·04
pH_i from γ- and β-ATP	7·24 ± 0·12	7·29 ± 0·16	7·47 ± 0·11
$[Mg^{2+}]_i$ from β-ATP and PME 1 (mM)	0·35 ± 0·04	0·64 ± 0·11	0·34 ± 0·03
pH_i from PME 1	7·14 ± 0·03	7·30 ± 0·08	7·36 ± 0·07

$[Mg^{2+}]_i$ and pH_i were estimated as described in Table 1.

the largest result of the three methods. This discrepancy is due to the assumed pH_i of 7·2 in eqn (1), and experimental error from the chemical shift of α-ATP, the right hump of which contains nicotinamide–adenine dinucleotide.

In experiments examining the effects of ouabain, pH_i and $[Mg^{2+}]_i$ were also estimated from the chemical shifts of the β-and γ-ATP peaks, and the chemical shifts of β-ATP and PME 1 (Table 2). Similar changes in pH_i were observed using both methods of estimation. Also, both methods revealed reversible increases in $[Mg^{2+}]_i$ (statistically significant) during application of ouabain, although the increases were smaller compared with those estimated from the chemical shifts of α- and β-ATP peaks.

Depletion of $[Mg^{2+}]_i$

Figure 5 shows typical spectra seen in Mg^{2+}- and Ca^{2+}-free solution in the presence of amiloride. After observing the control spectrum, both Mg^{2+} and Ca^{2+} were removed from the control solution (containing 1 mM EDTA), and 1 mM amiloride was added ($n = 3$). In all three preparations, these procedures gradually shifted the β-ATP peak to the lower frequency (rightward), and made it too broad to determine the exact chemical shift. The chemical shifts of the β-ATP peak in Fig. 5 (traces *c* and *d*) are probably around 17·5 p.p.m. (100 min exposure, estimated $[Mg^{2+}]_i$ ~30 μM) and 17·8 p.p.m. (150 min exposure, $[Mg^{2+}]_i$ ~19 μM), respectively. It has been predicted theoretically that when the β-peak resonates about the middle between the free- and Mg^{2+}-binding ATP peak positions, a broad peak would be observed due to the slow chemical exchange rate (Nageswara Rao, 1984). The γ-peak had a clear rightward shift (approximately 0·35 p.p.m. in 150 min), while the α-peak showed a smaller shift (0·18 p.p.m.) in the same direction (Fig. 5, trace *d*). The pH_i estimated from the PME 1 peak was approximately 7·25 after 150 min exposure to Mg^{2+}-free, Ca^{2+}-free solution.

We have previously shown similar spectra in the absence of amiloride (Nakayama & Tomita, 1990). However, the rightward shifts observed in Mg^{2+}-free, Ca^{2+}-free solutions were larger in the absence of amiloride (γ-peak, 0·44 p.p.m.; α-peak, 0·26 p.p.m., both in 100 min; Nakayama & Tomita, 1990). As amiloride only partially inhibited the depletion of $[Mg^{2+}]_i$, this implies the presence of an amiloride-insensitive Mg^{2+} pathway.

Intracellular Rb^+

Amiloride has variable inhibitory potencies on Na^+–K^+ ATPase in different tissues (Soltoff & Mandel, 1983; Dörge, Beck, Rick, Nagel & Thurau, 1990). If amiloride inhibits the Na^+–K^+ pump and significantly changes the intracellular Na^+ concentration in the smooth muscle of taenia caeci, $[Mg^{2+}]_i$ might be increased indirectly through Na^+–Mg^{2+} exchange. To examine the possible inhibitory effect of amiloride on the Na^+–K^+ pump, the intracellular Rb^+ was measured using ^{87}Rb NMR (at 5 min intervals). In Fig. 6*A*, the upper spectral peaks were obtained by

