Calcium–magnesium interactions in pancreatic acinar cells

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ABSTRACT **Although the role of calcium (Ca^{2+}) in the signal transduction and pathobiology of the exocrine pancreas is firmly established, the role of magnesium (Mg^{2+}) remains unclear. We have characterized the intracellular distribution of Mg^{2+} in response to hormone stimulation in isolated mouse pancreatic acinar cells and studied the role of Mg^{2+} in modulating Ca^{2+} signaling using microspectrofluorometry and digital imaging of Ca^{2+}- or Mg^{2+}-sensitive fluorescent dyes as well as Mg^{2+}-sensitive intracellular microelectrodes. Our results indicate that an increase in intracellular Mg^{2+} concentrations reduced the cholecystokinin (CCK) -induced Ca^{2+} oscillations by inhibiting the capacitive Ca^{2+} influx. An intracellular Ca^{2+} mobilization, on the other hand, was paralleled by a decrease in $[Mg^{2+}]_i$, which was reversible upon hormone withdrawal independent of the electrochemical gradients for Mg^{2+}, Ca^{2+}, Na^+, and K^+, and not caused by Mg^{2+} efflux from acinar cells. In an attempt to characterize possible Mg^{2+} stores that would explain the reversible, hormone-induced intracellular Mg^{2+} movements, we ruled out mitochondria or ATP as potential Mg^{2+} buffers and found that the CCK-induced $[Mg^{2+}]_i$ decrease was initiated at the basolateral part of the acinar cells, where most of the endoplasmic reticulum (ER) is located, and progressed from there toward the apical pole of the acinar cells in an antiparallel fashion to Ca^{2+} waves. These experiments represent the first characterization of intracellular Mg^{2+} movements in the exocrine pancreas, provide evidence for possible Mg^{2+} stores in the ER, and indicate that the spatial and temporal distribution of intracellular Mg concentrations profoundly affects acinar cell Ca^{2+} signaling.—Mooren, F. C., Turi, S., Günzel, D., Schlue, W.-R., Domschke, W., Singh, J., Lerch, M. M. Calcium–magnesium interactions in pancreatic acinar cells. *FASEB J.* 15, 659–672 (2001)**

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In contrast to the rapid intracellular calcium changes in response to external stimuli, the role of which in various signal transduction events is now firmly established (1–4), the importance of intracellular magnesium changes is largely unknown. Although Mg^{2+} is a known cofactor for multiple enzymatic reactions and its intracellular concentration has been shown to respond to hormonal stimuli—albeit in a much slower fashion than the corresponding Ca^{2+} concentrations—its role in regulating physiological or pathophysiological processes has remained speculative. Important similarities and differences regarding the physiology of the two divalent cations calcium and magnesium have been established. Intracellular calcium concentrations are generally kept at very low levels, and these low intracellular calcium levels (of ~100 nM) not only require the active maintenance of a 10,000-fold calcium gradient across the plasma membrane, but are also a prerequisite for the intracellular messenger role of calcium (5). Conversely, whereas magnesium has been shown to be involved in the modulation of various ion channels and transporters (6, 7) and this modulating effect appears to be of great pathophysiological and clinical relevance (8), its gradient across the plasma membrane is very small. It has been assumed that the intracellular magnesium concentration is, analogous to the intracellular calcium concentration, tightly controlled and regulated by agonist-dependent signaling events. However, only recent advances in the development of microanalytical methods, such as Mg^{2+}-sensitive fluorescent probes (9) and Mg^{2+}-selective microelectrodes (10), have made investigations into the intracellular Mg^{2+} regulation possible (11–14) and enabled experiments on Mg^{2+} changes in response to hormone agonists in either culture cell systems (15, 16) or primary cell isolates (17–19).

In this study we have investigated the biological role of Mg^{2+} in the exocrine pancreas, an organ system in which rapid intracellular Ca^{2+} changes determine the

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onset and course of physiological (1) as well as pathophysiological (20) events. Using isolated mouse pancreatic acini—intact secretory units of living exocrine cells—we used Mg^{2+}-sensitive fluorescent indicators as well as intracellular ion-selective microelectrodes to investigate the spatial and temporal changes in $[Mg^{2+}]_i$ in response to physiological and pathological stimuli. We have further determined their correlation to and interference with the intracellular calcium changes in pancreatic acinar cells. Our results indicate that intracellular Mg^{2+} concentrations in the exocrine pancreas are regulated in a stimulus-controlled manner. Moreover, they can counteract effects in the Ca^{2+} signal transduction pathway; the 'slower' uptake and release of Mg^{2+} from intracellular, most probably ER-associated stores, occur in a manner that is completely antiparallel to the 'rapid' Ca^{2+} release and reuptake in acinar cells.

MATERIALS AND METHODS

Materials

Magfura-2/AM, fura-2/AM, fluo-3/AM, BCECF, and BAPTA/AM were from Molecular Probes (Eugene, Oreg.). Cell-Tak was obtained from Collaborative Research Inc. (Bedford, Mass.). All other reagents were of the highest purity available and purchased from Sigma (St. Louis, Mo.) unless indicated otherwise.

Preparation of pancreatic acini

All animal experiments were approved by and conducted under the guidelines of the animal welfare committee of Münster University. Adult male white mice (NMRI) weighing between 20 and 25 g were obtained from Charles River (Sulzfeld, Germany), kept in Nalgene shoe box cages in a 12 h:12 h light:dark cycle with unlimited access to standard chow and water, and adjusted to laboratory conditions over the course of 1 wk. Pancreatic acini were prepared by a modified collagenase protocol as previously reported (21, 22). In brief, after a 12 h fast the animals were killed and the pancreas was rapidly removed, minced into small pieces, and placed into buffer (pH 7.4) containing NaCl (130 mM); KCl (5 mM); HEPES (10 mM); KH_2PO_4 (1.2 mM); $CaCl_2$ (1 mM); $MgSO_4$ (1 mM); glucose (10 mM); and collagenase (100 U/ml Type V, Sigma, München, Germany). After 10 min incubation at 37°C under continuous shaking (120 cycles/min), the digested tissue was washed 3 times in 10 ml buffer without the presence of collagenase and again shaken 10 times to dissociate the acini. Acini were filtered through muslin gauze, centrifuged at 400 rpm for 3 min, and washed twice more in buffer solution containing 4% bovine serum albumin (BSA). After the second wash, acini were suspended in 8 ml of buffer containing soybean trypsin inhibitor (0.1 mg/ml) and BSA (0.2% w/v). A stock suspension of acini was kept on ice for up to 4 h without significant reduction in cell viability (>95%) as assessed by trypan blue exclusion.

Loading of acini with ion-sensitive probes

For microfluorometric experiments acini were loaded with 5 μM of the ester of either magfura-2 or fura-2 at room temperature for 20 min for measurements of either $[Mg^{2+}]_i$ or $[Ca^{2+}]_i$, respectively. Wherever calcium and magnesium measurements were performed simultaneously, magfura-loaded acini were subjected to a second incubation with the calcium-sensitive probe fluo-3 (in combination with 0.001% Pluronic) for an additional 35 min at 35°C. Acini were then washed twice by centrifugation and preincubated for 30 min to allow complete de-esterification of the probes. Loaded cells were plated onto glass coverslips coated with Cell-Tak (1 $\mu l/cm^2$) and kept at room temperature for 30 min before use. In experiments where the uptake of magfura-2 into intracellular calcium stores was quantified, the nonesterified dextran derivative of magfura-2 was microinjected into individual acinar cells using an Eppendorf microinjection instrument.

Measurements of cytosolic free $[Mg^{2+}]_i$, $[Ca^{2+}]_i$ and pHi

Plated cells were mounted in a perfusion chamber (internal volume 0.28 ml) and placed on the stage of a Nikon Diaphot-TMD inverted microscope equipped with a Fluor X 100 oil immersion objective. Acini were continuously perfused with medium at a flow rate of 3 ml min^{-1} at room temperature. Fluorochrome excitation (340 and 380 nm for magfura-2 and fura-2, 488 nm for fluo-3, 495 and 440 nm for BCECF) was achieved using a Xenon lamp in series with two chopper-linked monochromators that were coupled to the microscope via fiber optics. Emitted light was collected behind a band-pass filter (509 nm for magfura-2 and fura-2, 535 nm for fluo-3, 538 nm for BCECF) by either a photomultiplier or an intensified CCD camera. In control background, fluorescence was found to be negligible. Ratios obtained from the dual excitation wavelengths probes magfura-2 and fura-2 were converted into magnesium and calcium concentrations by the formula given by Grynkiewicz et al. (23): $[Mg^{2+}]_i$ and $[Ca^{2+}]_i = (R - R\ min)/(R\ max - R) \times K_D \times (I\ ion\text{-}free/I\ ion\text{-}saturated)_{380\ nm}$, in which R min and R max are the minimal and maximal ratios obtained in either ion free or ion saturated solutions, respectively. K_D is the dissociation constant of magfura-2 for magnesium or of fura-2 for calcium, and I is the fluorescence intensity at the 380 nm wavelength of the free dye divided by the saturated ion-dye complex. All calibration parameters were obtained in separate experiments.

In a different approach, $[Mg^{2+}]_i$ was determined using a magnesium-sensitive intracellular microelectrode. The preparation of Mg^{2+}-sensitive microelectrodes was carried out as described by Hintz et al. (24). In brief, double-barreled microelectrodes were pulled from theta-style borosilicate glass (Clark, Reading, England). One of the two channels was silanized (40 min exposure to hexamethyldisilazan vapor at 40°C, followed by 2 h baking at 200°C); the second channel was prevented from being silanized by continuous perfusion with compressed air (1.5 bar). The tip of the silanized channel was filled with the Mg^{2+} sensor based on the neutral carrier ETH 5214 (Fluka, Buchs, Switzerland) and then backfilled with 100 mM $MgCl_2$; the other channel was filled with 3 M KCl and served as an intracellular reference electrode. All potentials were measured against the potential of an extracellular reference electrode (agar bridge and Ag/AgCl cell), using a two-channel voltmeter with differential amplifier (FD 223, WPI, Berlin, Germany). All output signals were AD-converted and recorded continuously on a PC. Before and after an experiment, the microelectrodes were calibrated in solutions containing 10, 2.5, 0.5, and nominally 0 mM $MgCl_2$ at an ionic background simulating intracellular conditions (in mM: KCl, 110; NaCl, 10; HEPES, 10, pH adjusted to 7.3 with KOH). Calibration curves were fitted with the Nicolsky-Eisenman equation, as described by Günzel and Schlue (25).

