Extracellular Mg^{2+} regulates the intracellular Na^+ concentration in rat sublingual acini

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Abstract The intracellular free Na^+ concentration ($[Na^+]_i$) increases during muscarinic stimulation in salivary acinar cells. The present study examined in rat sublingual acini the role of extracellular Mg^{2+} in the regulation of the stimulated $[Na^+]_i$ increase using the fluorescent sodium indicator benzofuran isophthalate (SBFI). The muscarinic induced rise in $[Na^+]_i$ was approximately 4-fold greater in the absence of extracellular Mg^{2+}. When Na^+ efflux was blocked by the Na^+,K^+-ATPase inhibitor ouabain, the stimulated $[Na^+]_i$ increase was comparable to that seen in an Mg^{2+}-free medium. Moreover, ouabain did not add further to the stimulated $[Na^+]_i$ increase in an Mg^{2+}-free medium suggesting that removal of extracellular Mg^{2+} may inhibit the Na^+ pump. In agreement with this assumption, ouabain-sensitive Na^+ efflux and rubidium uptake were reduced by extracellular Mg^{2+} depletion. Our results suggest that extracellular Mg^{2+} may regulate $[Na^+]_i$ in sublingual salivary acinar cells by modulating Na^+ pump activity.

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Key words: Salivary gland; Intracellular free Na^+ concentration; Na^+,K^+-ATPase; Magnesium

1. Introduction

The current fluid secretion model states that transepithelial Cl^- movement acts as the driving force for fluid secretion. Secondary active uptake of Cl^- across the basolateral membrane requires an inwardly directed Na^+ chemical gradient [1,2]. This Na^+ gradient is generated by the Na^+,K^+-ATPase which maintains the $[Na^+]_i$ at approximately 15 mM in resting sublingual acinar cells [3]. During muscarinic stimulation, $[Na^+]_i$ increases in salivary acinar cells as a consequence of increased activity of $Na^+/K^+/2Cl^-$ cotransport and Na^+/H^+ exchange [3–6]. This increase in $[Na^+]$ activates the Na^+,K^+-ATPase in order to maintain the inward Na^+ gradient during stimulation. The magnitude of the agonist-induced $[Na^+]_i$ increase is an important element in the modulation of fluid and electrolyte secretion in these cells. The Na^+,K^+-ATPase inhibitor ouabain blocks saliva formation by collapsing the Na^+ gradient necessary for Cl^- uptake [7]. Thus the inwardly directed Na^+ chemical gradient created by the Na^+ pump plays a central role in driving the fluid secretion process.

It is well known that Na^+,K^+-ATPase activity requires Mg^{2+} [8–10]. Apart from forming an essential substrate for the Na^+ pump, MgATP, Mg ions are also required for the transport of K^+ and Na^+ [10]. It has been demonstrated that Mg ions act at intracellular site(s). In human lymphocytes and atrial cells extracellular Mg^{2+} is required to maintain pump

activity [11,12]. However, it is not clear whether Mg^{2+} acted in these studies at an external site of the Na^+ pump or if extracellular Mg^{2+} removal depleted the cells of internal Mg^{2+} and so reduced pump activity.

Preliminary studies indicate that the extracellular Mg^{2+} concentration regulates the $[Na^+]_i$ in sublingual acinar cells, Mg^{2+} removal dramatically increasing the $[Na^+]_i$ during muscarinic stimulation [13]. The intracellular $[Mg^{2+}]$ is unchanged by removal of extracellular Mg^{2+} during stimulation [14], suggesting that Mg^{2+} may act extracellularly to enhance the stimulated $[Na^+]_i$ response. In the present study we examined the mechanisms by which extracellular Mg^{2+} may influence $[Na^+]_i$ regulation. Our results confirm that extracellular Mg^{2+} regulates $[Na^+]_i$; the intracellular $[Na^+]$ increased with decreased external $[Mg^{2+}]$ during muscarinic stimulation. This increase in $[Na^+]_i$ was apparently the result of decreased Na^+ efflux, suggesting that external Mg^{2+} controls $[Na^+]_i$ by modulating the activity of Na^+,K^+-ATPase.