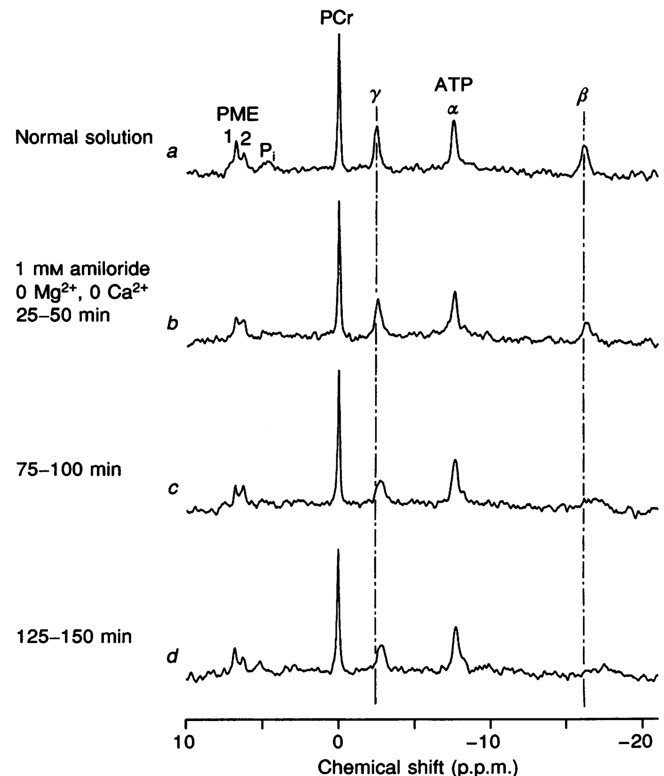

Figure 5. Effects of amiloride during exposure to Mg^{2+}- and Ca^{2+}-free solution

Amiloride (1 mM) was added to Mg^{2+}- and Ca^{2+}-free solution: Mg^{2+} and Ca^{2+} were isosmotically replaced by Na^+, and 1 mM EDTA added. The vertical lines correspond to the initial chemical shifts of the γ- and β-ATP peaks, respectively.

Fourier transformation of the raw NMR signals, including both extracellular and intracellular Rb^+. The smooth muscle preparation was loaded with Rb^+ in the absence of Ca^{2+}. In the first 5 min after addition of Rb^+ (5 mM) the NMR signal was considered to be obtained only from the extracellular solution (accumulation of Rb^+ would be negligible in Fig. 6*A a*). The intracellular Rb^+ was thus, estimated by subtracting the NMR signals accumulated in the first 5 min from each signal obtained subsequently (lower spectral peaks in Fig. 6*A*). Figure 6*B* shows the changes in intracellular Rb^+. After Rb^+ loading over 150 min, amiloride (1 mM) was applied for 100 min, but the intracellular Rb^+ was almost unchanged (Fig. 6*B*). In contrast, following 150 min addition of ouabain (10 μM), the intracellular Rb^+ concentration decreased to 19% of its original value and the withdrawal of ouabain for 150 min