Statistical analysis

Data shown in the figures are representative of 6 or more individual experiments in each group. Wherever applicable, data points represent means ± standard errors of the means (SE). Differences between groups were compared using the Student's t test and P values <0.05 were considered significant.

RESULTS

Because it has been shown that the presence of Mg^{2+} at either the extracellular or intracellular face of the plasma membrane can modulate ion transport systems including Ca^{2+} channels and pumps, we studied the effect of a perturbation of extracellular $[Mg^{2+}]_e$ on cholecystokinin-octapeptide (CCK) -evoked Ca^{2+} signals in pancreatic acinar cells. When acinar cells were exposed to concentrations of the secretagogue CCK that elicit a submaximal secretory response (10 pM), they responded with a characteristic pattern of calcium oscillations as indicated by the transient spiking pattern of fura-2 fluorescence in the cytosol. Neither an acute decrease nor an acute increase of the extracellular magnesium concentration had any significant effect on the frequency of intracellular calcium oscillations in response to CCK (**Fig. 1*A*, *B***). On the other hand, when acini were preincubated for 60 min in the presence of different extracellular magnesium concentrations, CCK evoked a profound effect on the frequency of calcium transients. Under near physiological $[Mg^{2+}]_e$ of 1.0 mM (Fig. 2*A*) calcium oscillations in response to CCK were recorded at a frequency of 1 min^{-1}. A preincubation in magnesium-rich buffer ($[Mg^{2+}]_e$, 5 mM) resulted in a decrease of this frequency to ~0.6 min^{-1} (Fig. 2*C*); a further increase of $[Mg^{2+}]_e$ to 10 mM reduced the frequency of calcium oscillations to ~0.3 spikes min^{-1} (Fig. 2*D*) and often resulted in a complete termination of the oscillations. In contrast, a decrease in the $[Mg^{2+}]_e$ to 0.2 mM increased the frequency of calcium oscillations to ~1.1 min^{-1} (**Fig. 2*B***).

Because Mg^{2+} can potentially interact with a variety of Ca^{2+} transport mechanisms at either the plasma membrane or at the membrane of intracellular calcium stores, it was necessary to elucidate in what manner it may affect calcium oscillations. Calcium oscillations are assumed to result from a rapid emptying and refilling of intracellular calcium stores. The filling state of these stores is therefore the net result of calcium leakage from the stores and the activity of the sarcoplasmic reticulum Ca^{2+}-ATPase. To determine whether Mg^{2+} was affecting this filling state of calcium stores, acini were incubated with the calcium ionophore ionomycin in a calcium-free solution. This results in a rapid leakage of Ca^{2+} from the stores and allows for the quantification of stored calcium. A preincubation in the presence of the different Mg^{2+} concentrations that had resulted in marked changes in Ca^{2+} oscillations, however, had no effect on the ionomycin-induced release of Ca^{2+} from the cells (data not shown).

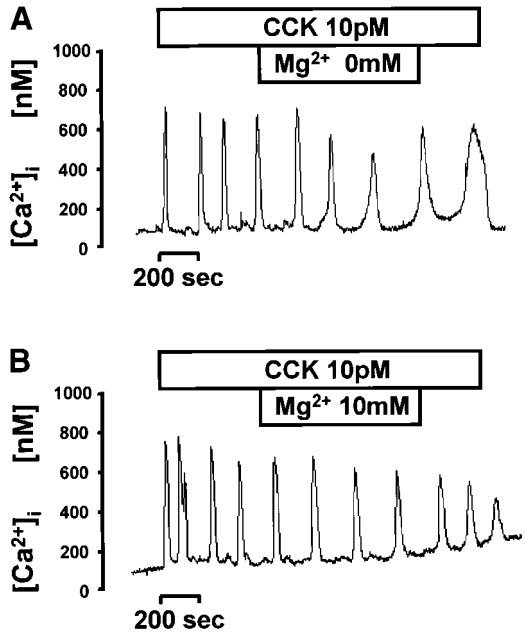

Figure 1. Isolated mouse pancreatic acinar cells were prepared by collagenase digestion, loaded with the calcium-sensitive dye fura-2, and stimulated with 10 pM CCK. Spectrofluorometric calcium recordings were performed on single cells at an excitation wavelength of 340/380 nm and an emission wavelength of 509 nm. Data shown in this or the subsequent figures are representative of a minimum of 6 or more individual experiments. Note that neither an acute decrease (*A*) nor an acute increase (*B*) of the extracellular magnesium concentration had a significant effect on CCK-evoked calcium oscillations.

One of the alternative mechanisms for the interference of Mg^{2+} with calcium oscillations, that of a calcium influx into acinar cells via the plasma membrane, was tested using the manganese influx technique. For this assay the manganese-induced quenching of fura-2 fluorescence at the isosbestic wavelength of 360 nm was used as an indicator of calcium movements across the plasma membrane (26). A reduction of $[Mg^{2+}]_e$ in the preincubation buffer from 1 mM to 0.2 mM enhanced the manganese-induced quenching at 360 nm significantly (**Fig. 3**), whereas an increase in $[Mg^{2+}]_e$ to 10 mM greatly reduced manganese bleaching. This result indicates that one mechanism by which magnesium interacts with calcium signaling involves inhibition of the capacitive calcium influx pathway in pancreatic acinar cells.

The question remained as to whether changes in $[Mg^{2+}]_e$ or changes in $[Mg^{2+}]_i$ could account for this effect. The observation that an extended period of preincubation was required before increased Mg^{2+} levels in the buffer affected CCK-induced calcium oscillations suggests that the latter is the case. To study the effect of $[Mg^{2+}]_i$ we used the magnesium-sensitive probe magfura-2. Preincubation for 60 min in buffer containing either 0.2 mM, 1.2 mM, or 10 mM magnesium did indeed result in different intracellular magnesium concentrations (n=22) of 0.41 ± 0.07 mM, 0.56 ± 0.08 mM, or 1.25 ± 0.29 mM, respectively.

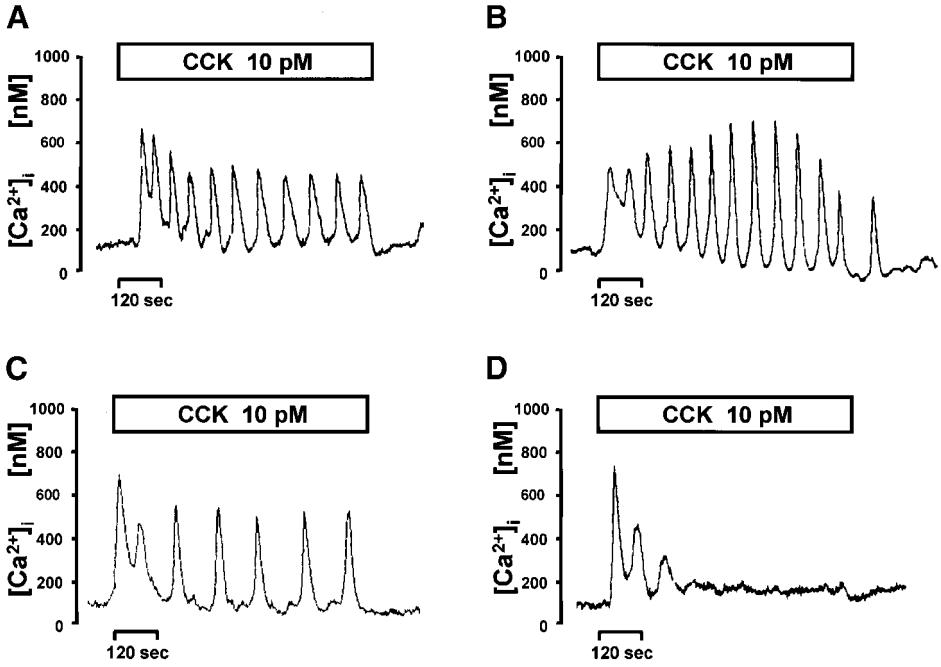

Figure 2. Isolated acinar cells were incubated in buffer containing different Mg^{2+} concentrations for 60 min. Calcium transients were recorded as denoted in the Fig. 1 legend. Under control conditions ($[Mg^{2+}]_e$ 1.0 mM) the frequency of CCK-induced (10 pM) calcium oscillations was ~1/min (*A*). Low $[Mg^{2+}]_e$ of 0.2 mM increased the frequency of calcium oscillations (*B*) whereas high $[Mg^{2+}]_e$ of 5 mM decreased the frequency of the oscillations (*C*). Oscillations often ceased completely when cells were incubated in buffer containing 10 mM Mg^{2+} (*D*).

Within a preparation of acini that was preincubated at the identical $[Mg^{2+}]_e$, the $[Mg^{2+}]_i$ of individual cells still varied to some degree, probably due to differences between individual acinar cells in regulating magnesium uptake. We made use of these individual differences between acinar cells and directly correlated the basal $[Mg^{2+}]_i$ with the frequency of calcium oscillations after loading cells simultaneously with a calcium-sensitive probe (fluo-3) together with a magnesium-sensitive probe (magfura-2) and exposing the cells to different extracellular magnesium concentrations for 60 min. We found an almost linear correlation between the intracellular magnesium concentration and the suppression of CCK-induced calcium oscillations (**Fig. 4**).

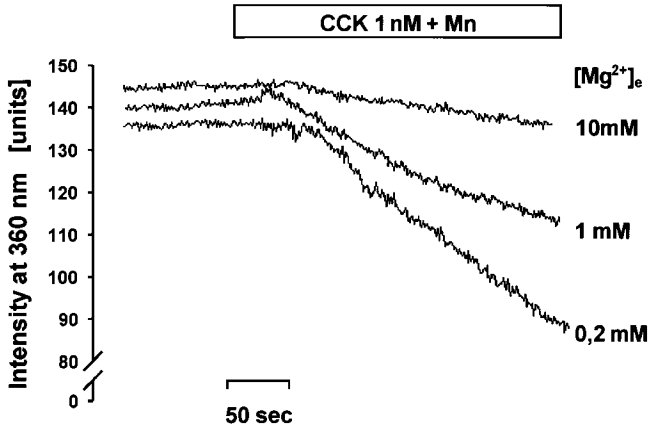

Figure 3. Calcium influx in response to CCK stimulation (1 nM) was studied after 60 min of preincubation of acinar cells in buffer containing various Mg^{2+} concentrations. Ca^{2+} entry was measured as the bleaching rate of fura-2 by manganese (Mn) at the isosbestic wavelength of 360 nm. After preincubation in the presence of low $[Mg^{2+}]_e$, the bleaching rate was found to be increased in comparison to control conditions ($[Mg^{2+}]_e$=1 mM), which indicates an increased Ca^{2+} entry. In contrast, after preincubation at high $[Mg^{2+}]_e$ of 10 mM, Ca^{2+} entry was found decreased.

