

Na\(^+\)-dependent Release of Mg\(^{2+}\) from an Intracellular Pool in Rat Sublingual Mucous Acini

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Magnesium ions are essential cofactors for many cell functions including enzymatic reactions and transmembrane ion movements (1, 2). In this latter role, Mg\(^{2+}\) is important for regulating Na\(^+\) and Ca\(^{2+}\) pump activity. The inward-directed chemical gradients created by these pumps are especially critical in the modulation of fluid secretion by salivary acinar cells. These physiological processes require that the [Mg\(^{2+}\)]\(_i\) be controlled within a narrow range. However, the mechanism(s) by which the intracellular free Mg\(^{2+}\) concentration is regulated is still poorly understood. Mg\(^{2+}\) is not passively distributed, the [Mg\(^{2+}\)]\(_i\) can be more than 100 times lower than the concentration predicted from its electrochemical equilibrium. Acutely raising or lowering the external [Mg\(^{2+}\)] has little effect on the total magnesium content of most cells suggesting that the plasma membrane has a very low Mg\(^{2+}\) permeability (3, 4). In cells artificially loaded with an elevated [Mg\(^{2+}\)]\(_i\), a plasma membrane Na\(^+\)/Mg\(^{2+}\) exchanger is activated that shuts down when the [Mg\(^{2+}\)] returns to resting levels (5, 6). These results suggest that the primary function of Na\(^+\)/Mg\(^{2+}\) exchange is to maintain the resting [Mg\(^{2+}\)]\(_i\). Consistent with the plasma membrane having a low Mg\(^{2+}\) permeability, the [Mg\(^{2+}\)]\(_i\), changes observed during stimulation in many cell types does not reflect increased Mg\(^{2+}\) movement across the plasma membrane, but results from mobilization of Mg\(^{2+}\) from an intracellular pool (7, 8).

The total intracellular magnesium content consists of cytosolic free Mg\(^{2+}\), cytosolic bound magnesium, and magnesium stored within organelles. More than 90% of the cellular magnesium is in the bound form (9, 10). Cytosolic free Mg\(^{2+}\) accounts for ~6% of the total cytosolic magnesium content in hepatocytes (11) and ~3% in murine S49 lymphoma cells (12). It has been suggested that ATP and RNA play a key role in the Mg\(^{2+}\) buffering system (1, 10). Muscarinic stimulation of salivary acinar cells greatly enhances ATP consumption (13). However, inhibition of Na\(^+\)/K\(^+\)-ATPase does not influence the muscarinic-stimulated increase in [Mg\(^{2+}\)]\(_i\), in sublingual acinar cells (7) suggesting that this increase in [Mg\(^{2+}\)]\(_i\) does not result from the liberation of Mg\(^{2+}\) during ATP consumption. Therefore, release of Mg\(^{2+}\) from an intracellular organelle is apparently responsible for the agonist-induced [Mg\(^{2+}\)]\(_i\) increase seen in salivary acinar cells.

Muscarinic stimulation induces a marked increase in [Ca\(^{2+}\)]\(_i\), that subsequently triggers an increase in [Na\(^+\)]\(_i\), in sublingual acini (14, 15). The agonist-induced increases in [Na\(^+\)]\(_i\), and [Ca\(^{2+}\)]\(_i\), occur simultaneously with the mobilization of Mg\(^{2+}\) from an intracellular pool (7). When the increase in either [Na\(^+\)]\(_i\), or [Ca\(^{2+}\)]\(_i\), is prevented, the stimulated increase in [Mg\(^{2+}\)]\(_i\) is blunted as well (7). These results suggest that muscarinic-induced Mg\(^{2+}\) mobilization is both Na\(^+\)- and Ca\(^{2+}\)-dependent; however, the nature of this Na\(^+\) and Ca\(^{2+}\) dependence is unknown. In the present study, the role of Na\(^+\) and Ca\(^{2+}\) in regulating intracellular Mg\(^{2+}\) movement in rat sublingual acini was examined. Although both muscarinic receptor activation and Ca\(^{2+}\) mobilization were sufficient for stimulating the release of Mg\(^{2+}\) from the intracellular pool, neither was required. In contrast, we found that an increase in [Na\(^+\)]\(_i\), is not...
only sufficient but also necessary to mobilize Mg$^{2+}$ from a quinidine-sensitive pool.