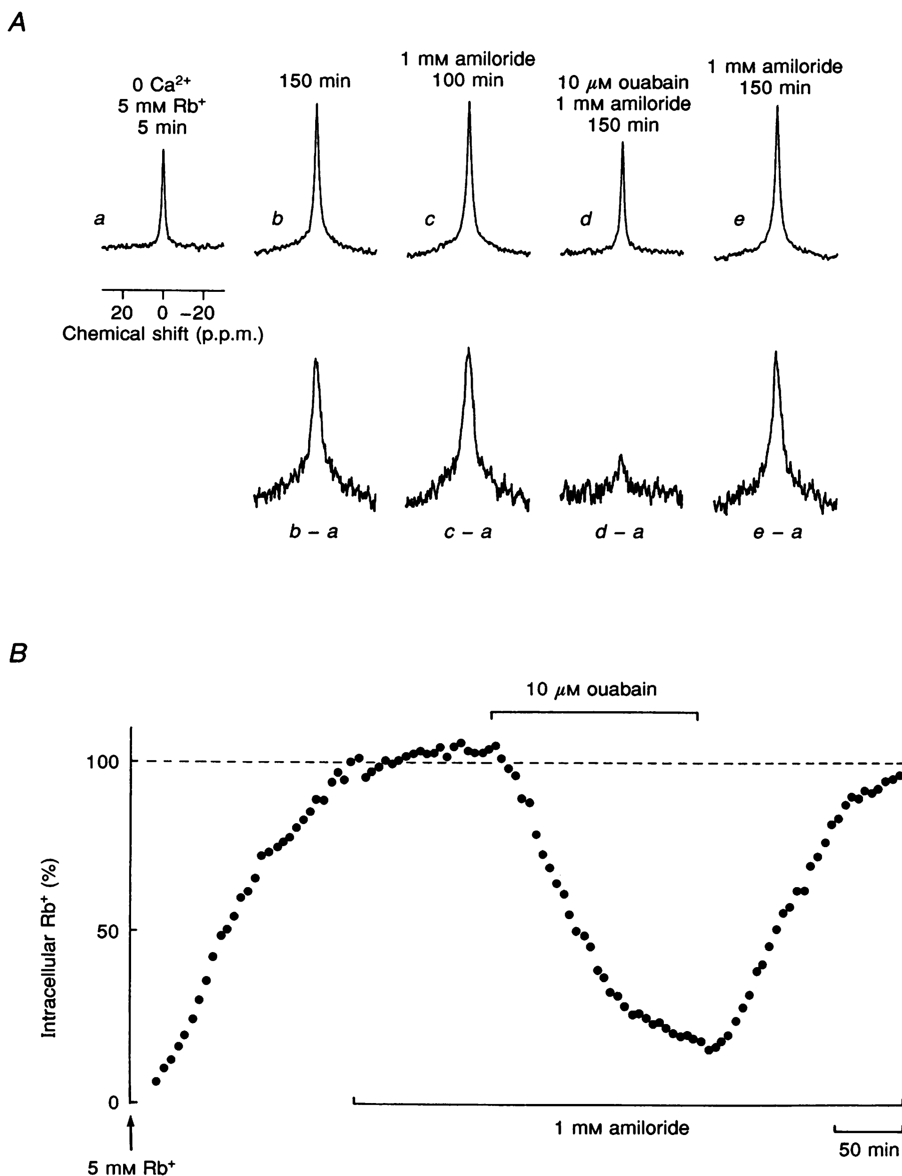

Figure 6. Changes in intracellular Rb^+

A, in the absence of Ca^{2+}, 5 mM Rb^+ was added for 150 min and Rb^+ gradually accumulated in the tissue. After the tissue had been saturated (150 min), 1 mM amiloride was applied for 100 min. Ouabain (10 μM) was then added for 150 min. In the presence of amiloride, ouabain was subsequently washed out (150 min). The upper spectral peaks were obtained from Fourier transformations of raw signals. The peak integral corresponds to the total (intracellular and extracellular) Rb^+ in the sample tube. Spectrum *a*, obtained from the first 5 min, was assumed to be due to the extracellular Rb^+ in the sample tube, when intracellular accumulation of Rb^+ was presumed to be very slight. The intracellular Rb^+ concentration was estimated by subtraction of spectrum *a* from each spectrum. The lower row of peaks in *A* are shown using an increased gain. The time course of the changes in intracellular Rb^+ is shown in *B*. The intracellular Rb^+ concentration just before the application of amiloride was taken as 100%.

almost fully restored this concentration in the presence of amiloride (the final concentration was 96% of the control value). In two other preparations, qualitatively similar responses were observed upon application of amiloride and ouabain.

DISCUSSION

In the present study, we investigated the regulation of intracellular free Mg^{2+} using amiloride and ouabain, representative inhibitory drugs for Na^+ transporters and Na^+–K^+ pumps, respectively. Application of either of the two drugs significantly and reversibly increased $[Mg^{2+}]_i$ (Fig. 3).

