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ROLE OF CALCIUM IN EXOCRINE PANCREATIC SECRETION

III. COMPARISON OF CALCIUM AND MAGNESIUM MOVEMENTS IN RABBIT PANCREAS

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SUMMARY

1. Calcium and magnesium movements in the isolated rabbit pancreas and in rabbit pancreas fragments are compared in a qualitative and quantitative way.

2. At the basal secretion rate calcium and magnesium are present in the secreted fluid in concentrations of about 30 % of their concentrations in the bathing medium.

3. Addition of 10^{-6} M carbachol to the bathing medium results in enzyme secretion accompanied by calcium and magnesium release, in divalent cation-free medium as well as in a complete medium.

4. The secretion of each divalent cation is the sum of two components: an extracellular flux and a flux of protein-associated cations, the so-called secretory flux.

5. The extracellular flux is proportional to the concentration of the divalent cation in the bathing medium. The secretory flux is not dependent on the presence of the divalent cation in the bathing medium, but is proportional to the amount of protein secreted. About 25 nmol of each cation is secreted per mg protein.

6. Ca^{2+} and Mg^{2+} can be nearly completely separated from the digestive enzymes by gel filtration. They equilibrate completely with their radioactive isotopes added to the sample just before elution, indicating that the cations are rapidly exchangeable after secretion.

7. Efflux studies on rabbit pancreas fragments, pre-loaded with $^{45}Ca^{2+}$, show a carbachol-stimulated $^{45}Ca^{2+}$ efflux (the stimulatory flux) in addition to a release of amylase. Fragments pre-loaded with $^{28}Mg^{2+}$ do not show carbachol stimulation of the tracer efflux.

8. These studies indicate that calcium and magnesium behave quite similarly with respect to the extracellular and secretory fluxes. The absence of a stimulatory flux for magnesium, suggests that the increase of the cytoplasmic calcium concentration plays a specific role in the stimulus-secretion coupling of pancreatic enzyme secretion.

INTRODUCTION

Information about the role of calcium in exocrine pancreatic secretion may be obtained from studies of the calcium movements, before and after stimulation.

Previously it has been shown that the calcium content of the pancreatic fluid is provided by at least two distinct calcium movements [1–4]. In addition, strong evidence has been obtained for a role of calcium in the stimulus-secretion coupling of pancreatic enzyme secretion [5–12]. In a previous paper [13] we have identified three distinct calcium movements in the isolated rabbit pancreas in vitro: (a) an extracellular route for calcium and other small molecules and ions (extracellular calcium flux); (b) a release of calcium across the apical membrane along with the digestive enzymes (secretory calcium flux); (c) a calcium flux across the serosal membrane (stimulatory calcium flux). All three fluxes are influenced by cholinergic agents.

The involvement of calcium in the stimulus-secretion coupling of pancreatic enzyme secretion may be reflected by one of these calcium movements, which should then be specific for calcium. Magnesium, the only other divalent cation in the incubation medium which is secreted into the juice [3, 14–16], is not supposed to play a role in the stimulus-secretion coupling [5]. Therefore, we have compared the pattern of the calcium movements with that of the magnesium movements in the isolated pancreas and pancreas fragments before and after stimulation of the enzyme secretion.

It is concluded that the extracellular and secretory fluxes are not specific for calcium, since they are completely parallelled by corresponding fluxes for magnesium. However, no stimulatory flux is observed for magnesium, suggesting that the involvement of calcium in the stimulus-secretion coupling of pancreatic enzyme secretion is reflected by the stimulatory calcium flux.

MATERIALS AND METHODS

The materials, the preparation of the rabbit pancreas, the incubation medium, the fraction collection and the assay procedures are the same as previously described [13, 17]. $^{28}MgCl_2$ is obtained from Brookhaven National Laboratory, Associated Universities, Inc., Upton, L.I., N.Y. 11973, U.S.A.

