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SECRETAGOGUE-EVOKED CHANGES IN INTRACELLULAR FREE MAGNESIUM CONCENTRATIONS IN RAT PANCREATIC ACINAR CELLS

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SUMMARY

1. This study employs the fluorescent dye Mag-Fura-2 acetoxymethyl ester (AM) to measure intracellular free magnesium concentration $[Mg^{2+}]_i$ in isolated rat pancreatic acinar cells. Initially a number of protocols were investigated to develop optimal loading conditions for the dye Mag-Fura-2 AM. The procedure yielding cells which showed minimal dye loss and no adverse compartmentalization was adopted for subsequent experiments.

2. The mean resting $[Mg^{2+}]_i$ is $1{\cdot}39 \pm 0{\cdot}08$ mM ($n = 39$).

3. Acetylcholine (ACh), cholescystokinin-octapeptide (CCK_8), carbamylcholine chloride evoked marked reduction in $[Mg^{2+}]_i$ in pancreatic acinar cells compared to resting values in the absence of secretagogues. The ACh-evoked decrease in $[Mg^{2+}]_i$ was abolished by pre-treatment with atropine. In contrast, noradrenaline, adrenaline and histamine had no significant effect on $[Mg^{2+}]_i$.

4. In acinar cells loaded with the Ca^{2+}-sensitive dye, Fura-2 acetoxymethyl ester (AM), ACh stimulation resulted in a marked elevation in intracellular free Ca^{2+} concentration $[Ca^{2+}]_i$. This response was blocked by pre-treatment with atropine.

5. Atomic absorption spectrophotometry was used to measure Mg^{2+} levels in effluent samples from pancreatic segments. Stimulation of pancreatic segments with ACh resulted in a marked elevation in Mg^{2+} concentrations (net efflux). On removal of ACh, Mg^{2+} concentration returned to resting level followed by a small net influx of Mg^{2+} into pancreatic tissue.

6. The results demonstrate that secretagogue-evoked alteration in $[Mg^{2+}]_i$ may occur concurrently with Mg^{2+} release from pancreatic tissue.

INTRODUCTION

It was the increasing conviction that calcium played a vital role as second messenger which first led to attempts at quantifying cytosolic free calcium in living cells (Rose & Loewenstein, 1975). Following initial work with the photoprotein aequorin, much improved Ca^{2+}-sensitive indicators were developed and now a variety of dyes exist for the imaging of intracellular Ca^{2+} (Grynkiewicz, Poenie & Tsien, 1985; Rink, 1988). More recently a number of new fluorescent dyes have been

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synthesized which are highly sensitive to other ions including Na^+, K^+, H^+ and Mg^{2+} (Alvarez-Leefmans, Girvaldez & Gamiño, 1987). These dyes are now being used to answer questions and complement other methods of research where understanding has thus far fallen short through lack of such specific bioprobes.

In numerous cell types it has long been understood that Mg^{2+} performs highly important functions, not least of which is its role as co-factor to over 300 enzymes (Gunther, 1981; Wacker, 1968; Flatman, 1984). Although Mg^{2+} is obviously important in cellular processes it has proved difficult to monitor accurately $[Mg^{2+}]_i$ in living cells without causing cellular damage (Chandler, Serratos, Rasgado & Sjondin, 1986). In the exocrine pancreas alteration to extracellular Mg^{2+} concentration ($[Mg^{2+}]_o$) has been shown to regulate secretagogue-evoked secretion, possibly by mediating changes in $[Ca^{2+}]_i$ (Lennard, Francis & Singh, 1989; Francis, Lennard & Singh, 1990). It therefore seemed highly desirable to investigate $[Mg^{2+}]_i$ with the new Mag-Fura-2 AM dye under both steady-state conditions and in the presence of known secretagogues. In order to do this it was first necessary to develop a suitable method for loading rat pancreatic acinar cells with Mag-Fura-2 AM as no studies have yet been reported which include a suitable loading protocol for this tissue. The first section of this study therefore, describes the development of a technique to provide cells containing suitably high levels of cytosolic Mag-Fura-2 AM for subsequent investigation of $[Mg^{2+}]_i$. The latter section of this study is an investigation of the effects of a number of secretagogues upon acinar cells previously loaded with Mag-Fura-2 AM adopting the most successful protocol. The technique of atomic absorbance spectrophotometry has also been employed to investigate net Mg^{2+} movement across the cell membranes of pancreatic tissue.