**EXPERIMENTAL PROCEDURES**

**Materials—**Earle’s minimal essential medium was purchased from Life Technologies, Inc. Fura-2/AM, mag-fura-2/AM, SBFI/AM, sodium green, tetraacetate (tetraacetate), and BAPTA/AM were from Molecular Probes, Eugene, OR. Collagenase (type CLSPA) was purchased from Worthington. Hyaluronidase (type I-S), bovine serum albumin (type V), carbachol, monensin, nigericin, ionomycin, N-methyl-n-glucamine, and gramicidin D were from Sigma. All other chemicals were of the highest grade available.

**Preparation of Sublingual Mucous Acini—**Sublingual mucous acini were prepared from male, Wistar strain rats (150–250 gm, Charles River, Kingston facility, NY) as described previously (14). Rats were killed by exsanguination after exposure to CO$_2$ and sublingual glands removed and placed in ice-cold digestion medium which consisted of Earle’s minimal essential medium containing 1% bovine serum albumin, 50 units/ml collagenase, and 0.02 mg/ml hyaluronidase. The glands were finely minced in 2 ml of the digestion medium and then placed in 10 ml of the same medium, incubating at 37°C in a Dubnoff shaker with continuous gassing with 95% O$_2$, 5% CO$_2$ (humidified), and agitation (80 cycles/min). The mince was dispersed by gently pipetting 10 times with a 10 ml plastic pipette at 15 min intervals. After 45 min of digestion, the preparation was centrifuged at 400 g for 30 s, the supernatant was discarded and replaced with fresh digestion medium. After a total of 1.5 h of digestion, the preparation was washed three times with a physiological salt solution (PSS) containing 0.01% bovine serum albumin and resuspended in the same medium. The PSS consisted of (mM): 110 NaCl, 25 NaHCO$_3$, 20 HEPES, 10 glucose, 5.4 KCl, 1.2 CaCl$_2$, 0.8 MgSO$_4$, 0.4 KH$_2$PO$_4$, 0.33 Na$_2$HPO$_4$, adjusted to pH 7.4 with NaOH. For the nominally Ca$^{2+}$-free solution, CaCl$_2$ was omitted. The Na$^+$-free solution, Na$^+$ was replaced with N-methyl-n-glucamine.

**Determination of [Ca$^{2+}$]$_i$—**The intracellular free Ca$^{2+}$ concentration was determined by using the Ca$^{2+}$-sensitive fluorescent indicator fura-2 as described previously (14). Briefly, dispersed sublingual acini were loaded with fura-2 by incubating in 2 μM fura-2/AM for 30 min at 23°C, rinsed twice with PSS containing 0.01% bovine serum albumin, and resuspended in the same medium. Acini were attached to a coverslip mounted to the bottom of a perfusion chamber on the stage of a Nikon inverted microscope interfaced to a SPEX AR-CM fluorometer (Edison, NJ). A Nikon fluor X40 1.3-NA oil immersion objective was used to isolate five to eight acinar cells using a pinhole turbule. Fluorescence ratios, obtained by exciting the dye at 340 and 380 nm and collecting the 475-nm emission, were converted to [Ca$^{2+}$]$_i$ using dual-wavelength ratio dyes such as SBFI. Therefore, fluorescence ratio determinations were as described above for [Ca$^{2+}$]$_i$. The fluorescence ratios were converted to [Mg$^{2+}$]$_i$, by calibration in situ (7). [Mg$^{2+}$]$_i$, was calculated according to Grynkiewicz et al. (16) using 1.5 mM as the K$_s$ of magnesium.