Gel filtration of the secreted fluid is carried out as follows. The secreted fluid collected in the first hour after stimulation with 10^{-6} M carbachol is first dried under a N_2 stream and subsequently redissolved in 225 μl of a radioactive solution containing $^{45}CaCl_2$ or $^{28}MgCl_2$. Samples are taken for determination of protein (5 μl) and divalent cations (10 μl) and for radioactive counting (10 μl). Subsequently 150 μl of the solution is applied to a Sephadex G-25 coarse (Pharmacia, Uppsala, Sweden) column (80 × 5 mm, pasteur capillary pipette), which is equilibrated with 0.9 % NaCl. Aliquots of 150 μl of the 0.9 % NaCl solution are applied to the column. Equal fractions are collected and analyzed for protein (15 μl), divalent cations (100 μl) and radioactivity (15 μl).

RESULTS

Effects of carbachol on the extracellular fluxes

The calcium in the pancreatic fluid is mainly derived from two calcium fluxes: the extracellular calcium flux and the secretory calcium flux [1–4, 13], the latter consisting of protein-associated calcium. Both fluxes are stimulated upon triggering of the pancreatic enzyme secretion. Quantitatively these effects are dose and drug dependent (Schreurs et al., unpublished). A previous study [13], with $^{45}Ca^{2+}$ present

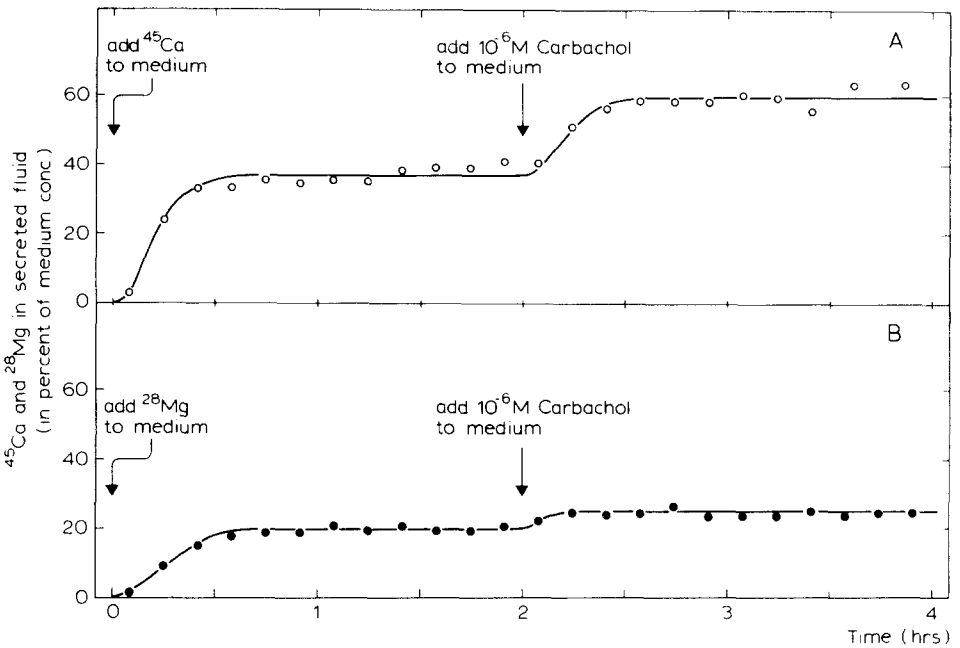

Fig. 1. Concentrations of $^{45}Ca^{2+}$ (A) and $^{28}Mg^{2+}$ (B) in the secreted fluid collected from the cannulated duct of the isolated rabbit pancreas, before and after stimulation with 10^{-6} M carbachol. The tracers are present in the bathing medium during the entire experimental period. Representative for five and two experiments, respectively.

in the bathing medium of the isolated rabbit pancreas, shows that the tracer equilibrates with the extracellular calcium flux within 30 min, but it does not equilibrate at all with the secretory calcium flux even after 2 h incubation. This indicates that the secretion of $^{45}Ca^{2+}$ represents, up to this moment, exclusively the extracellular calcium flux.

When the isolated rabbit pancreas is stimulated with carbachol, the $^{45}Ca^{2+}$ level after secretion is always higher than before stimulation, suggesting an irreversible increase of the extracellular route. With 10^{-5} M carbachol this increase is preceded by a transiently higher increase [13], whereas with 10^{-6} M carbachol the transient peak is absent (Fig. 1a).