$[Mg^{2+}]_i$ had increased by 0·48 mM 150 min after applying amiloride. Amiloride is known as a non-specific inhibitory drug for many Na^+ transporters. In smooth muscle 1 mM amiloride would inhibit a Na^+–H^+ exchanger (Kahn, Cragoe, Allen, Halligan & Shelat, 1990; Aickin, 1994), and subsequently affect pH_i. Such changes in pH_i may affect $[Mg^{2+}]_i$ regulatory mechanisms, as well as perturb estimation of $[Mg^{2+}]_i$ (standard estimation of $[Mg^{2+}]_i$ assumes a pH_i of 7·2). In the present study, we estimated pH_i in Ca^{2+}-free solutions using two methods: (1) from the chemical shifts of γ- and β-ATP; and (2) from the chemical shift of PME 1. This allowed $[Mg^{2+}]_i$ to be corrected for changes in pH_i. Although both corrected values of $[Mg^{2+}]_i$ were smaller than the $[Mg^{2+}]_i$ estimated using the standard method (from the chemical shifts of α- and β-ATP), all three methods revealed a significant increase in $[Mg^{2+}]_i$ 150 min after application of amiloride (Table 1). Similar results were obtained in the analysis for ouabain (Table 2).

Many factors which affect Na^+ transporters and pH seem to alter $[Mg^{2+}]_i$. In heart cells, for example, it has recently been reported that intracellular acidosis causes an increase in $[Mg^{2+}]_i$ probably through competition between H^+ and Mg^{2+} for intracellular, cation binding sites (Freudenrich, Murphy, Levy, London & Lieberman, 1992). A similar correlation between Mg^{2+} and pH_i has already been reported in smooth muscle cells of the guinea-pig taenia caeci (Nakayama *et al.* 1994). In this tissue we also suggested the presence of Na^+–Mg^{2+} exchange which maintains low $[Mg^{2+}]_i$ using the energy from the Na^+ gradient across the plasma membrane (Nakayama & Tomita, 1991; Nakayama *et al.* 1994). Many studies on cardiac muscle have suggested interaction between Na^+–H^+ and Na^+–Ca^{2+} exchange systems, and that intracellular acidosis causes a rise of $[Ca^{2+}]_i$ via changes in $[Na^+]_i$, and subsequently enhances the tension development (e.g. Vaughan-Jones, Lederer & Eisner, 1983; Kaila & Vaughan-Jones, 1987; Bountra & Vaughan-Jones, 1989). In some smooth muscles, contractions associated with intracellular acidification have been reported (e.g. Spurway & Wray, 1987; Taggart & Wray, 1993). It is pointed out that Na^+–Mg^{2+} exchange (Na^+-dependent Mg^{2+} transport) shares many properties with the Na^+–Ca^{2+} exchange (Flatman, 1991). Thus, as in the case of $[Ca^{2+}]_i$ regulation, intracellular acidosis may increase $[Mg^{2+}]_i$ through an increase in $[Na^+]_i$. On the other hand, inhibition of Na^+–H^+ exchange may facilitate intracellular acidosis, but prevent an increase in $[Mg^{2+}]_i$.