**Determinations of [Mg$^{2+}$]$_i$, [Ca$^{2+}$]$_i$, and Na$^+$—We previously found that mucaricin stimulation simultaneously increases [Ca$^{2+}$]$_i$, [Na$^+$], and [Mg$^{2+}$]$_i$.** In rat sublingual mucous acini (7, 14, 15), the agonist-induced [Mg$^{2+}$]$_i$ increase was inhibited when either extracellular Ca$^{2+}$ or Na$^+$ was removed to decrease the [Ca$^{2+}$]$_i$, and [Na$^+$], respectively, consistent with previously reported values (7, 14, 15). Therefore, [Ca$^{2+}$]$_i$, [Na$^+$], and [Mg$^{2+}$]$_i$ were determined using ion-sensitive fluorescent indicators. The resting levels of [Ca$^{2+}$]$_i$, [Na$^+$], and [Mg$^{2+}$]$_i$, were 56.5 ± 4.3 nm (n = 33), 16.7 ± 0.9 nm (n = 30), and 0.55 ± 0.01 nm (n = 65), respectively, using dual-wavelength ratio dyes such as SBFI. Therefore, fluorescence, obtained by exciting the dye at 500 nm and collecting the 530 nm emission, was normalized to the initial fluorescence in unsimulated acini and presented as arbitrary units.

**Determination of [Mg$^{2+}$]$_i$—**Sublingual acini were loaded with mag-fura-2 as previously reported (7) by incubation with 2 μM mag-fura-2/AM for 30 min at room temperature. Fluorescence ratio determinations were as described above for [Ca$^{2+}$]$_i$. The fluorescence ratios were converted to [Mg$^{2+}$]$_i$, by calibration in situ (7). [Mg$^{2+}$]$_i$, was calculated according to Grynkiewicz et al. (16) using 1.5 mM as the K$_s$ of magnesium.

**Fig. 1. Effects of gramicidin on the cytosolic free Ca$^{2+}$, Na$^+$, and Mg$^{2+}$ concentrations in BAPTA-loaded and unloaded sublingual acini.** Rat sublingual acini were loaded with ion-sensitive fluorescent indicators (fura-2, SBFI, or mag-fura-2). In some experiments acini were also loaded with the Ca$^{2+}$-selective chelator BAPTA (+ BAPTA). The dye-loaded acini were stimulated with 5 μM gramicidin D (Gram) at the times indicated by the arrows. Each trace is a representative response. Panel A, [Ca$^{2+}$]$_i$, — BAPTA, n = 6; + BAPTA, n = 5. Panel B, [Na$^+$]$_i$, — BAPTA, n = 5; + BAPTA, n = 6. Panel C, [Mg$^{2+}$]$_i$, — BAPTA, n = 5; + BAPTA, n = 5.

**Statistics—**All results are presented as means ± S.E. Traces are shown as the representative response of experiments from at least four separate cell preparations. Comparisons were made between different treatments using the unpaired Student’s t test. Differences were considered significant at p < 0.05.

**RESULTS**

The Na$^+$ Ionophore Gramicidin Increases [Ca$^{2+}$]$_i$, [Na$^+$], and [Mg$^{2+}$]$_i$.—We previously found that mucaricin stimulation simultaneously increases [Ca$^{2+}$]$_i$, [Na$^+$], and [Mg$^{2+}$]$_i$ in rat sublingual mucous acini (7, 14, 15). The agonist-induced [Mg$^{2+}$]$_i$ increase was inhibited when either extracellular Ca$^{2+}$ or Na$^+$ was removed to decrease the [Ca$^{2+}$]$_i$, and [Na$^+$], respectively, using ion-sensitive fluorescent indicators. The resting levels of [Ca$^{2+}$]$_i$, [Na$^+$], and [Mg$^{2+}$]$_i$, were 56.5 ± 4.3 nm (n = 33), 16.7 ± 0.9 nm (n = 30), and 0.55 ± 0.01 nm (n = 65), respectively, consistent with previously reported values (7, 14, 15). Fig. 1 (− BAPTA) shows that 5 μM gramicidin induced 49, 305, and 58% increases in [Ca$^{2+}$]$_i$, [Na$^+$], and [Mg$^{2+}$]$_i$, respectively, similar to receptor activation with the exception that the gramicidin-induced [Ca$^{2+}$]$_i$ increase was about 5-fold less (compare to the mucaricin-induced [Ca$^{2+}$]$_i$ increases shown in Figs. 6 and 7). The dependence of [Mg$^{2+}$]$_i$ on the increases in [Ca$^{2+}$]$_i$, and [Na$^+$], was assessed in sublingual acini loaded with BAPTA, a high capacity buffer that selectively binds Ca$^{2+}$.
about 10^3 over Mg^{2+} (20). Fig. 1 (+BAPTA) reveals that the
gramicidin-induced increase in [Ca^{2+}]], was blunted >75%,
whereas the gramicidin-induced increases in [Na^+]], and
[Mg^{2+}], were unchanged (343% increase for [Na^+]], and 62% increase
for [Mg^{2+}]]. These results suggested that the
gramicidin-induced increase in [Mg^{2+}], was independent of a rise in
the intracellular [Ca^{2+}]].