When instead of $^{45}Ca^{2+}$, $^{28}Mg^{2+}$ is added to the incubation medium, 10^{-6} M carbachol leads to a similar increase in the isotope content in the secreted fluid (Fig. 1b). The isotope levels before and the increases after stimulation vary considerably in individual experiments.

For the study of the quantitative relation between cation secretion and protein secretion, it simplifies matters to have a constant rather than a variable extracelluar flux. Hence we have used the lower carbachol concentration for stimulation. Under these circumstances the extracellular flux can be determined by extrapolation (see next section) without the use of radioisotopes.

Stimulation in a divalent cation-containing medium

At the basal secretion rate, the secreted fluid (200–800 μl/h) collected from the

cannulated duct of the isolated rabbit pancreas during incubation in normal Krebs-Ringer bicarbonate medium, has a protein output of 1–3 mg/h and a calcium and magnesium concentration of about 30 % of that in the medium. Fig. 2a shows the results of a typical experiment in which stimulation is brought about with 10^{-6} M carbachol. The divalent cation and protein concentrations, which are relatively constant before stimulation, show an immediate simultaneous increase after stimulation. Although the peak values of these three parameters occur in the same fraction, the protein concentration decreases faster than those of the divalent cations. In addition

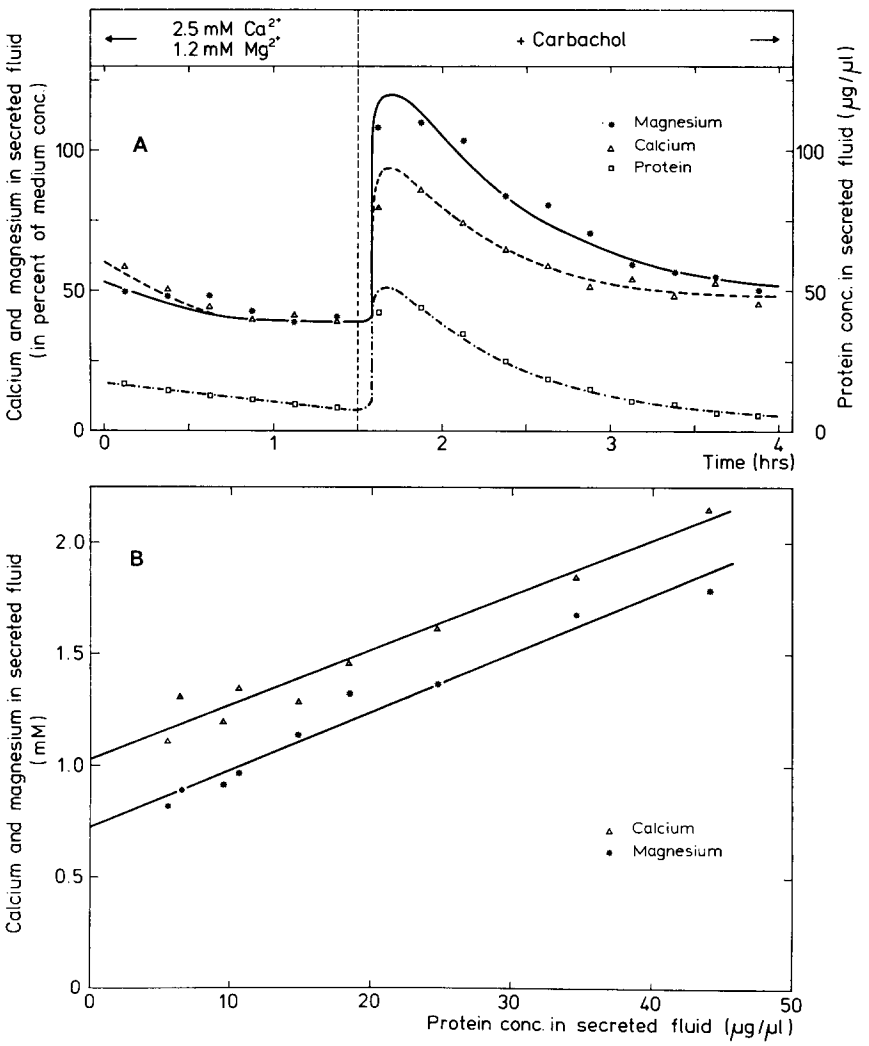

Fig. 2.(A) Protein, calcium and magnesium contents of the secreted fluid collected from the cannulated duct of the isolated rabbit pancreas, before and after stimulation with 10^{-6} M carbachol in normal medium. (B) Calculated regression lines for the relation between divalent cation and protein concentrations in the secreted fluid after stimulation with 10^{-6} M carbachol. Representative for six experiments.