2. Materials and methods

2.1. Chemicals and solutions

Collagenase (type CLSPA) was from Worthington Biomedical (Malvern, PA, USA). Hyaluronidase (type V), carbamyl choline (carbachol), ouabain, monensin, gramicidin D, nigericin, bovine serum albumin (BSA, type V) and N-methyl-D-glucamine were purchased from Sigma (St. Louis, MO, USA). Earle's minimal essential medium (MEM) was from Biofluids (Rockville, MD, USA). SBFI/AM and Pluronic F-127 were purchased from Molecular Probes (Eugene, OR, USA). The physiological salt solution (PSS) consisted of 110 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 0.8 mM $MgSO_4$, 0.33 mM NaH_2PO_4, 0.4 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM glucose, 20 mM HEPES and pH was adjusted to 7.4 with NaOH after gassing with 5% $CO_2/95\%$ O_2. For the Na^+-free solution, all Na^+ was replaced with N-methyl-D-glucamine, the pH was adjusted with Tris base, and an equimolar concentration of Cl^- was added using concentrated HCl.

2.2. Isolation of rat sublingual acini

Male, Wistar strain rats (Charles River, Kingston Facility, NY, USA, 150–250 g) were used for isolation of sublingual acini as described by Melvin et al. [5]. Rats were killed by exsanguination following exposure to CO_2 and the sublingual glands removed and minced in ice-cold digestion medium (Earle's MEM, 1% BSA, 50 U/ml of collagenase and 0.02 mg/ml of hyaluronidase). The minced glands in 10 ml of the digestion medium were incubated at 37°C in a Dubnoff shaker with continuous gassing (95% $O_2/5\%$ CO_2, humidified) and agitation (80 cycles/min). The sublingual mince was dispersed by gently pipetting 10 times with a 10 ml plastic pipette at 15 min intervals. After 45 min digestion, the suspension was centrifuged for 30 s at $50 \times g$ and the cells resuspended in 8 ml of fresh digestion medium for an additional 45 min. After a total of 1.5 h of digestion, the preparation was washed 3 times with PSS containing 0.01% BSA, and the acini resuspended in 1 ml PSS solution/gland. For the measurement of intracellular $[Na^+]$, dye-loaded acini were resuspended in PSS without BSA to promote attachment to a coverslip mounted in a perfusion chamber.

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2.3. Determination of [Na⁺]ᵢ

Acinar cells were loaded with SBFI and [Na⁺]ᵢ determined as previously described [3]. Briefly, solutions of SBFI/AM (2 mM) and the non-ionic detergent Pluronic F-127 (25%, w/v) were prepared in dimethyl sulfoxide, mixed in equal volumes, and added to the sublingual acinar suspension (final concentration 1 μM SBFI/AM). The acini were incubated with SBFI/AM for 1 h at room temperature. A pinhole turret was used to isolate five to eight acinar cells on the stage of a Nikon inverted microscope with a 40×, NA 1.3 oil immersion lens, and the fluorescence monitored with a microfluorometer (AR-CM, SPEX Industries, Edison, NJ, USA) at excitation wavelengths of 340 nm and 380 nm and an emission wavelength of 500 nm. Calibration of the SBFI fluorescence ratio was performed in situ as reported [5,15] using 2 μM gramicidin D, 5 μM monensin and 5 μM nigericin. The calibration solution for R_{max} contained 80 mM Na gluconate, 60 mM NaCl, 20 mM HEPES, 1.2 mM CaCl₂, 0.8 mM MgSO₄, pH 7.4. The R_{min} calibration solution replaced all Na⁺ salts with K⁺. The fluorescence ratio of the unbound dye versus the Na⁺-bound dye (β factor) was determined by monitoring the fluorescence at 380 nm and [Na⁺]ᵢ calculated as previously described by Grynkiewicz et al. [16] and Harootunian et al. [15]. The K_d of SBFI for Na⁺ used in these studies was 18 mM [17].