Muscarnic stimulation increases the [Mg^{2+}], by mobilizing
Mg^{2+} from an intracellular pool (7). To examine whether gramicidin
increases [Mg^{2+}], by a similar mechanism, acini were
exposed to gramicidin in a Mg^{2+}-free medium to eliminate Mg^{2+} influx. Fig. 2 shows that gramicidin increased the
[Mg^{2+}], 57 ± 14% in a Mg^{2+}-free solution (n = 5), comparable
to the magnitude of the Mg^{2+} increase seen in a Mg^{2+}-containing
medium (55 ± 3%, n = 5). This indicates that the gramicidin-
duced increase in [Mg^{2+}], is due to intracellular Mg^{2+}
mobilization.

Gramicidin causes depolarization of the plasma membrane.
We exposed acini to high extracellular K^+ to test the possibility
that depolarization stimulated the increase in [Mg^{2+}], in acini
exposed to gramicidin. Depolarization by this maneuver did not significantly alter the [Mg^{2+}], (n = 5; Fig. 3). Furthermore,
this cytosolic-like, low Na^+ medium (126 mM K^+ and 15 mM Na^+)
abolished the Na^+ gradient and eliminated the gramicidin-
duced increase in [Mg^{2+}], (Fig. 3). Thus, under experimental
conditions which prevented the ionophore-induced increase
in Na^+ (data not shown), gramicidin failed to mobilize intracellular Mg^{2+}. These results indicate that a Na^+ depend-
ent mechanism is involved.

We further explored the Na^+ dependence of the intracellular
Mg^{2+} response by activating Na+/H^+ exchange to increase the
[Na^+]. Acid loading salivary acinar cells increases Na+/H^+ exchange activity and increases the [Na^+], (15). Fig. 4
(−BAPTA) shows that acid-loading salivary acini by incu-
bating acini in 50 mM sodium propionate increased [Ca^{2+}],
224% (n = 5), [Na^+], increased approximately 73% (n = 5), and
[Mg^{2+}], increased about 24% (n = 9). Clamping the [Ca^{2+}],
near 30 nM (−BAPTA) totally blocked the propionate-induced increase in [Ca^{2+}], (n = 5); whereas, this treatment increased
[Na^+], 74% (n = 7) and increased [Mg^{2+}], 28% (n = 5), com-
parable to the results seen in acini not loaded with BAPTA. In
agreement with the gramicidin-induced increase in [Mg^{2+}],
these results indicate that intracellular Mg^{2+} release is Na^+ depend-
ent and does not require an increase in the [Ca^{2+}]].

Intracellular Na^+ Depletion Inhibits Mg^{2+} Mobilization.
Mag-fura-2-loaded acini were depleted of intracellular Na^+ for either 1
(n = 6) or 7 (n = 9) min by superfusing with Na^+-free PSS. Ten μM CCh
was added at the time indicated by the arrow. Each trace is a repre-
sentative response.
Na\(^+\)-dependent Mg\(^{2+}\) Mobilization

Further depletion of [Na\(^+\)], produced by exposing acini to the Na\(^+\)-free medium for 7 min suppressed the CCh-induced increase in [Mg\(^{2+}\)], >75\% (n = 9; p < 0.001).

Fig. 6 displays the association between Na\(^+\) depletion and the stimulated increases in [Ca\(^{2+}\)], [Na\(^+\)], and [Mg\(^{2+}\)]. Here, depletion of intracellular Na\(^+\) did not reduce the CCh-stimulated increase in [Ca\(^{2+}\)], (n = 5) but significantly inhibited the increases in both [Na\(^+\)], and [Mg\(^{2+}\)], (n = 5). These results indicate that agonist-stimulated mobilization of intracellular Mg\(^{2+}\) does not directly involve an increase in [Ca\(^{2+}\)], whereas the increase in the [Na\(^+\)], is required.