TABLE I

PARAMETERS RELATING DIVALENT CATION AND PROTEIN SECRETION

Parameters of the regression lines are calculated according to the least-squares method. Mean values values with standard errors of the mean. *A*, the amount of protein-associated cation (nmol per mg protein); *B*, the cation concentration in absence of any protein (mM); *r*, correlation coefficient, which measures the degree of fit of the given points to the least-squares straight line; *n*, number of experiments.

	A	*B*	*r*	*n*
Normal medium				
Calcium	21 ± 2.0	1.11 ± 0.06	0.96 ± 0.02	6
Magneisum	24 ± 2.8	0.69 ± 0.08	0.97 ± 0.02	7
Ca^{2+}- and Mg^{2+}-free medium				
Calcium	31 ± 3.1	0.19 ± 0.05	0.95 ± 0.02	5
Magnesium	33 ± 3.9	0.14 ± 0.05	0.98 ± 0.01	5

the relative stimulation of the protein secretion is much larger than that of calcium and magnesium, indicating that the cations are not exclusively protein associated in the secreted fluid.

In Fig. 2b the concentrations of the divalent cations (y) are plotted against the protein concentration (x) for the period after stimulation during which the extracellular flux has become constant (see previous section). A linear relation of the type $y = Ax + B$ is obtained for individual experiments. The intercept B represents the cation concentration in the absence of any protein and the slope A the amount of protein-associated cation. The mean values of A and B are shown in Table I, together with the correlation coefficients (r), which measure the degree of fit of the given points to the least-squares straight lines.

Stimulation in a divalent cation-free medium

When the normal Krebs-Ringer bicarbonate medium is replaced by a divalent cation-free medium, there is a marked decrease in the secretion of calcium and magnesium, while the protein secretion is only slightly affected (Fig. 3a).

When 10^{-6} M carbachol is added to the divalent cation-free medium, the protein secretion increases and is still accompanied by a simultaneous increase of the calcium and magnesium secretion. This indicates that these cations do not originate directly from the bathing medium, but rather from endogenous cation pools.

Plotting the concentrations of the divalent cations vs. the protein concentration, as described in the previous section, again yields a linear relation for individual experiments (Fig. 3b). The values of B, however, are close to zero, while the values of A show little change. The mean values of A and B, together with the correlation coefficients (r), which measure the degree of fit of the given points to the least-squares straight lines are shown in Table I.

Gel filtration of the secreted fluid

The foregoing experiments clearly show the existence of a protein-associated component in the secretion of divalent cations, which must originate from an endo-