2.4. Determination of stimulated rubidium uptake

Rubidium content was estimated using a rubidium lamp on an atomic absorption spectrophotometer (Perkin-Elmer PE 3030). Isolated sublingual acini were pre-incubated for 2 min at 23°C in rubidium-containing medium in which 5.4 mM KCl was substituted with equimolar RbCl. All data for stimulated rubidium uptake were corrected by subtracting the ouabain-insensitive, non-specific "binding" that occurred during this 2 min pre-incubation period (3.46 ± 2.18 μg rubidium/mg protein, n = 4). Stimulated rubidium uptake was monitored following the addition of 10 μM carbachol. Uptake was terminated after 15 min of stimulation by layering a 0.5 ml aliquot of the acinar suspension on 0.25 ml of oil (F-50; General Electric Company, Waterford, NY, USA) and centrifuging at 13000 rpm for 5 s in an Eppendorf 5415C microcentrifuge. After centrifugation of the acinar pellet through oil, the medium and oil were removed by suction. The cell pellet was then homogenized with a Teflon minihomogenizer in 100 μl of 0.2% KCl. The homogenized pellet was centrifuged at 12000 rpm for 5 min and the supernatant used for rubidium determination. The pellet was resuspended in 0.5 ml of 1% SDS for 24 h and protein content determined as described by Smith et al. [18] using bovine serum albumin as standard. Preliminary experiments determined the time course for rubidium uptake during muscarinic stimulation. The $t_{1/2}$ was 10.8 ± 1.9 min with maximum uptake of 49.8 ± 4.5 μg rubidium/mg protein (n = 5).

2.5. Statistical analysis and data presentation

All data shown are representative of at least 4 independent experiments using different cell preparations. All of the values are expressed as the mean ± S.E.M. Statistical differences were analyzed by Student's t-test for unpaired samples. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Extracellular Mg²⁺ removal increases the [Na⁺]ᵢ response to muscarinic stimulation

In the present study, resting [Na⁺]ᵢ was 15 mM in SBFI-loaded acini (n = 22), similar to our previous results [3]. Fig. 1 shows that muscarinic stimulation (10 μM carbachol), on average, increased [Na⁺]ᵢ at an initial rate of 14.8 ± 1.6 mM/min to a new intracellular equilibrium of 39.7 ± 4.0 mM after 5 min in a 0.8 mM Mg²⁺-containing physiological salt solution (n = 5). Conversely, in a nominally Mg²⁺-free solution the initial rate was almost 3-fold greater (42.6 ± 6.1 mM Na⁺/min) and [Na⁺]ᵢ increased after 5 min to 114.1 ± 16.6 mM (n = 5), an approximately 4-fold greater stimulated increase in the [Na⁺]ᵢ. The stimulated [Na⁺]ᵢ was expressed as the [Na⁺]ᵢ after 5 min stimulation minus the initial, resting [Na⁺]ᵢ.

Non-linear regression analysis of the increase in [Na⁺]ᵢ after 5 min stimulation at different external [Mg²⁺] indicates that the half-maximal effect for extracellular [Mg²⁺] was 0.33 ± 0.03 mM Mg²⁺.

3.2. Extracellular Mg²⁺ removal inhibits Na⁺ efflux

3.2.1. Na⁺ efflux is inhibited by extracellular Mg²⁺ removal. To determine whether Na⁺ efflux was inhibited by removal of extracellular Mg²⁺, the intracellular [Na⁺] was increased by stimulating acini with carbachol in a physiological Mg²⁺-containing medium (0.8 mM). After 2 min stimulation, muscarinic receptors were antagonized with atropine to initiate recovery of [Na⁺]ᵢ in either the presence or absence of extracellular Mg²⁺. Fig. 2 shows that in the presence of extracellular Mg²⁺ atropine induced net Na⁺ efflux, [Na⁺]ᵢ nearly recovering to the resting level in 7 min. Conversely, removal of extracellular Mg²⁺ prevented the antagonist-induced recovery of [Na⁺]ᵢ.