The sustained increase in [Mg\(^{2+}\)], induced by CCh is also contingent upon the [Na\(^+\)]. Fig. 7 shows that after three minutes CCh stimulation [Ca\(^{2+}\)], increased 423\% (n = 5), [Na\(^+\)], increased 126\% (n = 6), and [Mg\(^{2+}\)], increased approximately 45\% (n = 7). Removal of Na\(^+\) after 3 min stimulation did not alter the sustained increase in [Ca\(^{2+}\)]. Nonetheless, [Mg\(^{2+}\)], decreased rapidly in parallel with changes in [Na\(^+\)]. These results are in accord with the hypothesis that the muscarinic-induced mobilization of the intracellular Mg\(^{2+}\) pool is mediated by a Na\(^+\)-dependent transport mechanism.

Inhibition of Mg\(^{2+}\) Release by Quinidine—The Na\(^+\) dependence of both the CCh- and the gramicidin-induced discharge of Mg\(^{2+}\) from the intracellular pool suggests that a Na\(^+\)/Mg\(^{2+}\) exchange mechanism may be involved. The Na\(^+\)/Mg\(^{2+}\) exchangers in several cell types are sensitive to quinidine (5, 6, 21). Fig. 8 shows that both the CCh-induced and gramicidin-induced increases in [Mg\(^{2+}\)], were inhibited by quinidine. Acini were pretreated with 0.5 mM quinidine for approximately 1 min to permit the inhibitor to enter the cell (22). Acini were then stimulated with either gramicidin or CCh in the continued presence of quinidine. The [Mg\(^{2+}\)], responses were dramatically inhibited by quinidine, whereas Fig. 9 demonstrates that CCh-stimulated [Ca\(^{2+}\)], (control, n = 5; + quinidine, n = 5) and [Na\(^+\)], (control, n = 9; + quinidine, n = 7) increases were essentially unchanged suggesting that quinidine blocked Na\(^+\)-dependent release of Mg\(^{2+}\) from an intracellular pool.

DISCUSSION

We previously observed in rat sublingual acini that muscarinic stimulation induces Mg\(^{2+}\) mobilization from an intracellular pool and this increase in [Mg\(^{2+}\)] is both Na\(^+\)- and Ca\(^{2+}\)-dependent (7). The [Na\(^+\)], is tightly coupled to the [Ca\(^{2+}\)], in salivary acinar cells (14, 15, 23). In the present study we examined the interdependence of [Mg\(^{2+}\)], on Na\(^+\) and Ca\(^{2+}\). Our results clearly demonstrate that Mg\(^{2+}\) release is mediated by a Na\(^+\)-dependent ion transport mechanism, and the receptor-stimulated rise in [Ca\(^{2+}\)], activates this mechanism indirectly by increasing the [Na\(^+\)].

The muscarinic-stimulated increase in [Ca\(^{2+}\)], is sufficient but not necessary for Mg\(^{2+}\) mobilization. In fact, it appears that liberation of the intracellular Ca\(^{2+}\) pool does not release Mg\(^{2+}\) but induces uptake of Mg\(^{2+}\) by the Ca\(^{2+}\) pool, most likely to maintain the charge balance of this pool (24). The results displayed in Fig. 1 revealed that the gramicidin-induced increase in [Na\(^+\)], caused the release of Mg\(^{2+}\) from an intracellular pool. In acini loaded with the Ca\(^{2+}\)-selective chelator BAPTA, the gramicidin-induced increases in [Na\(^+\)], and [Mg\(^{2+}\)], were not altered by Ca\(^{2+}\) chelation. These results disassociate the increase in [Ca\(^{2+}\)], from the increase in [Mg\(^{2+}\)], and indicate that Ca\(^{2+}\) acted indirectly to mobilize internal Mg\(^{2+}\) by increasing the [Na\(^+\)].