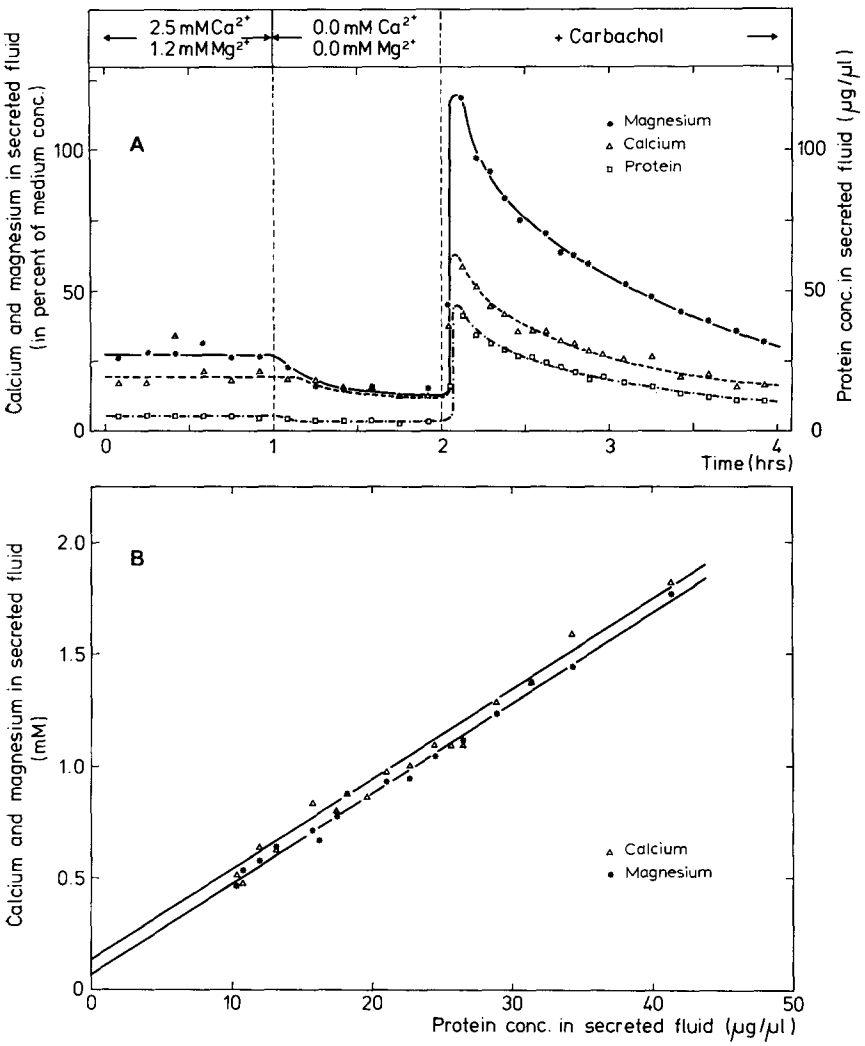

Fig. 3.(A) Protein, calcium and magnesium contents of the secreted fluid collected from the cannulated duct of the isolated rabbit pancreas, before and after stimulation with 10^{-6} M carbachol in a medium free of divalent cations. (B) Calculated regression lines for the relation between divalent cation and protein concentrations in the secreted fluid after stimulation with 10^{-6} M carbachol. Representative for five experiments.

genous pool. Previously [13] we have already shown that this cation pool exchanges poorly with the Ca^{2+} in the incubation medium. This suggests that the divalent cations are either strongly bound to the enzyme proteins or else that they are loosely bound to the protein but sequestered inside the granules.

In order to distinguish between these two possibilities, we have subjected samples of the secreted fluid to gel filtration. This procedure separates freely diffusible and protein-bound cations. The elution patterns (Fig. 4) show that the divalent cations are virtually completely separated from the bulk protein. Only a small fraction of the