To study whether agonist-evoked changes in intracellular calcium concentrations were paralleled by comparable intracellular magnesium changes, we used the same magfura-2-based technique to quantify $[Mg^{2+}]_i$ in individual acinar cells. Addition of CCK (100 pM) to isolated acinar cells resulted in an initial increase of the magfura-2 fluorescence ratio, followed by a sustained decrease, with a return of the ratio to prestimulatory values after CCK was removed from the medium (**Fig. 5*A***). This time course of the magfura-2 fluorescence signal could suggest that an initial increase in $[Mg^{2+}]_i$ is followed by a decrease in the free magnesium concentration and a return to basal levels after withdrawal of the stimulus. When, instead of using maximal CCK concentrations, we exposed acinar cells to a submaximal concentration of CCK (10 pM) that produced Ca^{2+} oscillations, we found that in 30–40% of the cells the decrease in magfura-2 ratio progressed in a stepwise manner, and the frequency of these steps corresponded to the frequency of agonist-evoked calcium oscillations (Fig. 5*B*). This observation suggests that the magfura-2 signal could have reflected not only changes in $[Mg^{2+}]_i$, but also a detection of calcium transients, which would have been accounted for by the known low-calcium affinity of magfura-2. The y-axes in Figs. 5–7*B*, 11, and 12 therefore denote the magfura ratio rather than $[Mg^{2+}]_i$ in order to document potential interferences of the signal by calcium. Because of this potential codetection of calcium signals by magfura-2 (which although unlikely in the low calcium conditions of resting cells, would have compromised our $[Mg^{2+}]_i$ measurements in the presence of the micromolar calcium concentrations reported in agonist-stimulated cells; ref 27–29), we have validated our methods to

determine intracellular magnesium in a number of ways.

To differentiate between the detection of calcium and magnesium by magfura-2, we used chelating agents with different affinities to the two cations. Whereas BAPTA binds to calcium with higher affinity than to magnesium, EDTA chelates both ions with comparable affinities. Moreover, the on rate of EDTA is slower than that of BAPTA, which facilitates the diffusion of calcium ions. Loading acinar cells with EDTA-AM resulted in an agonist-evoked initial increase of the magfura-2 signal that was of lower amplitude than in controls (**Fig. 6*A***) and, subsequently, in a slower and less pronounced decrease of the magfura-2 ratio. Loading acini with BAPTA-AM, on the other hand, completely abolished the initial increase of magfura-2 fluorescence as well as the stepwise reduction of the magfura-2 ratio, which in turn was replaced by a precipitous decline (Fig. 6*B*). The result of this experiment suggested that the magfura-2 signal in the presence of the more calcium-selective chelator BAPTA specifically reflected an intracellular magnesium signal. An alternative explanation could still have been a decrease of free calcium within intracellular stores because magfura-2 had been used as a low-affinity indicator to detect calcium in intracellular stores (30–32). In the respective experiments, however, longer loading periods and higher loading concentrations of magfura-2-AM were used in order to saturate intracellular esterases and thus allow the magfura-2-ester to leave the cytosol intact and enter calcium stores. Since our aim was to detect cytosolic magnesium rather than store-confined calcium, we loaded cells with magfura-2 at lower concentrations and for shorter periods of time.

To test what proportion of magfura-2 had remained cytosolic rather than being taken up into intracellular stores, we permeabilized the plasma membrane with 5 μM digitonin and quantified dye fluorescence at 360 nm. This low digitonin concentration has been found to permeabilize the plasma membrane without affecting the membrane of intracellular ion stores (33). We found that >95% of magfura-2 had remained in the cytosol and only a negligible amount of the probe had entered intracellular stores (**Fig. 7*A***). In another attempt to rule out the detection of a calcium leak from intracellular stores rather than a decrease of cytosolic magnesium by the magfura-2-fluorescence, we used the dextran derivative of magfura-2, which is nonpermeant for the plasma membrane or the calcium-store mem-

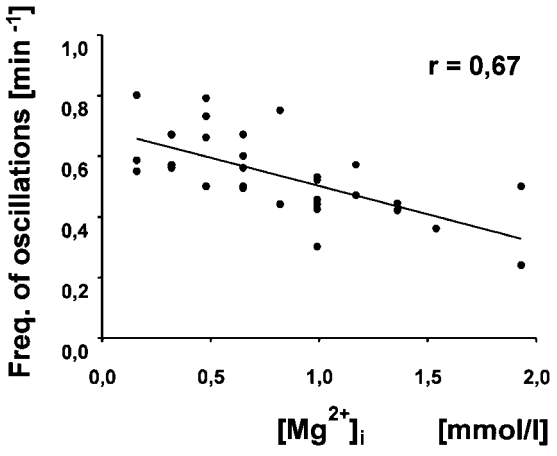

Figure 4. Relationship between the prestimulatory basal $[Mg^{2+}]_i$ and the frequency of CCK-evoked (10 pM) calcium oscillations in isolated pancreatic acinar cells. Cells were coloaded with magfura-2 and fluo-3. $[Mg^{2+}]_i$ was determined as magfura-2 ratio (excitation wavelength 340/380 nm and emission wavelength 509 nm) and $[Ca^{2+}]_i$ simultaneously as fluo-3 fluorescence (excitation wavelength 488 nm and emission wavelength 535 nm). Note the near linear relationship between intracellular magnesium levels and the decline in the frequency of calcium oscillations.

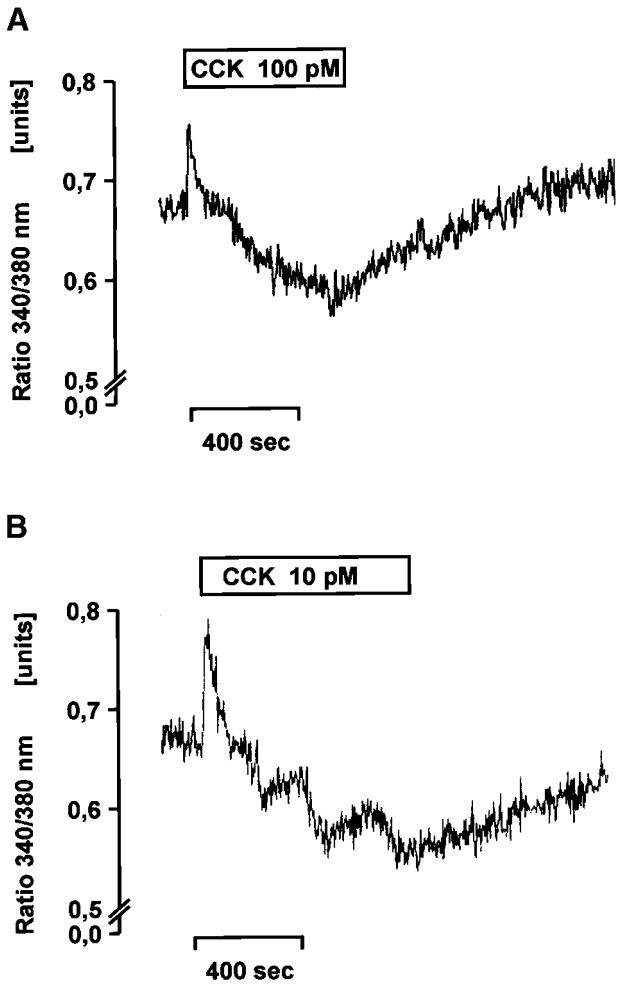

Figure 5. Effect of CCK stimulation on the magfura-2 ratio in single pancreatic acinar cells. Spectrofluorometric magnesium recordings were performed at an excitation wavelength of 340/380 nm and an emission wavelength of 509 nm. *A)* 100 pM CCK evoked an initial peak of the magfura-2 ratio, followed by a sustained decrease. The magfura-2 ratio returned to prestimulatory values after CCK withdrawal. *B)* After the initial peak, acinar cells responded to 10 pM CCK with a stepwise decrease of the magfura-2 ratio in 30–40%. The decrease of the magfura-2 ratio was again reversible after CCK withdrawal. Note that the *y* axis of this figure as well as of Figs. 6, 7B, 11, and 12 denotes the ratio of the magfura signal rather than $[Mg^{2+}]_i$ in order to document potential interference by intracellular calcium.

brane, and microinjected it directly into the cytosol of acinar cells. The CCK-induced magfura-2 signal in microinjected cells (Fig. 7*B*) was identical to the signal obtained from cells that had been loaded by incubation with magfura-2-AM (Fig. 5*A*). This finding again indicates that the magfura-2 signal reflected cytosolic magnesium concentrations rather than the calcium in intracellular stores. We finally used an electrophysiological approach to confirm the nature of the magfura-2 signal in response to CCK stimulation and in the presence of BAPTA. Ion-selective microelectrodes were inserted into single acinar cells as well as tissue fragments in order to record changes in $[Mg^{2+}]_i$ simultaneous with the membrane potential.

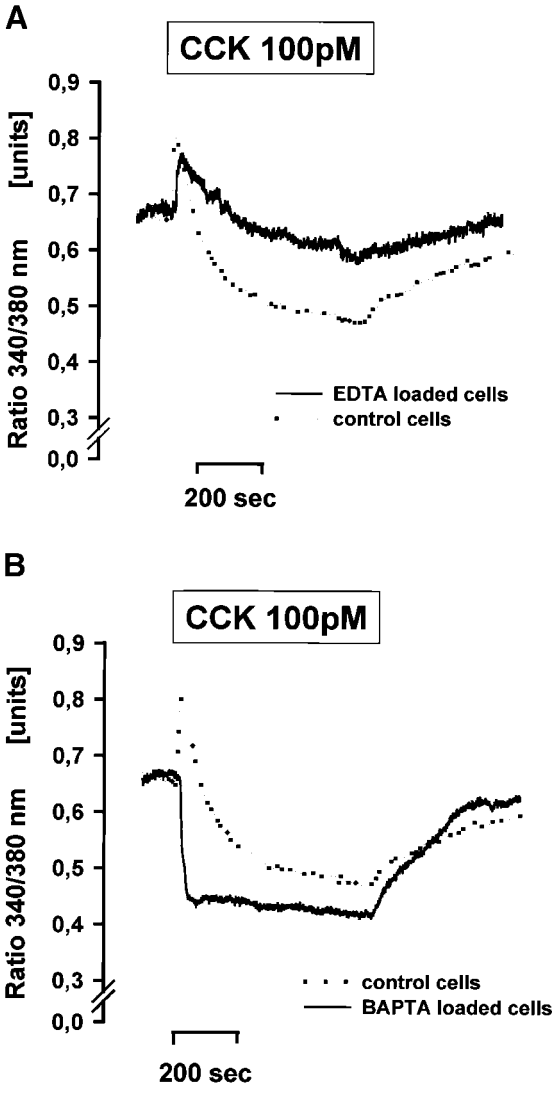

Figure 6. Effect of the calcium chelators EDTA (*A*) and BAPTA (*B*) on the CCK-evoked (100 pM) changes of the magfura-2 ratio. Acinar cells were loaded with magfura-2 and either EDTA-AM or BAPTA-AM simultaneously. Magfura-2 ratio changes in response to CCK were determined as indicated in the Fig. 4 legend. The changes in response to CCK in control cells (without EDTA or BAPTA) are indicated as a dotted line in both panels. In EDTA-loaded cells the CCK-evoked initial increase as well as the subsequent decrease of the magfura-2 signal was attenuated, whereas in BAPTA-loaded cells the initial peak was abolished and the ratio decline was found enhanced.