The plasma membrane of numerous cell types, including sublingual acinar cells, contains Na\(^+\)/Mg\(^{2+}\) exchangers (5, 6, 21, 25, 26) which utilize the Na\(^+\) gradient generated by Na\(^+\),K\(^{-}\)-ATPase as the energy source for extrusion of Mg\(^{2+}\). When the Na\(^+\) gradient was reduced about 4-fold by gramici-
increase in [Mg$^{2+}$] increase in [Na$^{+}$]

This possibility, and clearly demonstrated the release of Mg$^{2+}$ from an intracellular pool. Moreover, superfusing acinar cells in a cytosolic-like [Na$^{+}$] and [K$^{+}$] medium to prevent net movement of these ions upon exposure to gramicidin abolished the increase in [Mg$^{2+}$], inferring that Mg$^{2+}$ release requires an increase in the [Na$^{+}$]. In agreement with this observation, increasing [Na$^{+}$] by treating acini with sodium propionate produced an increase in [Mg$^{2+}$] similar to that seen with gramicidin (Fig. 4). The effects of gramicidin on the increase in [Mg$^{2+}$] were not likely due to increased permeability of cations across the membranes of intracellular organelles considering gramicidin primarily affects the plasma membrane (27).

The muscarinic-stimulated mobilization of Mg$^{2+}$ is Na$^{+}$-dependent (7). Depletion of cytosolic Na$^{+}$ blunted the CCh-stimulated [Mg$^{2+}$], increase without significantly influencing the CCh-induced increase in [Ca$^{2+}$] (Figs. 6 and 7). Furthermore, suppressing Na$^{+}$ influx mediated by the Na$^{+}$/K$^{+}$/2Cl$^{-}$ cotransporter by replacing Cl$^{-}$ (28) with either gluconate or SCN$^{-}$ blunted the CCh-stimulated increase in [Mg$^{2+}$] (data not shown), clearly indicating that the muscarinic-stimulated Mg$^{2+}$ release, like the gramicidin-induced increase in [Mg$^{2+}$], required an increase in the [Na$^{+}$]. Thus, it appears likely that the increases in [Mg$^{2+}$], induced by both gramicidin and CCh were from the same intracellular pool.

In summary, our data indicated that increasing the intracellular [Na$^{+}$], was necessary for the mobilization of Mg$^{2+}$ from an intracellular pool by Ca$^{2+}$-mobilizing agonists. Bypassing receptors, with either gramicidin or Na$^{+}$-propionate, raised the intracellular [Na$^{+}$] and increased the [Mg$^{2+}$]. These results show that neither receptor activation nor Ca$^{2+}$ mobilization is required to discharge the intracellular Mg$^{2+}$ pool. Thus, the agonist-stimulated increase in [Mg$^{2+}$] involves a cascade of events including an initial increase in [Ca$^{2+}$], which activates Na$^{+}$ influx. The resultant increase in [Na$^{+}$] involves a cascade of events including an initial increase in [Ca$^{2+}$], which activates Na$^{+}$ influx. The resultant increase in [Na$^{+}$], activates Na$^{+}$/Mg$^{2+}$ exchangers located in the membrane of an intracellular pool.

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FIG. 8. Quinidine inhibits the CCh- and gramicidin-induced increases in the cytosolic free Mg$^{2+}$ concentrations. Mg-fura-2-loaded acini were superfused with 0.5 mM quinidine beginning 2 min prior to stimulation in either Mg$^{2+}$-containing (+Mg$^{2+}$) or Mg$^{2+}$-free (−Mg$^{2+}$) media. At the time indicated by the arrows, either 10 μM CCh (panels A and B) or 5 μM gramicidin (Gram, panel C) was applied. Each trace is a representative response (n ≥ 5 for all treatments).

FIG. 9. Quinidine does not inhibit the CCh-induced increases in the cytosolic free Ca$^{2+}$ and Na$^{+}$ concentrations. Rat sublingual acini were loaded with ion-sensitive fluorescent indicators (fura-2 or sodium green). Dye-loaded acini were exposed to 0.5 mM quinidine for approximately one min and then stimulated with 10 μM CCh in PSS at the time indicated by the arrows. Upper panel, fura-2-loaded acini were superfused with quinidine and then stimulated in the continued presence of quinidine. Lower panel, sodium green-loaded acini were superfused with quinidine and then stimulated in the continued presence of quinidine. Each trace is a representative response. Upper panel, [Ca$^{2+}$], n = 5; lower panel, [Na$^{+}$], n = 7.
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