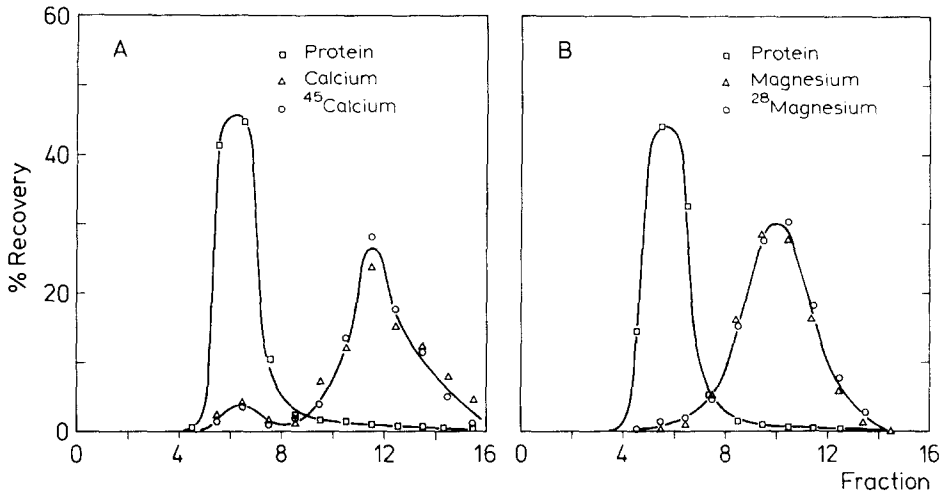

Fig. 4. Gel filtration on Sephadex G-25 coarse of samples of the secreted fluid collected from the cannulated duct of the isolated rabbit pancreas. Radioactive tracers are added to the samples just before their application to the column. (A) Elution pattern of samples to which $^{45}Ca^{2+}$ is added. (B) Elution pattern of samples to which $^{28}Mg^{2+}$ is added. Mean values of three experiments.

calcium (about 20 % of all protein-associated calcium) remains associated with the protein, while magnesium is completely separated. The easy exchangeability of the cations is also illustrated by the fact that both calcium and magnesium are completely equilibrated with their tracers, applied to the samples just before elution.

Efflux studies with pancreas fragments

The preceding experiments on the isolated rabbit pancreas have been carried out in order to obtain more information about the extracellular and secretory fluxes of calcium and magnesium. Previous studies with pancreas slices, pre-loaded with $^{45}Ca^{2+}$, show an increased $^{45}Ca^{2+}$ efflux upon stimulation [6–8]. We have shown that this so called stimulatory calcium flux is mainly localized over the serosal membrane [13]. Since this flux cannot be studied in the isolated rabbit pancreas, we have used rabbit pancreas fragments, which permit simultaneous measurement of the secretion to the stimulatory and secretory compartment of the tissue.

The pancreas fragments are pre-loaded with $^{45}Ca^{2+}$ for 2 h, washed and transferred to a series of plastic counting vials with tracer-free medium in order to determine the $^{45}Ca^{2+}$ efflux rate. Fig. 5 shows a continuously decreasing $^{45}Ca^{2+}$ efflux rate, reaching a relatively constant value after about 2 h.

When 10^{-5} M carbachol is added to the efflux medium, there is an immediate, large increase in the $^{45}Ca^{2+}$ efflux rate, confirming previous findings [6–8, 13]. The normal efflux rate is restored in about 30 min after stimulation.

When similar experiments are carried out with $^{28}Mg^{2+}$ instead of $^{45}Ca^{2+}$, the initial efflux rate is comparable to that of $^{45}Ca^{2+}$. When however 10^{-5} M carbachol is added to the efflux media, the $^{28}Mg^{2+}$ efflux rate is not effected at all, but remains relatively constant during the rest of the experiment. In both experiments there is the usual increase of the amylase release by carbachol.