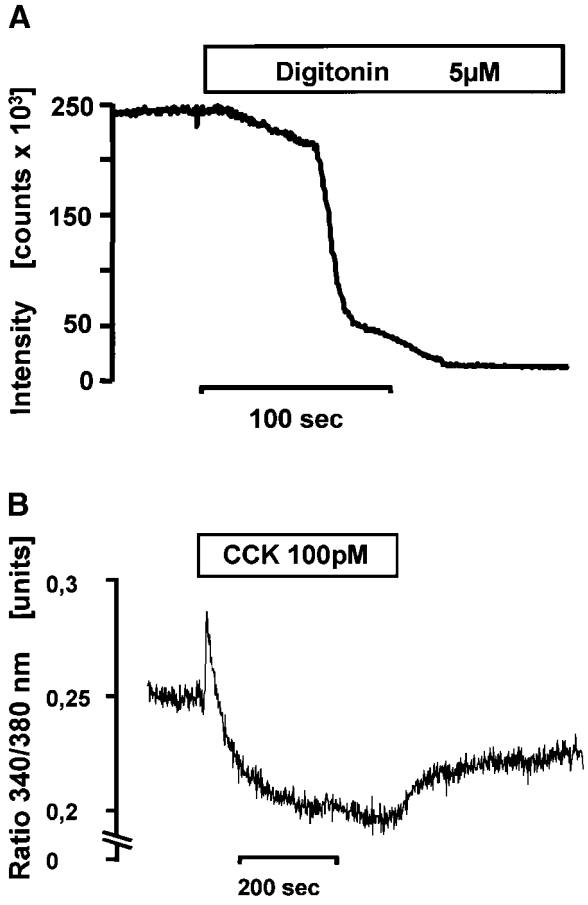

Figure 7. Experimental approaches to define the intracellular localization of magfura-2. *A)* Permeabilization with low concentration of digitonin (5 µM) led to a rapid release of >95% of the total fluorescence, which indicates a predominantly cytosolic localization of magfura-2 and excludes a significant uptake into intracellular stores. *B)* When the dextran derivative of magfura-2 was microinjected directly into the cells in order to ensure a strictly cytosolic localization, the subsequent stimulation with CCK (100 pM) evoked the same biphasic change of the magfura-2 ratio as in magfura-2-loaded cells (see Fig. 5*A*). This indicates that ratio changes represented changes in cytosolic magfura-2 concentrations and rules out a participation of intracellular ion stores.

The basal mean $[Mg^{2+}]_i$ as determined by the magnesium-sensitive electrode (n=45) was 0.58 mM (pMg 3.24±0.26). After CCK stimulation $[Mg^{2+}]_i$ decreased by 0.11 ± 0.12 mM and, after withdrawal of the stimulus, returned to baseline levels (**Fig. 8**). By using this independent approach to quantify $[Mg^{2+}]_i$ in pancreatic acini, we were not only able to validate the magfura-2 ratio as a reliable indicator for intracellular magnesium measurements, but found that recordings in the presence of the Ca^{2+}-chelator BAPTA most accurately reflected absolute $[Mg^{2+}]_i$. All Mg^{2+} measurements in the ensuing experiments therefore used the magfura-2 technique in BAPTA-AM-loaded acini unless indicated otherwise. The basal $[Mg^{2+}]_i$ in pancreatic acinar cells obtained by this method (n=62) was 0.59 ± 0.13 mM and the corresponding decrease in

response to 100 pM CCK was 0.22 ± 0.1 mM. These results are in good agreement with the electrophysiological data obtained in this study.

To determine whether the changes in intracellular magnesium concentrations depended on the specific activation of the CCK receptor and its second messenger pathway, we tested other calcium mobilizing agents. In response to a maximal stimulating concentration of the acetylcholine analog carbachol (100 μM), $[Mg^{2+}]_i$ decreased in a manner completely parallel to that observed after CCK stimulation and returned to baseline levels after the withdrawal of carbachol. Neither the addition of carbachol to CCK-stimulated cells nor the addition of CCK to carbachol-stimulated cells caused a further decrease in $[Mg^{2+}]_i$ or changed the kinetics of the recovery (data not shown). A nonreceptor-dependent manner in which intracellular calcium mobilization can be affected is by inhibiting the Ca^{2+}-ATPase activity of calcium stores with, for example, thapsigargin. Thapsigargin therefore depletes intracellular calcium stores and this calcium depletion is followed by a subsequent Ca^{2+} influx into acinar cells. Incubation of acini with 1 μM thapsigargin resulted in a slow and only moderate decrease in $[Mg^{2+}]_i$. A subsequent stimulation with CCK, however, induced an additional and rapid decrease in $[Mg^{2+}]_i$ (**Fig. 9*A***). When we reversed the sequence of incubation, we found that exposure to thapsigargin did not induce any further decrease in $[Mg^{2+}]_i$ in addition to that evoked by CCK (Fig. 9*B*). However, in both types of experiments, thapsigargin treatment prevented the recovery of the magfura-2 signal after withdrawal of the stimulus.

The rapid disappearance of significant amounts of magnesium from the acinar cell cytosol might be caused by two alternative mechanisms. The first would involve a magnesium efflux from acinar cells across the acinar cell membrane whereas the second would involve a magnesium uptake into intracellular stores. Provided a magnesium efflux from acinar cells had occurred, this efflux would most likely be affected by gradient changes across the plasma membrane of either magnesium itself or other ions. To test this possibility, we changed the ion concentrations in the extracellular buffer in a number of ways. Neither an increase to high magnesium concentrations (10 mM, not shown) nor incubation in magnesium-free buffer (**Fig. 10*A***) had any effect on intracellular magnesium changes in response to CCK stimulation.

Because a sodium-magnesium antiporter has been suggested to be operative in a variety of cells, we tested its potential role in regulating $[Mg^{2+}]_i$ in acini by replacing sodium with N-methyl-D-glucamine in the perfusion buffer. In addition, we omitted Ca^{2+} from the buffer to avoid an expected Ca^{2+} increase in response to the sodium depletion, which has been shown to be mediated by a sodium-calcium exchanger. Again, neither the presence nor the absence of sodium and calcium in the extracellular fluid had any significant effect on $[Mg^{2+}]_i$ changes in response to external stimuli (Fig. 10*B*). Similarly, neither the changes in membrane potential caused by exposure to high potassium concentrations (Fig. 10*C*) nor the complete omis-

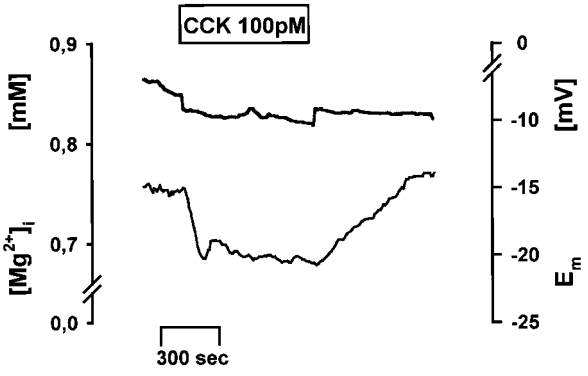

Figure 8. Simultaneous recordings of the membrane potential (upper curve, right scale) and the cytosolic magnesium concentration (lower curve, left scale) as determined by a triple-barreled microelectrode inserted into the cytosol of an isolated pancreatic acinar cell. Note the simultaneous decline in $[Mg^{2+}]_i$ together with the membrane depolarization in response to 100 pM CCK. Data shown are representative of 14 individual experiments.

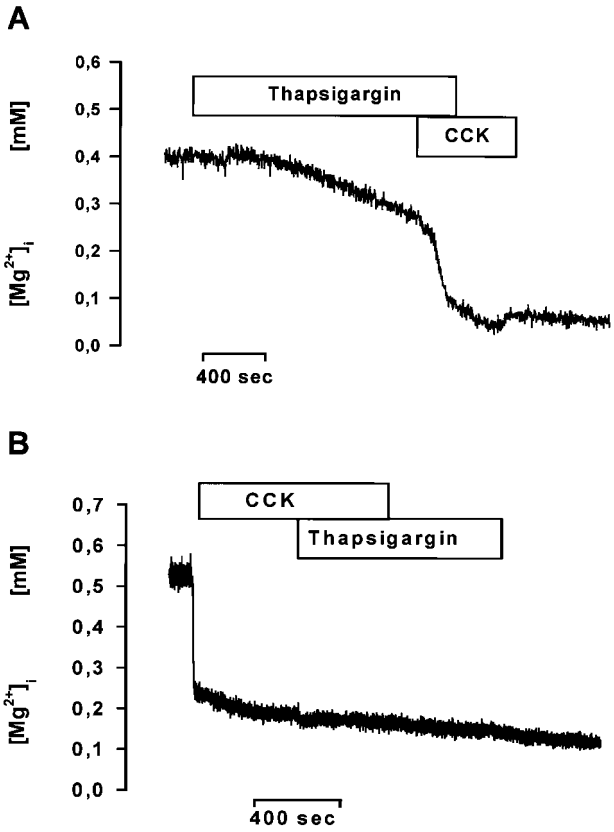

Figure 9. Incubation of magfura-2-loaded acinar cells with CCK and thapsigargin, an inhibitor of the sarcoplasmic reticulum ATPase. Thapsigargin induced a much slower decrease in $[Mg^{2+}]_i$ than that observed with CCK (*A*). The precipitous decline in $[Mg^{2+}]_i$ that followed exposure of acinar cells to CCK was not increased or affected by the addition of thapsigargin (*B*). Note that the *y* axis of this figure (as in Figs. 10 and 13) denotes intracellular magnesium concentrations because BAPTA-loaded cells were investigated to exclude interference by intracellular calcium.