A preliminary account of some aspect of this work was presented to the Physiological Society (Lennard & Singh, 1990).

METHODS

Sprague–Dawley rats of either sex and weighing between 200 and 300 g were used throughout this study. Animals were killed by a blow to the head followed by severance of the vertebral column. The pancreas was then rapidly removed and placed into modified Krebs–Ringer–HEPES (KRH) solution of the following composition (in mM): NaCl, 130; KCl, 5; HEPES, 20; KH_2PO_4, 1·2; $MgSO_4$, 2·0; glucose, 10·0; $CaCl_2$, 1·0; soyabean trypsin inhibitor, 0·1 mg ml^{-1}, bovine serum albumin (BSA), 0·2% (w/v) and pH 7·4 (Lennard *et al.* 1989). The pancreas was then dissociated to acinar cells with collagenase (Sigma) in two stages totalling 75 min by established methods described previously (Amsterdam & Jameson, 1972; Francis *et al.* 1990). Next acinar cells were centrifuged at 50 *g* for 4 min through modified KRH solution containing 0·5% BSA. In some experiments the acini were divided into two portions. One portion was used to measure $[Ca^{2+}]_i$ while the other was used to measure $[Mg^{2+}]_i$.

Measurement of $[Mg^{2+}]_i$ using Mag-Fura-2 AM

Aliquots of cells were taken and subjected to the various experimental protocols (see Table 1). Cell suspensions were centrifuged, resuspended and incubated for 20 or 40 min at a number of temperatures in media containing various concentrations of Mag-Fura-2 AM and Pluronic. After incubation, suspensions were centrifuged and re-eluted in fresh media containing 0·2% BSA and maintained at 37 °C. Cells were post-incubated for 40 or 60 min, centrifuged and resuspended in media which was BSA deficient. Samples were then placed into a Perkin-Elmer LS5 spectrofluorimeter for subsequent investigation.

Measurement of fluorescence

Suspensions of cells were excited at 335 nm and fluorescence was measured at 510 nm (Raju, Murphy, Levy, Hall & London, 1989).

EGTA at a concentration of 5 mM was added to samples from each loading protocol to chelate Mg^{2+} ions and thus detect the fluorescence signal from putative Mag-Fura-2 AM in the extracellular medium by quenching.

TABLE 1. Explanation of different protocol treatments

	Protocol number						
Treatment	1	2	3	4	5	6	7
Centrifugation through 0·5% BSA				×	×	×	×
2 μM-Mag-Fura-2 AM	×	×		×	×	×	×
4 μM-Mag-Fura-2 AM			×				
20 min loading 20 °C		×					
40 min loading 20 °C	×			×			
40 min loading 37 °C			×				
40 min loading 20/37 °C						×	
40 min loading 32/37 °C					×		×
Pluronic in loading medium					×		
40 min post incubation	×	×		×			
60 min post incubation						×	×
Associated problems with loading procedures							
Poor fluorescence		×		×		×	
Dye leaching/high extracellular	×	×	×				
Poor cell response to 10^{-5} M-ACh	×			×	×		
Compartmentalization			×				

Top section of table shows experimental protocol used to load pancreatic acinar cells with Mag-Fura-2 AM. Bottom section indicates problems found when assessing the success of each protocol. The best protocol was found to be No. 7.

To other samples digitonin was added (final bath concentration 2×10^{-4} M) to permeabilize the acinar cells and permit excess Mg^{2+} and Ca^{2+} into the cytosol. Once a new steady fluorescence level had been reached, 20 μl Triton X-100 was added to dissolve all cellular membranes and thus release Mag-Fura-2 AM which may have been trapped in intracellular organelles. Further release of Mag-Fura-2 AM in this way was seen as an increase in fluorescence levels. Table 1 shows a summary of results from the various protocols used in this study. It was decided to adopt protocol 7 as this ensured high fluorescence readings combined with minimal leaching of the dye from cells and zero compartmentalization.