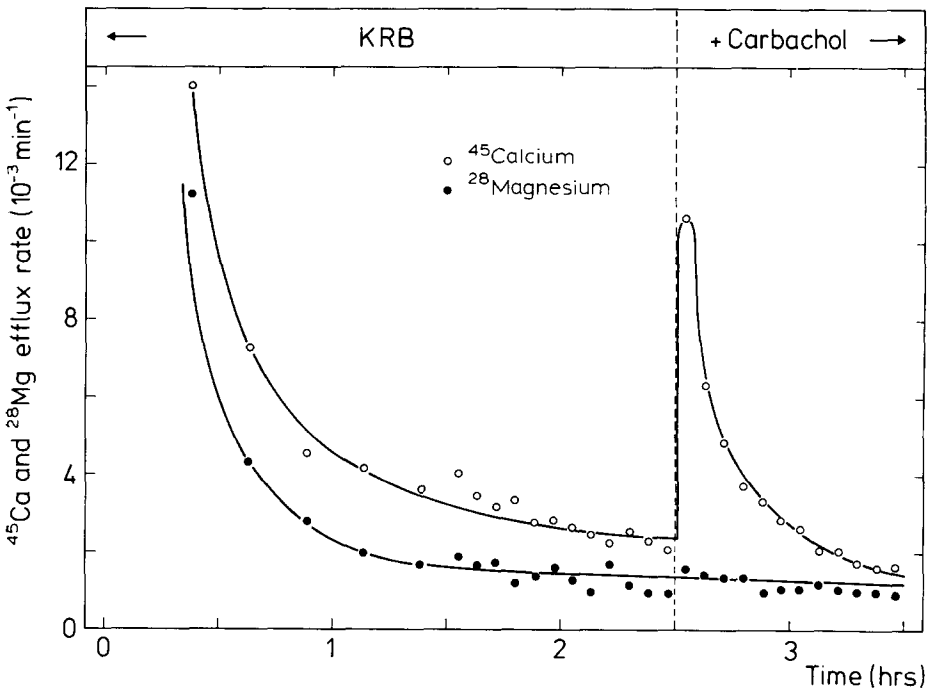

Fig. 5. Effects of 10^{-5} M carbachol on the efflux of $^{45}Ca^{2+}$ and $^{28}Mg^{2+}$ from pre-loaded rabbit pancreas fragments. Mean values of four experiments.

DISCUSSION

Ca^{2+} are closely involved in pancreatic enzyme secretion. They are not only present in the secreted fluid [1–4, 13, 18], but they may also play a role in the stimulus-secretion coupling of pancreatic enzyme secretion. This would involve an increased cytoplasmic calcium concentration (see review, ref. 5).

The involvement of calcium in the exocrine pancreatic secretion is at least partly reflected by the distinct calcium movements, which we have shown to exist in the rabbit pancreas in vitro [13]. These calcium movements consist of an extracellular route for calcium and other small molecules and ions (extracellular calcium flux), a release of calcium across the apical membrane along with the enzymes (secretory calcium flux) and a calcium flux across the serosal membrane (stimulatory calcium flux). The secretion into the pancreatic duct has also been reported for other divalent cations, such as zinc and magnesium [3, 14–16, 18]. Since magnesium, the only other divalent cation in the bathing medium, is not supposed to play a role in the stimulus-secretion coupling of pancreatic enzyme secretion [5], we have compared the three calcium movements with those of magnesium in a qualitative and quantitative way.

During basal secretion the concentrations of calcium and magnesium are 30 (S.E. 5.9, $n = 5$) and 33 (S.E. 5.5, $n = 5$) %, respectively, of the concentrations in the bathing medium. This means that the absolute magnesium concentration is about half that of calcium. Goebell et al. [2] and Zimmerman et al. [19] find for calcium

a nearly 1 : 1 ratio between secreted fluid and extracellular medium, which decreases when the flow rate is increased by secretin. Considering that the flow rate of the non-stimulated isolated rabbit pancreas is nearly equal to the maximal flow rate after secretin stimulation in vivo [20], our results are compatible with the results of these authors. Moreover, at the end of some of our experiments we notice a decrease in flow rate parallelled by an increase in calcium and magnesium concentration.

Stimulation with carbachol not only increases the enzyme secretion, but also the calcium and magnesium concentrations of the secreted fluid. The values, which are thus obtained, may even exceed those for the bathing medium. This suggests that these cations do not directly originate from the bathing medium. Despite the simultaneous increase of the protein and divalent cation concentrations in the secreted fluid upon stimulation, the divalent cations cannot be secreted entirely in association with the protein. Firstly, because there is no constant ratio between cation levels and protein level. Secondly, because a change in the divalent cation concentration of the incubation medium results in a parallel change of their concentrations in the secreted fluid, while enzyme secretion is only slightly affected (Fig. 3a). This means that calcium and magnesium must be secreted in at least two fractions, one associated with the digestive enzymes and the other as free ions, as has already been suggested for calcium [1–4].

Our observation of the similarity in the secretion of magnesium and of calcium has led us to the conclusion that there exists an extracellular and a secretory flux for both calcium and magnesium. The experiments in which $^{28}MgCl_2$ is present in the bathing medium show that the extracellular magnesium flux is fully represented by the secretion of $^{28}Mg^{2+}$, since this tracer does not exchange with the protein-associated magnesium.

We have examined the relation between the secretion of divalent cations and protein after stimulation with 10^{-6} M carbachol, without the use of radioactive isotopes. Plotting the divalent cation concentrations (y) vs. the protein concentration (x) yields a linear relation of the type $y = Ax + B$ for both cations in individual experiments. The contribution of the secretory flux (A) is 21 (S.E. 2.0, $n = 6$) and 24 (S.E. 2.8, $n = 7$) nmol/mg protein for calcium and magnesium, respectively. The extracellular flux (B) amounts to 1.11 (S.E. 0.06, $n = 6$) and 0.69 (S.E. 0.08, $n = 7$) mM.