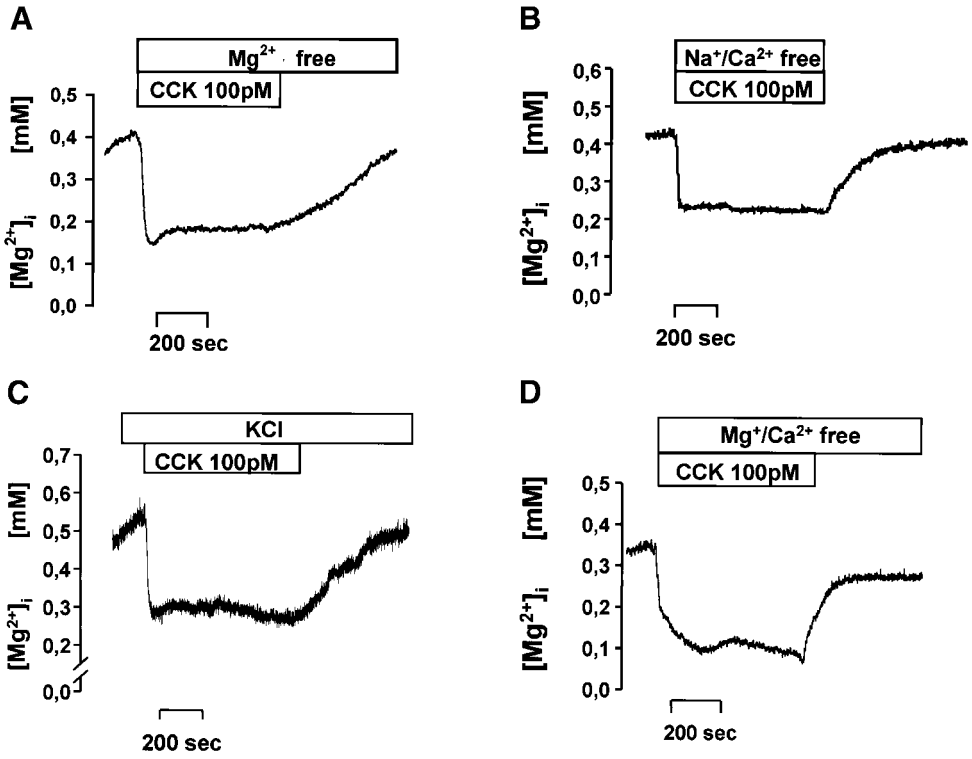

Figure 10. Effect of electrochemical gradient for different ions on $[Mg^{2+}]_i$ after CCK stimulation. Isolated acinar cells were loaded with BAPTA-AM and $[Mg^{2+}]_i$ changes in response to CCK stimulation (100 pM) were measured as indicated in the Fig. 4 legend. Neither the omission of Mg^{2+} (*A*), the omission of Na^+ and Ca^{2+} (*B*), the omission of K^+ (*C*), nor the omission of Mg^{2+} and Ca^{2+} (*D*) from the buffer changed the kinetics of intracellular magnesium changes in response to CCK.

sion of magnesium and calcium from the extracellular medium had any noticeable effect on the CCK-induced $[Mg^{2+}]_i$ changes in pancreatic acinar cells (Fig. 10*D*).

Our use of the calcium chelator BAPTA during magfura-2 measurements could potentially have masked the effect of calcium on intracellular magnesium movements because BAPTA could have served as calcium reservoir, releasing its bound calcium after the period of CCK stimulation had ended. To exclude this hypothesis, we performed a series of experiments in which acini were not loaded with BAPTA, but exposed to calcium-free buffer for 100 s before CCK stimulation during CCK stimulation and for 500 s after CCK had been withdrawn. In fura-2 calcium experiments we observed that this treatment is sufficient to deplete intracellular calcium. In magfura-2 experiments, however, this manner of calcium depletion in the absence of BAPTA affected neither the basal $[Mg^{2+}]_i$ nor the magnesium changes in response to CCK (**Fig. 11*A***). In a different approach to rule out the influence of calcium on intracellular magnesium changes, we used lanthanum, which is known to block calcium entry into acinar cells when used in micromolar concentrations. Exposure of acinar cells to 250 μM lanthanum in the absence of BAPTA again had no significant effect on $[Mg^{2+}]_i$ changes in response to CCK (Fig. 11*B*).

To test whether CCK stimulation is at all associated with a magnesium efflux from acinar cells, we measured $[Mg^{2+}]_e$ in acinar cell preparations of high cell density (cytocrit 35%) after exposure to CCK, using a magnesium-sensitive electrode. Even in the presence of low extracellular magnesium concentrations (0.35 mM), which were chosen to better detect magnesium efflux from the cells, the increase in $[Mg^{2+}]_e$ after 20 min of incubation ($\sim 0.01 \pm 0.001$ mM, $n=16$) was the same in CCK-stimulated as in control cells.

Once magnesium movements across the acinar cell membrane had been ruled out as a mechanism involved in regulating $[Mg^{2+}]_i$ changes in response to secretory stimuli, we attempted to characterize potential intracellular magnesium buffers. One of the known intracellular buffering mechanisms that would have explained the disappearance of magnesium from the cytosol of acinar cells is its binding to intracellular ligands such as ATP, ADP, phosphates, and calmodulin. If, however, such a binding of magnesium were involved in the CCK-induced $[Mg^{2+}]_i$ changes, this mechanism would be affected by intracellular pH alterations (34). When we used magfura-2-loaded cells in the absence of BAPTA and determined the intracellular pH with the pH-sensitive fluorescent probe BCECF, the addition of ammonium chloride to the acinar preparation induced a rapid intracellular alkalinization, which was followed by a reacidification after removal of NH_4Cl (**Fig. 12*A***). The NH_4Cl-induced alkalinization/acidification sequence, however, had no effect on intracellular magnesium concentrations (Fig. 12*B*). Continuous incubation with either amiloride (0.1 mM, data not shown) or N-methyl-D-glucamine (see above),

which both alter intracellular pH (35), had no effect on CCK-induced Mg^{2+} changes.

An intracellular organelle capable of storing large amounts of magnesium is the mitochondrion, and magnesium is involved in a number of enzymatic processes that are confined to mitochondria (36, 37). To test whether mitochondria represent the critical magnesium stores responsible for the uptake of cytosolic magnesium, we interfered with the mitochondrial metabolism in various ways. Neither uncoupling of the mitochondrial respiratory chain by incubation with carbonyl cyanide p-(trifluoromethoxy)-phenylhydrazone (FCCP) (**Fig. 13*A***), inhibition of the mitochondrial transition pore with cyclosporin (Fig. 13*B*), nor incubation with phenylarsine, which is known to release mitochondrial magnesium most likely via the transition pore (not shown), had any effect on stimulus-evoked $[Mg^{2+}]_i$ changes in pancreatic acinar cells.

To further characterize the nature and site of intracellular magnesium stores, we performed localization experiments using fluorescence imaging of ion-specific probes. When we used the calcium-specific dye fura-2, we found 76% of cells that were stimulated with 100 pM CCK to respond with an increase of cytoplasmic calcium that was initiated at the apical or luminal pole of the acinar cell, which contains the zymogen granules, and progressed from there to the basolateral portion of the respective cell (**Fig. 14*A***). This result agrees with earlier publications in which this characteristic and spatially distinct pattern of Ca^{2+} mobilization in response to CCK was initially reported (27, 28). The temporal delay between the initiation of the Ca^{2+} signal at the apical pole and the subsequent Ca^{2+} increase in the basolateral region was calculated to be 0.38 ± 0.04 s after CCK stimulation and 0.29 ± 0.01 s after carbachol stimulation. In ~20% of cells, no regional differences in the release of calcium were detectable. On the other hand, when we used magfura-2 and BAPTA-loaded cells to image regional magnesium changes by fluorescence microscopy, the cytosolic magnesium signal began to decrease in the basolateral region of 67% of acinar cells, and this decrease progressed to the apical pole (Fig. 14*B*). The temporal delay from the initial basolateral decrease to the subsequent apical decline of $[Mg^{2+}]_i$ was calculated

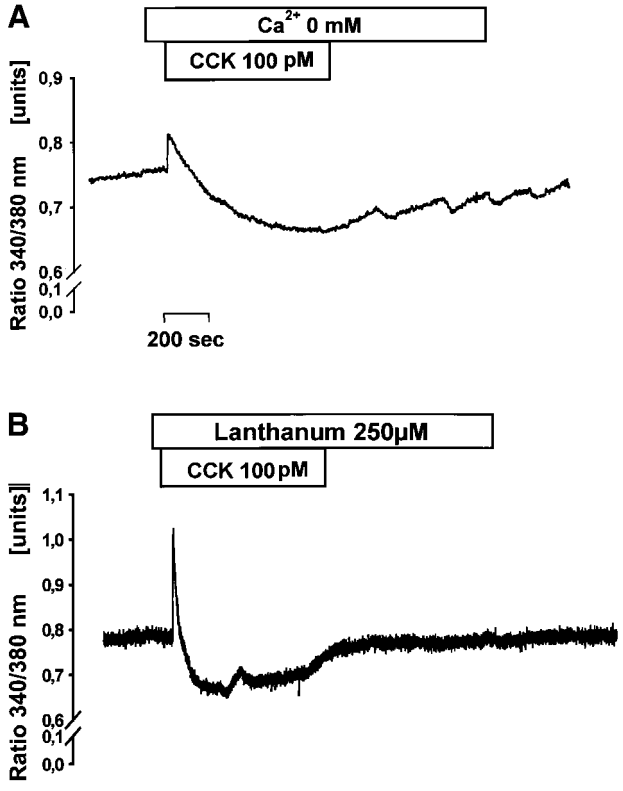

Figure 11. Effect of cellular calcium depletion on the magfura-2 ratio changes in response to CCK. Because BAPTA might potentially serve as an additional intracellular calcium buffer, experiments (*A, B*) were performed in the absence of BAPTA. Cells were stimulated with CCK (100 pM) in either nominally calcium free buffer (*A*) or in medium containing 250 μM lanthanum (*B*) in order to exclude Ca^{2+} in response to CCK stimulation. Both experimental conditions resulted in similar magfura-2 recordings as seen in the presence of extracellular calcium (see Fig. 5*A*). These experiments indicate that the CCK-evoked $[Mg^{2+}]_i$ changes are independent of the presence of calcium.