In the present experiments, however, we found that the increase in $[Mg^{2+}]_i$ during application of amiloride was not accompanied by intracellular acidosis, but rather an increase in pH_i. Measurements of intracellular Rb^+ revealed that amiloride did not inhibit the Na^+–K^+ pump. These results suggest that the increase in $[Mg^{2+}]_i$ by amiloride is due neither to changes in Na^+ gradient nor competition between H^+ and Mg^{2+}. Intracellular alkalinization observed during application of amiloride would, in fact, mask the increase in $[Mg^{2+}]_i$, to some extent, by absorbing Mg^{2+} in the common cation binding sites. Thus, it seems likely that amiloride directly blocks Na^+–Mg^{2+} exchange as it does other Na^+ transporters (Benos, 1988). In the absence of external Ca^{2+} and Mg^{2+}, $[Mg^{2+}]_i$ fell below 10 μM after 100 min (Nakayama & Tomita, 1990). Significant depletion of $[Mg^{2+}]_i$ was also observed, even when amiloride was added (Fig. 4), or when Na^+ was removed (K^+ substitution) from the external solution (Nakayama & Tomita, 1991). We can postulate the presence of another Mg^{2+} pathway which is amiloride insensitive (or less sensitive) and blocked by Ca^{2+}, and which provides a passive route for Mg^{2+} movement. This assumption is also consistent with the fact that removal of external Ca^{2+} results in a larger increase in $[Mg^{2+}]_i$ following application of amiloride to Mg^{2+}-containing solutions. In ferret red blood cells, it has been reported that 1 mM or higher concentrations of amiloride only partially inhibit Mg^{2+} efflux (and uptake) and the maximum inhibition is 60–70% of the total flux (Flatman & Smith, 1990, 1991). This also suggests the presence of two Mg^{2+} pathways in these cells.

The reversible increase in $[Mg^{2+}]_i$ after addition of ouabain is not accompanied by intracellular acidosis, but rather pH_i increases in the presence of the drug (Table 2). In contrast to the effects of amiloride, ouabain (10 μM) significantly reduced intracellular Rb^+ (measured as an index of Na^+–K^+ pump activity), suggesting that there is a corresponding increase in $[Na^+]_i$. The time course of the depletion and restoration of the intracellular Rb^+ upon application and wash-out of ouabain follows the time course of the increase and decrease in $[Mg^{2+}]_i$. Also, the concentration of high-energy phosphates slowly decreased during application and wash-out of ouabain, irrespective of the changes in $[Mg^{2+}]_i$. As extracellular Na^+ was maintained at a physiological concentration, a Na^+-dependent Mg^{2+} pump driven by ATP hydrolysis (Lüdi & Schatzmann, 1987; Frenkel, Graziani & Schatzmann, 1989) seems unlikely to play a dominant role in the guinea-pig taenia caeci. The results strongly support the presence of Na^+–Mg^{2+} exchange driven by the Na^+ gradient with the ouabain-induced increase in $[Mg^{2+}]_i$ being secondary to an increase in $[Na^+]_i$, perhaps through the following processes: (1) $[Mg^{2+}]_i$ may increase through a

reversed mode of Na^+–Mg^{2+} exchange, or (2) Mg^{2+} extrusion through Na^+–Mg^{2+} exchange may be reduced, so that the exchange system cannot deal with the increased Mg^{2+} influx in the absence of Ca^{2+}. The present results however, do not differentiate between these two possibilities. Also, as with Na^+–Ca^{2+} exchange, Na^+–Mg^{2+} exchange might be inactivated by intracellular Na^+ (Hilgemann, 1990). Owing to this possible downregulation, the presence of a Na^+-dependent Mg^{2+} pump cannot be fully ruled out.

Since ATP is an important substance for buffering intracellular Mg^{2+}, a decrease in [ATP] would elevate $[Mg^{2+}]_i$. Such an effect of changing [ATP] has been reported in cardiac (Headrick & Willis, 1989) and skeletal muscles (Westerblad & Allen, 1992). In the present experiments, however, changes in [ATP] consistent with this mechanism were not observed: the cellular high-energy phosphates slowly decreased throughout. In chicken red blood cells, it has been reported that amiloride inhibited the phosphorylation, induced by Mg^{2+} loading, of a 230 kDa membrane protein (Günther & Vormann, 1986). In the smooth muscle of the taenia, inhibition of various protein phosphorylations by amiloride and its derivatives has been shown (Ozaki, Moriyama, Karaki, Kohama & Cragoe, 1989). ATP may be involved in Mg^{2+} regulation via phosphorylation of Mg^{2+} transporters (DiPolo & Beaugé, 1988; Flatman, 1991). Also, apart from the phosphorylation mechanism, a high requirement of ATP ($K_D > 3$ mM) for activation of Na^+–Ca^{2+} exchange has been reported (Collins, Somlyo & Hilgemann, 1992). If a similar regulating mechanism exists in Na^+–Mg^{2+} exchange, its activity would be affected by physiological changes in the ATP concentration in smooth muscle (Nakayama *et al.* 1988; Ishida & Paul, 1990).