Cells loaded by protocol 7 were scanned at excitation wavelengths of 300–400 nm and fluorescence was measured at 510 nm. Excitation scans were repeated in the presence of digitonin (2×10^{-4} M) and then digitonin (2×10^{-4} M) plus EGTA (5 mM) (Fig. 1). The large changes in fluorescence at 335 nm and 370 nm between Mg^{2+} saturated (in the presence of digitonin) and zero Mg^{2+} conditions were indicative of good loading technique and enabled subsequent $[Mg^{2+}]_i$ calculation. These experiments were repeated using Mn^{2+} (5 mM) instead of EGTA. Mn^{2+} quenched fluorescence to background levels and did not lead to the reciprocal shift in fluorescence curves seen when using EGTA. It was, therefore, decided to employ EGTA for all experiments.

Scans were also made using the cell-impermeant Mag-Fura-2 AM Mg^{2+}/EGTA media ranging from zero Mg^{2+} to 20 mM-Mg^{2+} (Fig. 1*B*). From these scans a dissociation constant of 1·5 mM was calculated. This was in agreement with the findings of Raju *et al.* (1989) and was, therefore, adopted for later calculations of $[Mg^{2+}{}_i]$.

Measurement of $[Ca^{2+}]_i$

Two millilitre aliquots of the cell suspension loaded with 2 μM-Fura-2 were placed in quartz cuvettes in a Perkin-Elmer LS5 spectrofluorimeter and continuously stirred. Samples were excited at 340 nm for Fura-2 and the resulting fluorescence was measured at 510 nm. At the end of each

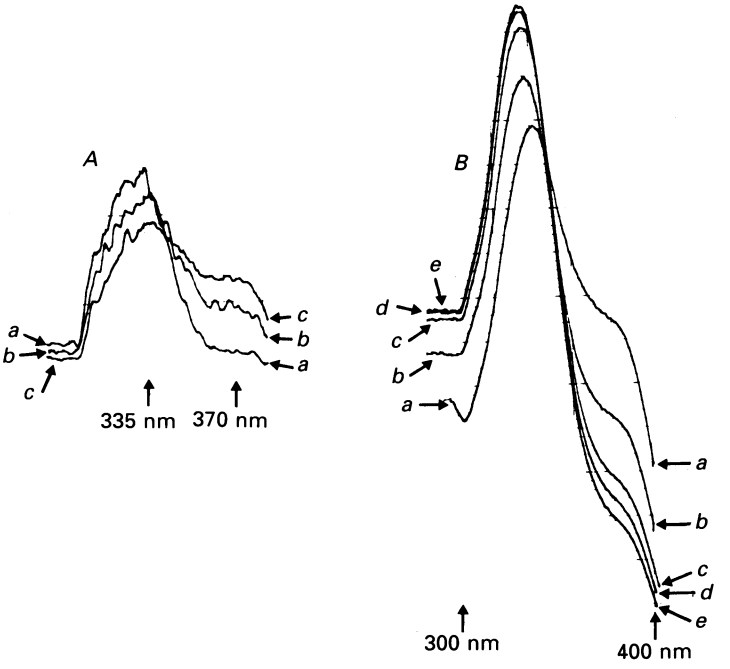

Fig. 1. *A*, excitation scan of pancreatic acinar cells loaded with Mag-Fura-2 AM in normal medium (*a*), medium containing digitonin (*b*) and medium containing digitonin and EGTA (*c*). Emission wavelength 510 nm. *B*, excitation scan of Mag-Fura-2 AM free dye in medium containing zero (*a*), 1 mM (*b*), 5 mM (*c*), 10 mM (*d*), and 20 mM-Mg^{2+} (*e*). Emission wavelength was 510 nm.

experiment maximum and minimum fluorescence values were obtained by the respective addition of digitonin (50 μl of a 5 mM solution), and 5 mM-EGTA. Final $[Ca^{2+}]_i$ was calculated by the method described by Tsien, Pozzan & Rink (1982).