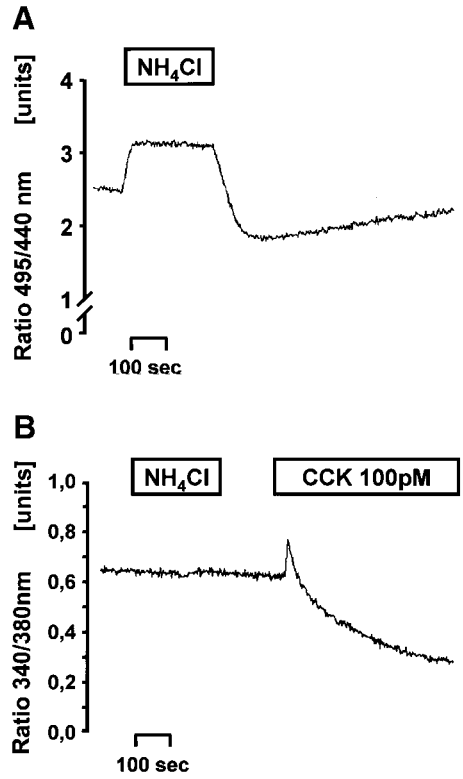

Figure 12. Effect of intracellular pH alterations on magfura-2 ratio changes in response to CCK. *A)* Control experiments using cells that were loaded with the pH-sensitive dye BCECF (in the absence of BAPTA) demonstrated that perfusion with NH_4Cl lead to a rapid alkalinization of the cells as indicated by an increase of the 490/440 nm ratio. Withdrawal of NH_4Cl allowed for a rapid reacidification of the cytosol. *B)* NH_4Cl perfusion had, however, no effect on the magfura-2 ratio in acinar cells and thus excluded ATP as a potential magnesium buffer. The magfura-2 response to subsequent stimulation with CCK demonstrated that cells had remained viable and fully functional.

to be 0.49 ± 0.12 s after CCK stimulation (n=15) and 0.60 ± 0.12 s after carbachol stimulation (n=16). A spatially inhomogeneous disappearance of the Mg signal from the cytosol, indicated by a more prominent decrease of the magfura-2 fluorescence from the basolateral rather than from the apical region, was found in >90% of all imaged acinar cells.

DISCUSSION

Interactions between the two divalent cations calcium and magnesium on the cellular level have remained a controversial issue. That this debate continues is due to the incompatibility of certain results obtained in different cell types and under different experimental conditions that do not seem to fit into a general concept. Whereas some authors have reported a potentiation of intracellular calcium signals in the presence of increased Mg^{2+} concentrations (38), others found that high Mg^{2+} concentrations can impair the cellular calcium response (39–43). Our present data, which were obtained from isolated pancreatic acini and by single-cell measurements, agree with those of the latter investigators because they indicate that increased Mg^{2+} concentrations have a direct effect on the frequency and amplitude of Ca^{2+} oscillations in response to CCK or carbachol stimulation. An interference of high $[Mg^{2+}]_i$ with fura-2, the fluorescent indicator we used to determine calcium concentrations, can be ruled out because Mg^{2+} does not affect the K_D of fura-2 over a

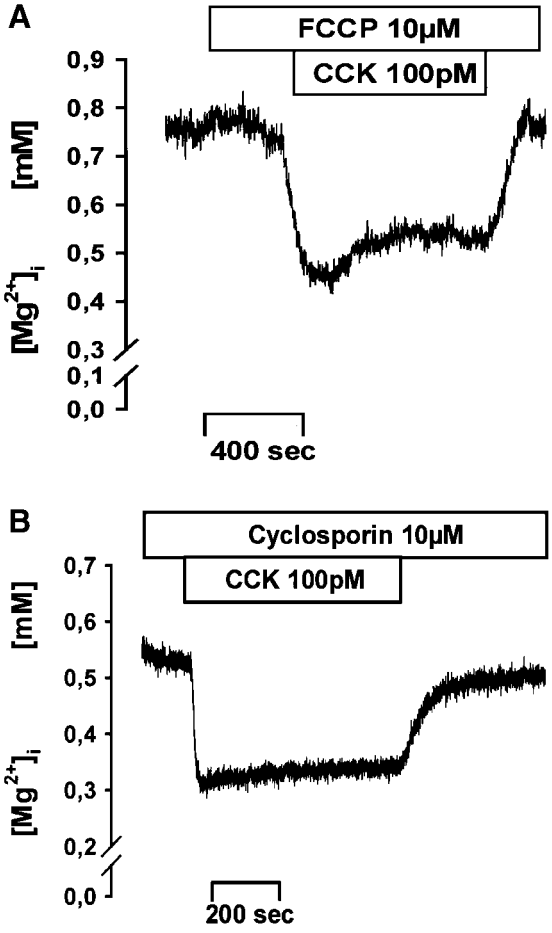

Figure 13. Drugs acting primarily on mitochondrial metabolism had no effect on the CCK-evoked $[Mg^{2+}]_i$ mobilization in single pancreatic acinar cells. *A)* Although FCCP, an uncoupler of the mitochondrial respiratory chain, induced a continuous calcium increase (as determined in fura-2-loaded cells, data not shown), it had no effect on the CCK-evoked Mg^{2+} movements in BAPTA-loaded acinar cells. *B)* Cyclosporin, an inhibitor of the mitochondrial transition pore, also failed to affect intracellular Mg^{2+} movements in response to CCK. These observations exclude mitochondria as a potential magnesium buffer.

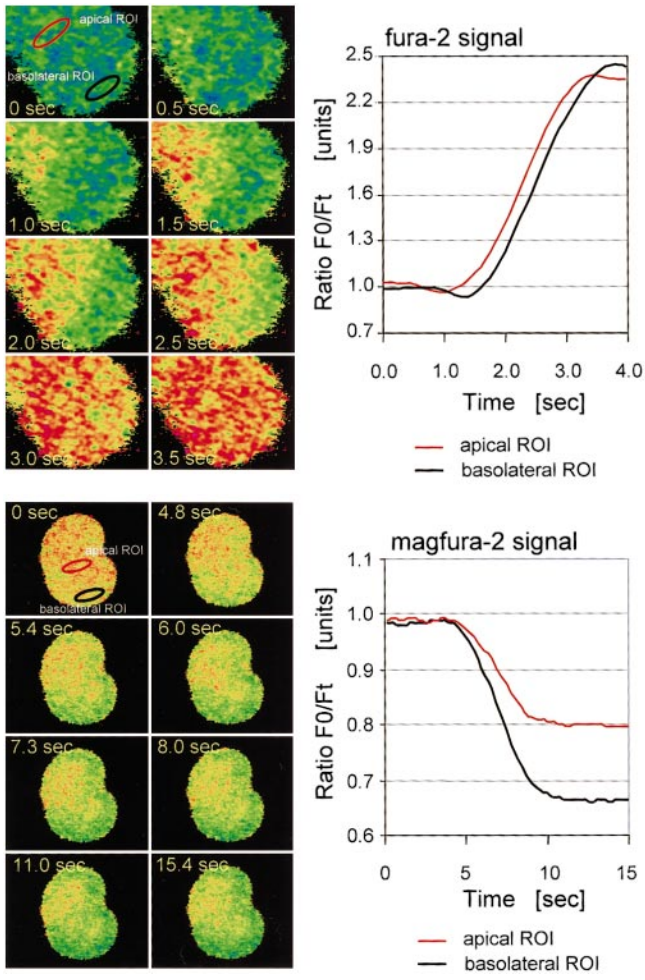

Figure 14. Comparison of the spatial distribution of the CCK-induced Ca^{2+} and Mg^{2+} response. Isolated acinar cells were loaded with fura-2 for digital calcium imaging and with magfura-2 for digital magnesium imaging as described in Materials and Methods and stimulated with CCK (100 pM). Whereas the fura-2 Ca^{2+} signal initially increased at the apical pole and subsequently spread from there toward the basolateral part of acinar cells (*A*), the CCK-induced decrease in Mg^{2+} was initiated at the basolateral part and spread from there to the apical pole of the acinar cell (*B*). Apical (red lines) and basolateral (black lines) regions of interest (ROI) for subcellular ion measurements are indicated in the digital images and according recordings. Data shown are representative of 12 calcium experiments and 27 individual magnesium experiments.

wide range of concentrations (44). Moreover, the manganese influx measurements in our study provide direct evidence that the capacitive calcium entry is the principal target of Mg^{2+} ions and that Mg^{2+} blocks this pathway by interference with Ca^{2+} permeation. An analogous interference of Mg^{2+} with the entry of Ca^{2+} into the cell has previously been shown for L-type calcium channels in myocytes (45, 46) and for a ligand-gated calcium influx pathway in rat pituitary lactotropes (47). Our current data further suggest that these magnesium effects depend on $[Mg^{2+}]_i$ changes rather than $[Mg^{2+}]_e$ alterations, because acute extracellular magnesium changes failed to affect intracellular calcium signaling and an extended period of incubation ($\geq$30 min) at high extracellular magnesium concentrations was required before any effects were detectable. By making use of the fact that individual cells from the same acinar preparation take up magnesium at different rates—a phenomenon not unlike the heterogeneous $[Mg^{2+}]_i$ loading rates previously found in tracheal gland cells (48)—we could correlate individual calcium signals with individual cytosolic magnesium concentrations, and have found a linear relationship between the two. This must be regarded as further evidence for a direct effect of $[Mg^{2+}]_i$ on the regulation of calcium signaling in acinar cells.

However, before a significant role of magnesium in the physiology of an exocrine gland like the pancreas can be assumed, it remains to be shown that intracellular magnesium concentrations respond to cellular status changes such as hormonal stimulation in a dynamic manner. Although attempts to elucidate the role of $[Mg^{2+}]_i$ in pancreatic acinar cell physiology have been made in the past (16, 17), they were often impeded by the unavailability of single-cell imaging techniques or the limitations of Mg^{2+}-specific probes. Even today, the most specific Mg^{2+}-sensing fluorescent probe available, magfura-2, has a residual low affinity to calcium, with a K_D of $\sim$20 μM (49), and intracellular calcium concentrations above 1 μM have been shown to be detectable using magfura-2. This cross-reactivity of magfura-2 with calcium should not affect the accurate determination of the basal intracellular magnesium concentrations under resting conditions, but might affect magnesium measurements in the presence of high calcium concentrations, i.e., after an agonist-evoked release of $[Ca^{2+}]_i$ from intracellular stores (27, 28). An additional source of interference by the low calcium affinity of magfura-2 consists in the accumulation of the probe in intracellular compartments such as calcium stores. Some authors have made use of these properties of magfura-2 to investigate calcium storage mechanisms (30–32). In this type of experiment longer incubation periods and higher loading concentrations were used to saturate the intracellular esterases that would have converted the fluorochrome-ester magfura-AM to membrane-impermeant magfura and would have prevented its uptake into calcium stores. In addition, cells were usually permeabilized to release the cytosolic portion of magfura and thus reduce background fluorescence (32). In our experiments where we used magfura-2 as a high-affinity probe for magnesium, we prevented a potential cross-reactivity with calcium by several means. To test our experimental and loading conditions, we either permeabilized acini with digitonin or injected the membrane-impermeant dextran derivative of magfura-2 directly into the cells.