The pH_i did not decrease, but, instead, increased during application of amiloride. One may suspect that 1 mM amiloride is not sufficient to block Na^+–H^+ exchange in the smooth muscle of the taenia caeci. However, in the presence of amiloride, removal of bicarbonate from the perfusate (Ca^{2+}-containing solution) significantly decreased pH_i (by 0·15–0·35 units with 0·5–1 mM amiloride after 50 min; authors' unpublished observation), suggesting that bicarbonate-dependent systems (Aickin, 1988; Wray, 1988) effectively regulated pH_i during simple application of amiloride. In rat uterine smooth muscle, it has been shown that transient decreases in pH_i are linked to spontaneous contraction (Taggart & Wray, 1993). Spontaneous contraction in the taenia ceased with 1 mM amiloride. This probably caused the small increase in pH_i observed in normal solution. On the other hand, amiloride did not increase pH_i in high-Mg^{2+} solution, probably because spontaneous activity is abolished by 12 mM Mg^{2+} (Nakayama & Tomita, 1991) before application of amiloride. Also, in Ca^{2+}-free solution, ouabain did not cause any intracellular acidosis, although it presumably reduced the Na^+ gradient. As we have little information about the ionic composition of the intracellular space, it is difficult to assess exactly how pH_i is regulated. Bicarbonate-dependent pH regulation is probably involved, as well as a reduction in lactate production and membrane depolarization. We can rule out the possibility that competition between H^+ and Mg^{2+} for common intracellular binding sites contributes to the increases in $[Mg^{2+}]_i$ from the fact that intracellular acidosis does not occur during application of either drug. Indeed, we observed a small alkalinization during application of the drugs (Tables 1 and 2). Given the effects of changes in pH_i on intracellular Mg^{2+} binding, the increase in $[Mg^{2+}]_i$ after inhibition of Na^+–Mg^{2+} exchange is likely to be larger than that measured here.

Mg^{2+} is known as a necessary cofactor for many enzyme reactions (Flatman, 1991). In the present study, we demonstrated that $[Mg^{2+}]_i$ can be changed by amiloride and ouabain, representative inhibitory drugs for Na^+ transporters and Na^+–K^+ pumps, respectively. There are a number of other drugs which are known to inhibit Mg^{2+} extrusion (e.g. imipramine, quinidine; Féray & Garay, 1988). These types of drugs are often used in animal experiments and also in clinical therapy. In normal solution, Mg^{2+} movement across the plasma membrane is thought to be very small compared to its intracellular buffering capacity. There is evidence that Ca^{2+} inhibits Mg^{2+} flux. Thus, we used Ca^{2+}-free solutions so that changes in $[Mg^{2+}]_i$ could be more clearly measured over a limited experimental time. However, even under physiological concentrations of Ca^{2+}, prolonged and chronic applications of such drugs may modulate (perhaps subtly) the cellular responses through changes in $[Mg^{2+}]_i$.

In conclusion, in the smooth muscle of the taenia caeci, the mechanisms for the observed increases in $[Mg^{2+}]_i$ following treatment with either amiloride or ouabain would appear to be a direct inhibition of Na^+–Mg^{2+} exchange or an indirect inhibition of this transporter by an increase in $[Na^+]_i$, respectively. Many other factors, such as pH_i or ATP, may also modulate these changes in $[Mg^{2+}]_i$.

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