Atomic absorption spectrophotometry

Small segments of rat pancreas (3–5 mg) weighing a total of about 80–100 g were placed into a Perspex flow chamber and superfused with Krebs–Henseleit solution at a flow rate of 1 ml min^{-1} and maintained at 37 °C. The Krebs–Henseleit solution comprised (in mM): NaCl, 103; KCl, 4·76; $CaCl_2$, 2·56; $MgCl_2.6H_2O$, 1·13; $NaHCO_3$, 25; NaH_2PO_4. $2H_2O$, 1·15; D-glucose, 2·8; sodium pyruvate, 4·9; sodium glutamate, 4·9; sodium fumarate, 2·7; pH 7·4 and continuously gassed with a mixture of 95% O_2, 5% CO_2.

Following an equilibration period of 40–45 min, effluent samples were collected at 2 min intervals. The tissue was then stimulated with either 10^{-6} or 10^{-5} M-ACh for a duration of 10 min and then returned to normal physiological salt solution. After each experiment tissues were blotted and weighed. The concentration of Mg^{2+} in the effluent from the tissues was determined using a Perkin-Elmer Atomic Absorbance Spectrophotometer (Model 2280). Samples were diluted 50–100 times and absorbance was read at 285 nm. Values obtained were measured against a calibration graph mark using known Mg^{2+} standards.

Statistical analysis

Data provided are expressed as means ± S.E.M. wherever possible. A paired Student's *t* test was used on data to assess statistical significance. Only *P* values of less than 0·05 were considered significant.

All reagents used were supplied by Sigma except for Mag-Fura-2 AM and Pluronic which were obtained from Molecular Probes Inc.

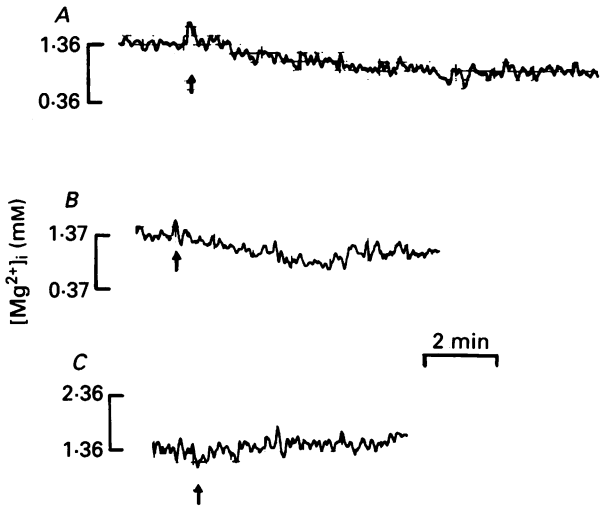

Fig. 2. Effect of 10^{-5} M-ACh upon $[Mg^{2+}]_i$ in pancreatic acinar cells loaded with Mag-Fura-2 AM in the absence (*A* and *B*) and presence (*C*) of 10^{-5} M-atropine. Note that the response in *B* shows some degree of recovery toward pre-stimulation levels. Time and concentration calibrations are shown by vertical and horizontal bars. Arrows denote points at which ACh was added to the cuvette. These traces are typical of eight to ten such experiments.

RESULTS

Fluorescence spectrofluorimetry

The mean resting $[Mg^{2+}]_i$ in rat pancreatic acinar cells was found to be 1·39 ± 0·08 mM ($n = 39$). When treated with ACh, mean $[Mg^{2+}]_i$ were seen to fall over a period of 3–6 min until a new steady resting level was reached. This value was typically 41·6 ± 5·3 % ($n = 10$) of the resting $[Mg^{2+}]_i$ and 52·5 ± 2·96 % ($n = 4$) in the presence of 10^{-5} M-ACh and 10^{-7} M-ACh, respectively (Fig. 2*A*). Occasionally, addition of ACh caused $[Mg^{2+}]_i$ to fall and then rise to pre-stimulation levels (Fig. 2*B*). It was found that ACh was without effect in acinar cell suspensions pre-treated for 3 min with 10^{-5} M-atropine (Fig. 2*C*). For comparison acinar cells were loaded with the Ca^{2+}-sensitive fluorescent dye, Fura-2 AM. In these preparations the addition of 10^{-7} M-ACh caused a sharp rise in $[Ca^{2+}]_i$ followed by a decline to pre-stimulation levels over a similar period of time to that taken for $[Mg^{2+}]_i$ to reach its lowest point