When stimulation is carried out in a divalent cation-free medium, again a linear relation is found. The contribution of the secretory flux (A) in this case is 31 (S.E. 3.1, $n = 5$) and 33 (S.E. 3.9, $n = 5$) nmol/mg protein for calcium and magnesium, respectively. These values are hardly significantly different ($P < 0.05$–0.1) from the corresponding ones in the preceding experiments, indicating that the cation content of the bathing medium does not influence the amount of protein-associated cations. This conclusion is supported by the absence of any exchange between each of these cation pools and the corresponding radioisotopes in the medium. On the other hand, the extracellular flux (B) is nearly zero in a divalent cation-free medium, 0.19 (S.E. 0.05, $n = 5$) and 0.14 (S.E. 0.05, $n = 5$) mM for calcium and magnesium, respectively, indicating that this flux depends directly on the concentrations of the cations in the medium. The very small remaining contribution of this flux may be due to leakage of divalent cations from the tissue in divalent cation-free media.

Our values of the amounts of the protein-associated divalent cations are close to those of Rutten [3], who found 42 nmol calcium and 33 nmol magnesium/mg

protein, after stimulation with different kinds and doses of drugs. Ceccarelli et al. [4] reported a value of about 50 nmol calcium/mg protein for guinea pig, while Sullivan et al. [18] found much lower values for pig: 3.3 nmol calcium and 1.3 nmol magnesium/mg protein.

Gel filtration of the secreted fluid shows that the divalent cations can be virtually completely separated from the protein under our conditions. In addition, there is a complete exchange of the cations with their tracers, added just before elution, indicating that a rapid exchange is possible between the cations after their secretion. In contrast, the protein-associated cation pools do not exchange with tracers added to the bathing medium, before and during secretion. These observations suggest that although the cations are loosely bound to the enzyme proteins, they cannot exchange as long as the enzymes are sequestered inside the zymogen granules. Since this sequestration ceases after secretion, the question arises why exchange is absent during the passage of secretory products through the ductular system, despite the fact that the concentration of divalent cations in the secreted fluid may temporarily exceed their bath concentrations. The absence of exchange may be explained by the fact that the time for divalent cations to equilibrate with the bathing medium (30 min, see Fig. 1) is relatively long with respect to the time (5 min) that secretory products stay within the ductular system. Whatever the role of the protein-associated divalent cations may be, it is clear that calcium and magnesium behave qualitatively and quantitatively in the same way with respect to their extracellular and secretory fluxes, which together supply the divalent cations present in the secreted fluid.

The comparison between calcium and magnesium with respect to the stimulatory flux is shown in Fig. 5. In the absence of stimulation the efflux from pancreas fragments is comparable for $^{45}Ca^{2+}$ and $^{28}Mg^{2+}$. Upon stimulation the $^{45}Ca^{2+}$ efflux rate shows a marked increase, but the $^{28}Mg^{2+}$ efflux remains constant, while the amylase release is comparable in both cases. Previously we have advanced evidence that the $^{45}Ca^{2+}$ efflux after stimulation must take place across the serosal membrane [13], and is due to the release of calcium from an intracellular store [17, 21]. The absence of a comparable increased $^{28}Mg^{2+}$ efflux strongly suggests that the increase of the cytoplasmic calcium concentration is a specific and essential step in the stimulus-secretion coupling of pancreatic enzyme secretion. Moreover, the ionophore A-23187, which is known to transport calcium and magnesium across biological membranes [22], only stimulates pancreatic enzyme secretion in the presence of calcium, which cannot be replaced by magnesium [17, 21].

The present study leads to the conclusion that calcium and magnesium behave very similar with respect to their extracellular and secretory fluxes. The presence of a stimulatory flux for calcium only suggests that this flux, due to a release of calcium from an intracellular store, represents calcium which plays a specific and essential role in the stimulus-secretion coupling of pancreatic enzyme secretion.

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