The results from these experiments indicated that $>$95% of magfura-2 was localized in the acinar cell cytosol and no cross-reactivity with calcium from intracellular stores had interfered with our magnesium measurements. To rule out interference by cytosolic calcium, we used ion chelators with different affinities to calcium and magnesium to differentiate between their cytosolic binding to magfura-2. In experiments where we used BAPTA-AM, a chelator that has a higher affinity to calcium than to magnesium, we found that the prominent initial rise in the magfura-2 ratio as well as oscillation-like changes during the subsequent decline of the ratio could be accounted for by magfura-2 detection of cytosolic calcium. The rapid decrease of the magfura-2 ratio in response to secretory stimuli, however, and the return of the ratio to prestimulatory levels after the stimulus was withdrawn appeared to reflect a disappearance of magnesium from the cytosol. These observations provided us with a tool to study dynamic $[Mg^{2+}]_i$ changes under different experimental conditions. As a control for our magnesium recordings in magfura-2 and BAPTA-AM-loaded acini, we used an electrophysiological approach that is completely independent of the limitations of fluorescent ion-sensitive probes and found that, indeed, our magfura-2 ratio recordings accurately reflected cytosolic magnesium concentrations.

We have thus confirmed by independent means that stimulation of pancreatic acinar cells with Ca^{2+} mobilizing agonists like CCK or carbachol elicits a rapid decrease of cytosolic Mg^{2+}, and that withdrawal of the stimulus is followed by a restitution of the initial cytosolic Mg^{2+} concentration. Though these observations are in contrast with results obtained from other cell types that were treated with a variety of stimuli (15, 50, 51), they agree with studies on the sublingual mucous gland (52). The decrease in $[Mg^{2+}]_i$ observed in pancreatic acinar cells in this study might theoretically be caused either by an export of magnesium across the plasma membrane or by a shift of magnesium from the cytosol into cellular stores. In subsequent experiments we found no evidence of a Mg^{2+} extrusion across the plasma membrane, because the cytosolic magnesium concentrations under resting conditions or in response to hormonal stimuli were not affected by changes of the electrochemical gradient for Mg^{2+} or by changes in membrane potential induced by diminishing the potassium equilibrium. In particular, the absence of any influence of magnesium-free buffer on the recovery of $[Mg^{2+}]_i$ after the withdrawal of the stimulus illustrates clearly that the intracellular magnesium movements are independent of the presence or absence of extracellular magnesium.

We could further rule out the involvement of a sodium-magnesium exchanger, although it has been suggested that this antiporter is involved in magnesium movements across the membrane of pancreatic acinar cells (53, 54). Those experiments, however, were done in the absence of a calcium chelator, and the perfusion of acinar cells with sodium-deficient medium resulted in a large increase of intracellular calcium via action of the Na^+/Ca^{2+} exchanger. This, in turn, would be expected to affect magfura-2 ratio recordings in a non-magnesium-specific manner (55). When we avoided the effect of sodium depletion on calcium movements by using a sodium- and calcium-deficient medium in addition to chelating calcium with BAPTA, we could solve the seemingly incompatible differences between the two studies. In additional experiments in which we measured the release of magnesium into the perfusion medium directly, we could confirm that agonist stimulation does not evoke a significant extrusion of magnesium from isolated acini. The experiments presented here therefore appear to reflect the mechanisms involved in intracellular magnesium regulation more accurately than earlier attempts when slices of pancreas were used (54).

Accordingly, we must assume that the decrease in $[Mg^{2+}]_i$ in response to CCK or carbachol is caused by an intracellular mechanism. A significant buffering of cytosolic magnesium by intracellular ligands such as ATP and calmodulin, or phospholipids like phosphatidylserine and phosphatidylcholine, was ruled out as a potential explanation because $[Mg^{2+}]_i$ was not affected by changes in intracellular pH. A second potential mechanism, that of a magnesium uptake into mitochondria (56), was ruled out in experiments in which we uncoupled oxidative phosphorylation or impaired the function of the mitochondrial transition pore, a large conductance ion channel that is known to permit Mg^{2+} movements between the cytosol and the mitochondrial matrix.

Support for an uptake into intracellular magnesium stores came from imaging studies in which we recorded the spatial and temporal resolution of magfura-2 ratio changes in response to agonist stimulation. The data from these studies have indicated that the disappearance of magfura-2 from the cytosol is initiated in the basolateral portion of acinar cells and progresses from there to the apical pole. Because this localized shift from the basolateral to the secretory compartment constitutes a completely antiparallel event to the known calcium release from intracellular stores, we studied calcium and magnesium changes in the same setup. In these imaging studies we have confirmed the initial calcium rise at the apical pole, which was followed by a progression to the basolateral portion of acinar cells as previously reported (27, 28), whereas the magfura-2 magnesium signal disappeared in the opposite direction. Moreover, this temporal polarity was paralleled by a sustained spatial gradient of cytosolic Mg^{2+} and $[Mg^{2+}]_i$ was found to be consistently lower close to the basolateral membrane. The initiation of the Mg decrease in the basolateral portion of acinar cells, where most of the endoplasmic reticulum is located, makes it seem most likely that $[Mg^{2+}]_i$ is predominantly taken up by stores that are located in the ER. Data that have indicated a spatial heterogeneity of $[Mg^{2+}]_i$ as well as the presence of cytosolic magnesium gradients have previously been reported from studies of the human tracheal gland (48). Additional support for the general existence of ER-based magnesium stores can be seen in studies that have investigated the bee photoreceptor and the rat sublingual gland (52, 57).

Our experiments indicate further that particularly the recovery of cytosolic magnesium concentrations after withdrawal of the secretory stimulus is independent of the presence or absence of calcium. It depends, however, on the activity of an ATPase as indicated by the fact that this release is thapsigargin sensitive.

The exact mechanisms by which Mg^{2+} enters and leaves the intracellular store require further study. One possible entry pathway might be the intracellular calcium release channels. Despite the ability of Mg^{2+} to inhibit intracellular calcium release channels, it may also pass through these channels. This kind of retrograde movement ought to be favored by the electrochemical gradient for Mg^{2+} across the ER membrane during secretagogue stimulation and has been shown to occur for other divalent cations such as Mn^{2+} in permeabilized hepatocytes (58).

The intracellular signaling events involved in the CCK-induced decrease of $[Mg^{2+}]_i$ in pancreatic acinar cells are currently under investigation. One critical process that appears to participate in this signaling cascade is the activation of protein kinase C. Another issue that requires further study is the role of agonist-evoked magnesium changes in the physiology and pathophysiology of the exocrine pancreas. Ongoing studies in which the early events involved in the onset of acute pancreatitis are being explored have already shown that the modulating effect of magnesium on intracellular calcium signaling can be therapeutically used to attenuate the severity of the disease in an animal model (59).

In conclusion, the results of this study have indicated that intracellular Mg^{2+} concentrations in the exocrine pancreas are regulated in a stimulus-controlled manner. Changes in $[Mg^{2+}]_i$ can counteract the effects in the Ca^{2+} signal transduction pathway, and the 'slower' uptake and release of Mg^{2+} from intracellular, most probably ER-associated stores, occur in a manner that is completely anti-parallel to the 'rapid' Ca^{2+} release and reuptake in acinar cells. These observations suggest that the role of Mg^{2+} in the physiology as well as the pathophysiology of the exocrine pancreas and its diseases warrants a completely new evaluation. FJ