3.2.2. Regulation of Na⁺,K⁺-ATPase activity by removal of extracellular Mg²⁺. Fig. 2 indicates that extracellular Mg²⁺ controls [Na⁺]ᵢ by regulating Na⁺ efflux. In salivary acinar cells the principal if not the only Na⁺ efflux pathway under physiological conditions is the Na⁺,K⁺-ATPase. Consistent with extracellular Mg²⁺ modulation of Na⁺ pump activity, Fig. 3 demonstrates that the [Na⁺]ᵢ increase in ouabain-treated acini was comparable in the presence or absence of extracellular Mg²⁺. In 5 such experiments in the presence of extracellular Mg²⁺, the initial rates and the magnitudes of the [Na⁺]ᵢ increases were 29.7 ± 5.5 mM Na⁺/min and 114.0 ± 8.7 mM Na⁺, respectively. Similarly, in the absence of extracellular Mg²⁺ the initial rates and the magnitudes were 27.3 ± 3.8 mM Na⁺/min and 116.2 ± 21.8 mM Na⁺, respectively. Because the effect of Mg²⁺-containing medium+ouabain on the stimulated [Na⁺]ᵢ response was similar to that seen in Mg²⁺-free medium+ouabain, extracellular Mg²⁺ removal most likely acted on the Na⁺ pump. Extracellular Mg²⁺ apparently regulates Na⁺ pump activity in resting cells as well. In the absence of stimulation, [Na⁺]ᵢ increased about 25% in 5 min

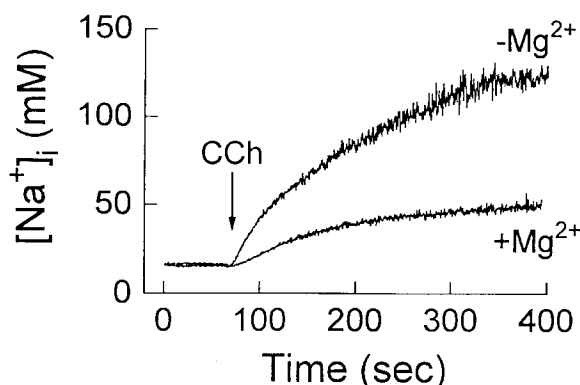Fig. 1. Extracellular Mg²⁺ removal increases the [Na⁺]ᵢ in rat sublingual mucous acini during muscarinic stimulation. Isolated rat sublingual mucous acini were loaded with SBFI and [Na⁺]ᵢ monitored as described in Section 2. An [Na⁺]ᵢ increase was stimulated in varying concentrations of external Mg²⁺ (0, 0.05, 0.1, 0.2, 0.4, 0.8 and 10 mM). Shown is the stimulation-induced [Na⁺]ᵢ increase in zero (−Mg²⁺) and 0.8 mM external Mg²⁺ (+Mg²⁺). Carbachol (10 μM CCh) was added at the time indicated by the arrow. Approximately 2 min prior to stimulation acini were switched from 0.8 mM external Mg²⁺ to the final Mg²⁺ concentration. Traces are representative of 5 separate experiments. The half maximal effect of external Mg²⁺ on the magnitude of the [Na⁺]ᵢ increase was 0.33 ± 0.03 mM Mg²⁺.
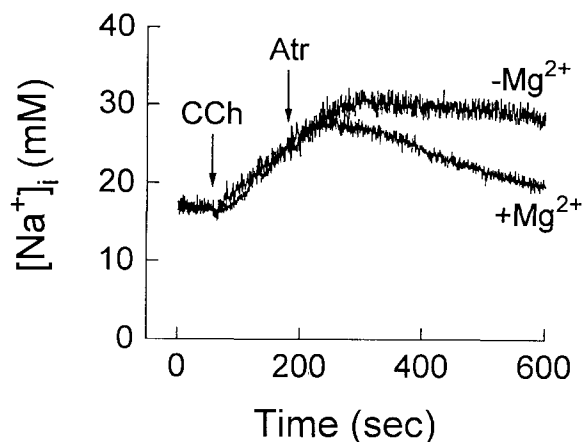
Fig. 2. Extracellular Mg^{2+} removal inhibits Na^+ efflux following muscarinic stimulation. $[Na^+]_i$ was monitored in sublingual acini as described in Fig. 1. At the time indicated by the first arrow 10 μM carbachol (CCh) was introduced. After 2 min stimulation in an Mg^{2+}-containing solution the superfusate was switched to a solution containing 10 μM atropine (Atr, second arrow) in the presence $(+Mg^{2+})$ or absence $(-Mg^{2+})$ of external Mg^{2+}. Seven min after atropine addition the $[Na^+]_i$ was 17.8 ± 1.2 mM in Mg^{2+}-containing medium $(n = 6)$ and 27.5 ± 3.9 in Mg^{2+}-free medium $(n = 5; P < 0.05)$.