(Fig. 3*A*). In preparations pre-treated with 10^{-5} M-atropine, 10^{-7} M-ACh failed to elicit an increase in $[Ca^{2+}]_i$ (Fig. 3*B*). Carbamylcholine chloride also caused a reduction in $[Mg^{2+}]_i$. This was seen to be similar in potency to ACh at the same concentration as 10^{-5} M-carbamylcholine chloride reduced $[Mg^{2+}]_i$ to $42 \pm 3{\cdot}5\%$ ($n = 5$).

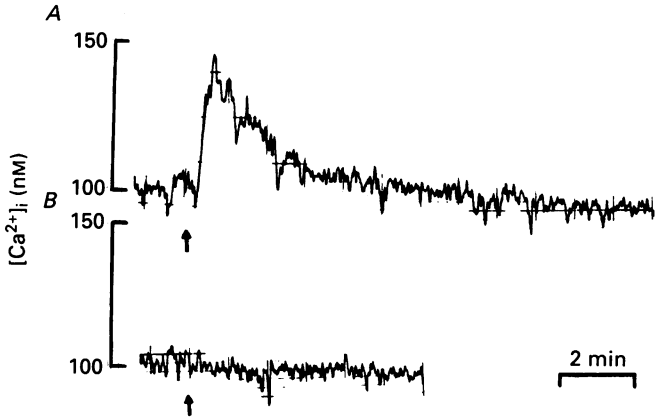

Fig. 3. Original chart recordings showing the effect of 10^{-7} M-ACh (added at arrows) on $[Ca^{2+}]_i$ in pancreatic acinar cells loaded with Fura-2 AM in the absence (*A*) and presence (*B*) of 10^{-5} M-atropine. These traces are typical of six such experiments. The resting $[Ca^{2+}]$ in this series of experiments was 110 ± 10 nm ($n = 8$). Time and concentrations are shown by horizontal and vertical calibrations.

In an attempt to show that the action of ACh on $[Mg^{2+}]_i$ was not an artifact, an analogue of the gut hormone CCK (CCK_8) was also employed. The results show that this secretagogue caused a reduction in $[Mg^{2+}]_i$ in acinar cells loaded with Mag-Fura-2 AM and the effects were seen to be dose dependent (Fig. 4*A–C*). Typically, $[Mg^{2+}]_i$ fell to $37 \pm 4{\cdot}5\%$ ($n = 5$), $45{\cdot}6 \pm 6\%$ ($n = 13$) and $78{\cdot}8 \pm 9{\cdot}7\%$ ($n = 5$) in the presence of 10^{-8}, 10^{-10} and 10^{-12} M-CCK_8, respectively. Cell suspensions were also challenged with other secretagogues including adrenaline, noradrenaline and histamine. Several concentrations of each substance were used (10^{-5}–10^{-7} M) but no consistent changes in $[Mg^{2+}]_i$ or $[Ca^{2+}]_i$ were observed.

Atomic absorption spectrometry

Since ACh stimulation resulted in marked alteration in $[Mg^{2+}]_i$ in pancreatic acinar cells it was therefore decided to measure net changes in Mg^{2+} concentrations in effluent samples following stimulation of pancreatic segments with the cholinergic agonist. The aim was to determine the direction of movement of Mg^{2+} in pancreatic acinar cells. Figure 5 shows the time course net changes in Mg^{2+} concentrations in effluent samples during stimulation of pancreatic segments with 10^{-6} M (△) and 10^{-5} m (▲) of ACh. ACh caused a marked and significant ($P < 0{\cdot}001$) efflux of Mg^{2+} into the effluent compared to pre-stimulation values. On removal of ACh Mg^{2+} efflux declined rapidly to resting level followed by a net influx of Mg^{2+} by pancreatic segments.