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REFERENCES

1. Petersen, O. H. (1992) Stimulus-secretion coupling: cytoplasmic calcium signals and the control of ion channels in exocrine acinar cells. *J. Physiol.* **448,** 1–5
2. Petersen, O. H., Burdakov, D., and Tepikin, A. V. (1999) Polarity in intracellular calcium signaling. *Bioessays* **21,** 851–860
3. Berridge, M., Lipp, P., and Bootman, M. (1999) Calcium signalling. *Curr. Biol.* **9,** R157–R159
4. Berridge, M. J. (1997) Elementary and global aspects of calcium signalling. *J. Exp. Biol.* **200,** 315–319
5. Carafoli, E. (1987) Intracellular calcium homeostasis. *Annu. Rev. Biochem.* **56,** 395–433
6. Birch, N. J. (1993) *Magnesium and the Cell,* Academic Press, New York
7. Flatman, P. W. (1984) Magnesium transport across cell membranes. *J. Membr. Biol.* **80,** 1–14
8. Sueta, C. A., Patterson, J. H., and Adams, K.F. Jr. (1995) Antiarrhythmic action of pharmacological administration of magnesium in heart failure: a critical review of new data. *Magnes. Res.* **8,** 389–401
9. Raju, B., Murphy, E., Levy, L. A., Hall, R. D., and London, R. E. (1989) A fluorescent indicator for measuring cytosolic free magnesium. *Am. J. Physiol.* **256,** C540–C548
10. Alvarez-Leefmans, F. J., Gamiño, S. M., and Rink, T. J. (1984) Intracellular free magnesium in neurones of Helix aspera measured with ion-selective micro-electrodes. *J. Physiol. (London)* **354,** 303–317
11. Dai, L. J., and Quamme, G. A. (1992) Cyclic nucleotides alter intracellular free Mg^{2+} in renal epithelial cells. *Am. J. Physiol.* **262,** F1100–F1104
12. Romani, A., Marfella, C., and Scarpa, A. (1992) Regulation of Mg^{2+} uptake in isolated rat myocytes and hepatocytes by protein kinase C. *FEBS Lett.* **296,** 135–140
13. Romani, A., and Scarpa, A. (1990) Hormonal control of Mg^{2+} transport in the heart. *Nature (London)* **346,** 841–844
14. Bond, M., Vadasz, G., Somlyo, A. V., and Somlyo, A. P. (1987) Subcellular calcium and magnesium mobilization in rat liver stimulated in vivo with vasopressin and glucagon. *J. Biol. Chem.* **262,** 15636–15642
15. Grubbs, R. D. (1991) Effect of epidermal growth factor on magnesium homeostasis in BC3HI myocytes. *Am. J. Physiol.* **260,** C1158–C1164
16. Ng, L. L., Davies, I. E., and Garrido, M. C. (1991) Intracellular free magnesium in human lymphocytes and the response to lectin. *Clin. Sci.* **80,** 539–542
17. Lennard, R., and Singh, J. (1991) Secretagogue-evoked changes in intracellular free magnesium concentrations in rat pancreatic acinar cells. *J. Physiol.* **435,** 483–492
18. Lennard, R., and Singh, J. (1992) Effects of secretagogue on intracellular free calcium and magnesium concentrations in rat pancreatic acinar cells. *J. Gen. Pharmacol.* **23,** 903–908
19. Zhang, G. H., and Melvin, J. E. (1992) Secretagogue-induced mobilization of an intracellular Mg^{2+} pool in rat sublingual mucous acini. *J. Biol. Chem.* **267,** 20721–20727
20. Krüger, B., Albrecht, E., and Lerch, M. M. (2000) The role of intracellular calcium signaling in premature protease activation and the onset of pancreatitis. *Am. J. Pathol.* **157,** 43–50
21. Williams, J. A., Korc, M., and Dormer, R. L. (1978) Action of secretagogues on a new preparation of functionally intact, isolated pancreatic acini. *Am. J. Physiol.* **235,** E517–E524
22. Lerch, M. M., Saluja, A. K., Dawra, R., Saluja, M., and Steer, M. L. (1993) The effect of chloroquine administration on two experimental models of acute pancreatitis. *Gastroenterology* **104,** 1768–1779
23. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260,** 3440–3450
24. Hintz, K., Günzel, D., and Schlue, W.-R. (1999) Na^{+}-dependent regulation of the free Mg^{2+} concentration in neuropile glial cells and P neurones of the leech Hirudo medicinalis. *Pfluegers Arch.* **437,** 354–362
25. Günzel, D., Durry, S., and Schlue, W.-R. (1997) Intracellular alkalinization causes Mg^{2+} release from intracellular binding sites in leech Retzius neurones. *Pfluegers Arch.* **435,** 65–73
26. Gukovskaya, A., and Pandol, S. (1994) Nitric oxide production regulates cGMP formation and calcium influx in pancreatic acinar cells. *Am. J. Physiol.* **266,** G350–G356
27. Kasai, H., Li, Y. X., and Miyashita, Y. (1993) Subcellular distribution of Ca^{2+} release channels underlying Ca^{2+} waves and oscillations in exocrine pancreas. *Cell* **74,** 669–677
28. Toescu, E. C., Lawrie, A. M., Petersen, O. H., and Gallacher, D. V. (1992) Spatial and temporal distribution of agonist-evoked cytoplasmic Ca^{2+} signals in exocrine acinar cells analysed by digital image microscopy. *EMBO J.* **11,** 1623–1629
29. Tsunoda, Y., Stuenkel, E. L., and Williams, J. A. (1990) Characterization of sustained $[Ca^{2+}]_i$ increase in pancreatic acinar cells and its relation to amylase secretion. *Am. J. Physiol.* **259,** G792–G801
30. Hofer, A. M., Landolfi, B., Debellis, L., Pozzan, T., and Curci, S. (1998) Free $[Ca^{2+}]$ dynamics measured in agonist-sensitive stores of single living intact cells: a new look at the refilling process. *EMBO J.* **17,** 1986–1995
31. Hofer, A. M., and Machen, T. E. (1994) Direct measurement of free Ca in organelles of gastric epithelial cells. *Am. J. Physiol.* **267,** G442–G451
32. Van de Put, F. H. M. M., and Elliott, A. C. (1996) Imaging of intracellular calcium stores in individual permeabilized pancreatic acinar cells. *J. Biol. Chem.* **271,** 4999–5006
33. Toescu, E. C., Gardner, J. M., and Petersen, O. H. (1993) Mitochondrial Ca^{2+} uptake at submicromolar $[Ca^{2+}]_i$ in permeabilised pancreatic acinar cells. *Biochem. Biophys. Res. Commun.* **192,** 854–859
34. Freudenreich, C. C., Murphy, E., Levy, L. A., London, R. E., and Lieberman, M. (1992) Intracellular pH modulates cytosolic free magnesium in cultured chicken heart cells *Am. J. Physiol.* **262,** C1024–C1030
35. Muallem, S., and Loessberg, P. A. (1990) Intracellular pH-regulatory mechanisms in pancreatic acinar cells. *J. Biol. Chem.* **265,** 12806–12819
36. Corkey, B. E., Duszynski, J., Rich, T. L., Matschinsky, B., and Williamson, J. R. (1986) Regulation of free and bound magnesium in rat hepatocytes and isolated mitochondria. *J. Biol. Chem.* **261,** 2567–2574
37. Diwan, J. J. (1987) Mitochondrial transport of K^{+} and Mg^{2+}. *Biochim. Biophys. Acta* **895,** 155–165
38. Yoshimura, M., Oshima, T., Matsuura, H., Ishida, T., Kambe, M., and Kajiyama, G. (1996) Potentiation of the intracellular Ca^{2+} response to arginine vasopressin by increased cytosolic-free Mg^{2+} in rat vascular smooth muscle cells. *Biochim. Biophys. Acta* **1312,** 151–7
39. Zhu, Z., Tepel, M., Spieker, C., and Zidek, W. (1995) Effects of extracellular Mg^{2+} concentration on agonist-induced cytosolic free Ca^{2+} transients. *Biochim. Biophys. Acta* **1265,** 89–92
40. Mooren, F. C., Geada, M. M., Singh, J., Stoll, R., Beil, W., and Domschke, W. (1997) Effects of extracellular Mg^{2+} concentration on intracellular signalling and acid secretion in rat gastric parietal cells *Biochim. Biophys. Acta* **1358,** 279–288
41. Francis, L. P., Lennard, R., and Singh, J. (1990) Cellular mechanism of action of magnesium on acetylcholine-evoked secretory response in the rat pancreas. *Exp. Physiol.* **75,** 669–680
42. Sebille, S., Pereira, M., Millot, J.-M., Jacquot, J., Delabroise, A.-M., Arnaud, M., and Manfait, M. (1998) Extracellular Mg^{2+} inhibits both histamine-stimulated Ca^{2+}-signaling and exocytosis in human tracheal secretory gland cells. *Biochem. Biophys. Res. Commun.* **246,** 111–116
43. Yu, H. J., Hypolite, J. A., Wein, A. J., and Levin, R. M. (1995) Effect of magnesium ions on rabbit detrusor contractility and intracellular free calcium. *Pharmacology* **51,** 186–94
44. Williams, D. A., and Fay, F. S. (1990) Intracellular calibration of the fluorescent calcium indicator fura-2. *Cell Calcium* **11,** 75–83
45. Agus, Z. S., Kelepouris, E., Dukes, I., and Morad, M. (1989) Cytosolic magnesium modulates calcium channel activity in mammalian ventricular cells. *Am. J. Physiol.* **256,** C452–C455
46. Lansman, J. B., Hess, P., and Tsien, R. W. (1986) Blockade of current through single calcium channels by Cd^{2+}, Mg^{2+} and Ca^{2+}. *J. Gen. Physiol.* **88,** 321–347
47. Kasahara, K., Tasaka, K., Masumoto, N., Nishizaki, T., Mizuki, J., Tahara, M., Miyake, A., and Tanizawa, O. (1993) High magnesium concentration inhibits ligand-stimulated calcium influx and hormone secretion in rat pituitary lactotropes with involve-

ment of intracellular free magnesium. *Biochem. Biophys. Res. Commun.* **197,** 92–99
48. Sebille, S., Millot, J.-M., Maizieres, M., Arnaud, M., Delabroise, A.-M., Jacquot, J., and Manfait, M. (1996) Spatial and temporal Mg^{2+} signaling in single human tracheal gland cells. *Biochem. Biophys. Res. Commun.* **227,** 743–749
49. Hurley, T. W., Ryan, M. P., and Brinck, R. W. (1992) Changes of cytosolic Ca^{2+} interfere with measurements of cytosolic Mg^{2+} using mag-fura-2. *Am. J. Physiol.* **263,** C300–C307
50. Gylfe, E. (1990) Insulin secretagogues induce Ca^{2+}-like changes in cytoplasmic Mg^{2+} in pancreatic β-cells. *Biochim. Biophys. Acta* **1055,** 82–86
51. Ishijama, S., Sonoda, T., and Tatibana, M. (1991) Mitogen-induced early increase in cytosolic free Mg^{2+} concentration in single Swiss 3T3 fibroblasts. *Am. J. Physiol.* **261,** C1074–C1080
52. Zhang, G. H., and Melvin, J. E. (1994) Intracellular Mg^{2+} movement during muscarinic stimulation. *J. Biol. Chem.* **269,** 10352–10356
53. Wisdom, D. M., Geada, M. M., and Singh, J. (1996) Characterization of a sodium-dependent magnesium efflux from magnesium-loaded rat pancreatic acinar cells. *Exp. Physiol.* **81,** 367–374
54. Günther, T., and Vormann, L. (1985) Mg^{2+} efflux is accomplished by an amiloride-sensitive Na^{+}/Mg^{2+} antiport. *Biochem. Biophys. Res. Commun.* **130,** 540–545
55. Wisdom, D., Salido, G. M., Baldwin, L. M., and Singh, J. (1996) The role of magnesium in regulating CCK-8 evoked secretory responses in the exocrine rat pancreas. *Mol. Cell. Biochem.* **154,** 123–132
56. Jung, D. W., and Brierley, G. P. (1994) Magnesium transport by mitochondria. *J. Bioenerg. Biomembr.* **26,** 527–535
57. Baumann, O., Walz, B., Somlyo, A. V., and Somlyo, A. P. (1991) Electron probe microanalysis of calcium release and magnesium uptake by endoplasmic reticulum in bee photoreceptors. *Proc. Natl. Acad. Sci. USA* **88,** 741–744
58. Hajnoczky, G., Lin, C., and Thomas, A. P. (1994) Luminal communication between intracellular calcium stores modulated by GTP and the cytoskeleton. *J. Biol. Chem.* **269,** 10280–10287
59. Turi, S., Hlouschek, V., Mooren, F. C., Schnekenburger, J., Domschke, W. Lerch, M. M. (2000) Effects of prophylactic magnesium treatment on the onset and course of acute experimental pancreatitis. *Gastroenterology* **118,** A430 (abstr.)

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