after the removal of extracellular Mg^{2+} from 16.0 ± 1.8 to 20.0 ± 2.6 mM $[Na^+]$ $(n = 4)$, equivalent to the effect of ouabain on $[Na^+]_i$ over the same time period (from 16.9 ± 1.1 to 22.3 ± 2.0 mM $[Na^+]$, $n = 4$).

3.2.3. Extracellular Mg^{2+} removal inhibits ouabain-sensitive rubidium influx. To more directly test the hypothesis that Na^+ pump activity is modulated by extracellular Mg^{2+} we monitored rubidium uptake. Rubidium acts as a K^+ surrogate on many K^+ transport pathways including the Na^+,K^+-ATPase. Fig. 4 shows that extracellular Mg^{2+} removal significantly inhibited the ouabain-sensitive component of rubidium uptake. The Na^+ pump inhibitor ouabain blocked rubidium uptake during muscarinic stimulation by nearly 80%. Removal of extracellular Mg^{2+} reduced the uptake of rubidium approximately 55%. Addition of ouabain to the Mg^{2+}-free medium further decreased rubidium uptake to levels comparable
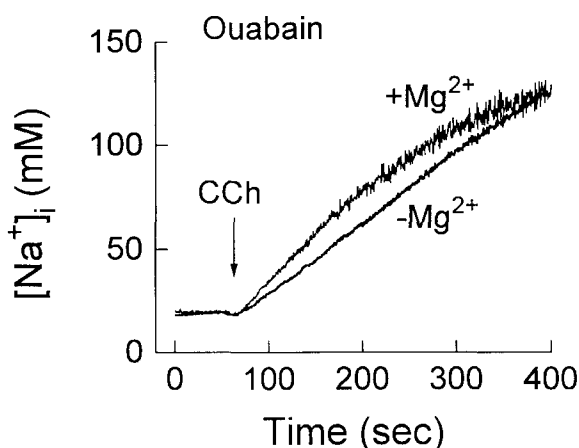

Fig. 3. Effects of extracellular Mg^{2+} and ouabain on the $[Na^+]_i$ response to stimulation. $[Na^+]_i$ was monitored in SBFI-loaded sublingual acini as described in Fig. 1. At the time indicated by the arrow, 10 μM carbachol (CCh) plus 1 mM ouabain was introduced. Acini were superfused with either Mg^{2+}-containing medium $(+Mg^{2+})$ or Mg^{2+}-free medium $(-Mg^{2+})$. Traces are representative of 4 separate experiments.
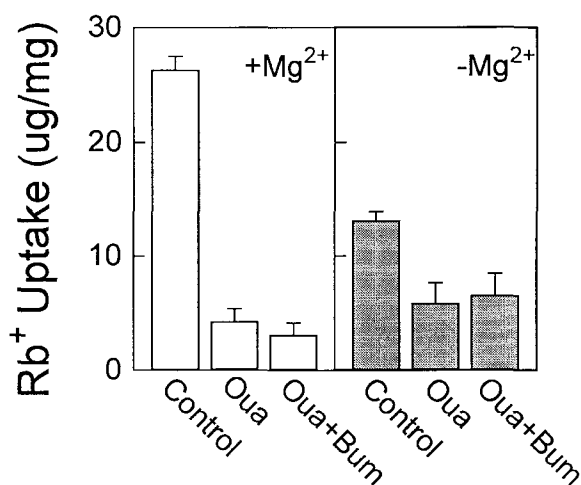