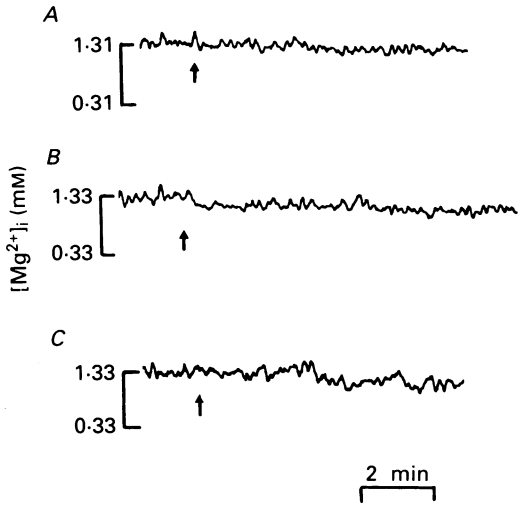

Fig. 4. The effects of 10^{-12} M (*A*), 10^{-10} M (*B*) and 10^{-8} M-CCK_8 (*C*) on $[Mg^{2+}]_i$ in pancreatic acinar cells loaded with Mag-Fura-2 AM. Time and concentration scales apply to all traces. Arrows denote point of addition of CCK_8. These traces are typical of five to eight such experiments.

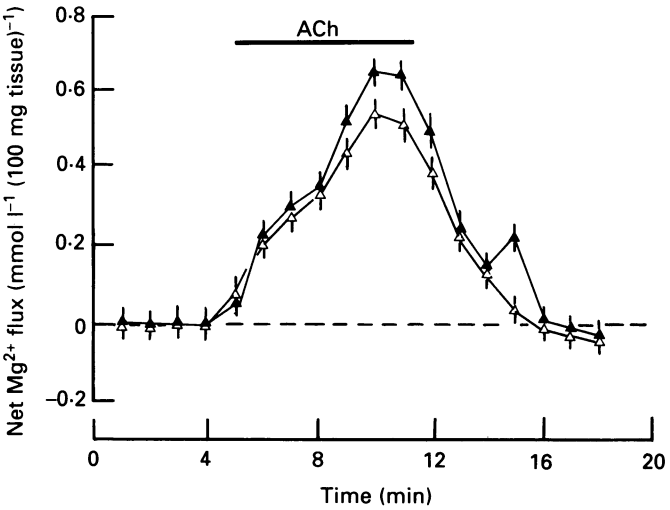

Fig. 5. Time course changes in Mg^{2+} concentration in effluent samples during stimulation of rat pancreatic segments with 10^{-5} M-ACh (▲) and 10^{-6} M-ACh (△). Each point is mean ± S.E.M. (vertical bars; $n = 3$–6). Note that the positive values represent a net efflux and negative values represent net influx of Mg^{2+}. The horizontal bar represents the duration of ACh stimulation.

DISCUSSION

The fluorescent indicator dye, Mag-Fura-2 AM is comparatively new and so far has been employed in few cell types. It was, therefore, important to develop a protocol for loading so that the associated problems, seen with the use of other dyes, could be

avoided (Madgaroli, Milani, Meldolesi & Pozzan, 1987; Moore, Becker, Fogarty, Williams & Fay, 1990). The protocol adopted here enabled sufficient dye to be loaded into cells to give good fluorescence readings. Loading times were short and no detectable compartmentalization was evident.