Fig. 4. Extracellular Mg^{2+} removal and ouabain inhibit muscarinic induced rubidium uptake. Rubidium uptake was monitored as described in Section 2. Acinar rubidium content was determined after 15 min of stimulation with 10 μM carbachol (CCh) in Mg^{2+}-containing $(+Mg^{2+})$ or Mg^{2+}-free $(-Mg^{2+})$ medium in the presence or absence of 1 mM ouabain $(Oua) \pm 0.02$ mM bumetanide (Bum). The number of individual experiments for each condition is ≥ 8. All manipulations are significantly different from the control Mg^{2+}-containing medium, $P < 0.05$.

to those seen in the presence of ouabain in an Mg^{2+}-containing solution. In contrast, the $Na^+/K^+/2Cl^-$ cotransport inhibitor bumetanide failed to produce a significant additional decrease in rubidium uptake (Fig. 4). The apparent insensitivity of rubidium uptake to bumetanide suggests that the oaubain-sensitive Na^+,K^+-ATPase is the primary rubidium uptake mechanism during stimulation, and secondly, that extracellular Mg^{2+} removal did not enhance cotransporter activity.

4. Discussion

A large inward-directed Na^+ chemical gradient is required to drive fluid and electrolyte secretion by salivary acinar cells [1,2]. In a preliminary report we found that removal of external Mg^{2+} during muscarinic stimulation increases the magnitude of the $[Na^+]_i$ response and thus collapses the Na^+ gradient in sublingual acini [13]. In the present study we further investigated this phenomenon in an attempt to identify the mechanism by which extracellular Mg^{2+} governs the intracellular $[Na^+]$ response to stimulation. Our results indicate that extracellular Mg^{2+} may regulate $[Na^+]_i$ by modulating Na^+,K^+-ATPase activity, pump activity increasing as the extracellular Mg^{2+} concentration increases. Consequently, the increase in $[Na^+]_i$ induced by extracellular Mg^{2+} removal is at least partially due to decreased Na^+ efflux.

It is unclear whether removal of extracellular Mg^{2+} may have augmented Na^+ uptake via Na^+/Mg^{2+} exchange as well. We have previously characterized an Na^+/Mg^{2+} exchanger located in the plasma membrane of rat sublingual acini, although it apparently contributes little to Na^+ uptake under physiological conditions. This exchanger is activated by elevated $[Mg^{2+}]_i$ and essentially shuts down when physiological $[Mg^{2+}]_i$ is regained [19]. When sublingual acini were loaded with Mg^{2+} to concentrations comparable to those observed during stimulation [14], Na^+-dependent Mg^{2+} efflux was enhanced (data not shown). Nevertheless, the predicted initial rate of Na^+ influx was only about 0.2% of the rate observed during stimulation in a Mg^{2+}-free solution (Fig. 1), assuming

that a stoichiometry of 3 Na^+:1 Mg^{2+} is operative in sublingual acini [20,21]. Consistent with Na^+/Mg^{2+} exchange contributing little to the $[Na^+]_i$ increase, we have previously shown that muscarinic stimulation does not increase exchanger activity, indeed, stimulation appears to down-regulate the Na^+/Mg^{2+} exchanger in sublingual acini [19]. Clearly, increased Na^+/Mg^{2+} exchanger activity cannot account for the response of $[Na^+]_i$ to extracellular Mg^{2+} removal seen in this study.

Few studies have examined the dependence of Na^+ pump activity on the extracellular Mg^{2+} concentration. In rat skeletal muscle and red blood cells, removal of Mg^{2+} did not inhibit transport mediated by the Na^+ pump. Conversely, extracellular Mg^{2+} appears to regulate Na^+ pump activity in a human atrial cell line [11] and human lymphocytes [12], although neither of these studies measured the $[Mg^{2+}]_i$. Campos and Beaugé [22] have shown that Mg-ATP acts as a substrate for the Na^+ pump whereas Mg^{2+} is an essential activator that binds to the enzyme independent of, but close to, the ATP binding site. Mg^{2+} binding and release at this site may be necessary for modulating the transport cycle by shifting the distribution of enzyme between transport conformations [23]. Direct interaction of Mg^{2+} is thus essential for the activation of Na^+,K^+-ATPases, although this requirement is associated with an intracellular site(s). In our previous studies the intracellular $[Mg^{2+}]$ increased during stimulation, even in the absence of extracellular Mg^{2+} [14], indicating that decreased $[Mg^{2+}]_i$ is not likely to be involved in the inhibition of the Na^+ pump. However, we cannot exclude the possibility that $[Mg^{2+}]_i$ decreased in a subcellular compartment close to the pump's intracellular Mg^{2+} binding site.

Alternatively, extracellular Mg^{2+} removal may acutely regulate Na^+,K^+-ATPase activity by modulating the intracellular concentration of a "messenger" such as calcium ions or protons. The Na^+,K^+-ATPase is known to be pH sensitive, the activity decreasing as the pH decreases. Extracellular Mg^{2+} removal enhanced the intracellular acidification induced by muscarinic stimulation (data not shown). However, proton concentrations in the physiological pH range have no direct effect on Na^+ pump rate [24]. Therefore, it seems improbable that the pH drop produced by Mg^{2+} depletion (about 0.1 pH unit) was sufficient to inhibit pump activity and produce a greater than 3-fold increase in $[Na^+]_i$ during stimulation.

Extracellular Mg^{2+} removal also increased the magnitude of the initial transient of the stimulated $[Ca^{2+}]_i$ increase by about 30% (approximately 220 nM in an Mg^{2+}-containing solution versus nearly 290 nM in an Mg^{2+}-free solution). This initial increase in $[Ca^{2+}]_i$ lasts about 30 s and is followed by a lower sustained increase in $[Ca^{2+}]_i$ that lasts as long as the agonist is present. Extracellular Mg^{2+} removal did not change the level of the sustained $[Ca^{2+}]_i$ increase (data not shown). Most studies suggest that elevated Ca^{2+} inhibits pump activity. However, $[Ca^{2+}]_i$ in the physiological range may produce either stimulation or inhibition, apparently depending on the isoform of the enzyme expressed [25]. Evidently Ca^{2+} does not directly regulate Na^+,K^+-ATPase activity but modulates the pump by activating Ca^{2+}-dependent pathways [26]. Therefore, the Mg^{2+}-dependent increase in $[Ca^{2+}]_i$ may transiently inhibit Na^+,K^+-ATPase activity in sublingual acinar cells.

4.1. Physiological significance

Magnesium deficiency has been previously linked to impaired K^+ and Na^+ regulation [27]. Although severe dietary Mg^{2+} deficiency is rare in affluent societies, hypomagnesemia is a common electrolyte imbalance during pregnancy, in critically ill hospitalized patients, and is frequently seen in the elderly and in alcoholics. It may not be necessary to dramatically reduce the extracellular $[Mg^{2+}]$ to alter gland function. Decreasing the extracellular Mg^{2+} concentration to 0.4 mM increased the magnitude of the stimulated $[Na^+]_i$ increase in the present study more than 30% above that seen at 0.8 mM, a value approximately equal to the normal blood plasma levels. Because the rate of transepithelial Cl^- movement (fluid secretion) is tightly coupled to the magnitude of the inward-directed Na^+ chemical gradient, a markedly reduced fluid production may occur with only a moderate Mg^{2+} deficiency.

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