The present study shows that the resting level of $[Mg^{2+}]_i$ in pancreatic acinar cells is in the low millimolar range. This finding is in keeping with observations of $[Mg^{2+}]_i$ in a number of other tissues studied by various methods (Cittadini & Scarpa, 1983; Flatman, 1984; Gupta & Gupta, 1984; Alvarez-Leefmans *et al.* 1987). It is, however, of great import that the secretagogues ACh, carbamylcholine chloride and CCK_8 can all promote a reduction in $[Mg^{2+}]_i$. It is highly improbable that our observations are merely artifactual. It is clear that the observed reduction in fluorescence levels cannot be attributed to a bleaching effect, induced by the excitation beam. Under control responses of 10 min, fluorescence emissions from cells did not fall significantly. Additionally, the dose dependency seen with secretagogues and the ability of atropine to block the ACh-evoked response would exclude the possibility of dye bleaching. Secondly, these responses cannot be attributed to Ca^{2+} interacting with the Mag-Fura-2 AM dye. Although Mag-Fura-2 AM does have an affinity for Ca^{2+} (Raju *et al.* 1989) $[Ca^{2+}]_i$ is insufficient to exert any marked effects upon this dye. Also it is well known, and has been shown in the present study, that $[Ca^{2+}]_i$ is raised above resting levels when cells are in the presence of secretagogues.

That $[Mg^{2+}]_i$ fell but then in some cases rose again in the presence of secretagogue is worthy of further discussion. $[Ca^{2+}]_i$ generally returned to pre-stimulation values only a few minutes after the onset of stimulation. There is much evidence to suggest that this ion is initially released from intracellular stores to be exported from the cells later (Merrit & Rubin, 1985; Muallem, Schoeffield, Finnel & Pandol, 1988; Pandol, Schoeffield, Sachs & Muallem, 1985). Hence, this explains a rise and fall in cytosolic Ca^{2+} levels. The static volume cuvette system used in the present study did not permit perfusion of the cells under observation. Once administered secretagogues and therefore their effects could not be withdrawn. As the exact mechanism of Mg^{2+} mobilization here is not known we can only reconcile the maintenance of reduced $[Mg^{2+}]_i$, seen in many of our experiments, to continued secretagogue stimulation.

Our atomic absorbance spectrophotometric results indicate that at least some of the reduction in $[Mg^{2+}]_i$ can be accounted for by its export from the cell. In this system it was possible to constantly perfuse the preparation and remove the secretagogue stimulus after a set time period. In the post-stimulation period, in the absence of a secretagogue, Mg^{2+} efflux did indeed fall and eventually led to a net uptake of Mg^{2+} by the pancreatic tissue. This presumably would have brought the $[Mg^{2+}]_i$ back to the pre-stimulation value.

At present it is only possible to speculate upon the role of secretagogue-induced change in $[Mg^{2+}]_i$. The many enzymes which are Mg^{2+} dependent show a Mg^{2+} optimum at which enzymatic activity is maximized (Gunther, 1981). The $[Mg^{2+}]_i$ changes seen in the present study could therefore act to increase or decrease enzymatic activity depending upon individual enzyme optima. As our study shows $[Mg^{2+}]_i$ reduction in response to secretagogues which also change $[Ca^{2+}]_i$, it seems pertinent to consider enzymes involved here. One important pool of Ca^{2+} utilized in the secretagogue-evoked event is located in the endoplasmic reticulum (Berridge &

Irvine, 1989). This pool is released through a caffeine-sensitive Ca^{2+} channel which is highly Mg^{2+} dependent (Meissner, Darling & Eveleth, 1986; Osipichuk, Wakui, Yule, Gallacher & Petersen, 1990). At millimolar Mg^{2+} concentrations this channel is inhibited yet below this range the open state probability is increased. The inference from the present study is that as $[Ca^{2+}]_i$ levels fall, after the initial secretagogue evoked rise, a reduction in $[Mg^{2+}]_i$ would serve to protract Ca^{2+} release from the endoplasmic reticulum pool. This would be by optimizing $[Mg^{2+}]_i$ for the caffeine-sensitive Ca^{2+} channel and therefore promoting the open state of the caffeine-sensitive Ca^{2+} channel.

In conclusion we have shown that $[Mg^{2+}]_i$ can be reduced by ACh, carbamylcholine chloride and CCK_8 and this occurs in tandem with Mg^{2+} release from pancreatic tissue. These responses are highly significant because numerous enzymes are dependent upon optimal Mg^{2+} concentrations. The release of the second messenger Ca^{2+} may be influenced by the reduction in Mg^{2+} seen here under secretagogue-evoked stimulation.

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