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ROLE OF CALCIUM IN EXOCRINE PANCREATIC SECRETION

VI. CHARACTERISTICS OF THE PARACELLULAR PATHWAY FOR DIVALENT CATIONS

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Summary

(1) The transepithelial permeability for Ca^{2+} and Mg^{2+} in the isolated rabbit pancreas has been studied.

(2) Values for the permeability of the unstimulated pancreas were obtained either by adding radioactive tracers to the bathing medium and measuring their concentration in the secreted fluid under steady-state conditions, or by analysis of the Ca^{2+} and Mg^{2+} concentrations in the secreted fluid after correction for protein-bound divalent cations.

(3) Both methods give almost the same results: 27 and 26% for Ca^{2+} and 21 and 18% for Mg^{2+}, respectively; both values being expressed as the percentage of the concentrations in the bathing medium.

(4) The amounts of Ca^{2+} and Mg^{2+}, appearing in the secretory fluid after correction for protein-bound cations, are linearly related to the extracellular Ca^{2+} and Mg^{2+} concentrations in the bathing medium, which indicates passive permeation. The two cations appear to pass through the paracellular route in their hydrated form.

(5) Stimulation with carbachol or pancreozymin causes an increase in the paracellular permeability. This increase is approximately equal for the two divalent cations. Its time dependence and magnitude depend on the concentration of the stimulant rather than on the type of stimulant.

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Introduction

The secreted fluid of the pancreas is isotonic with the plasma and the interstitial fluid. The concentrations of Na^+ and K^+ are equal to or somewhat higher than those in the extracellular fluid [1—5]. The concentrations of the divalent cations, Ca^{2+} and Mg^{2+}, however, are generally lower than those in the extracellular fluid [6—10].

From a previous study [9], it appears that the divalent cations in the fluid secreted by the isolated rabbit pancreas originate from two sources: one part is secreted along with the secretory proteins, whereas another part originates directly from the extracellular fluid. We have shown that the latter fraction (extracellular flux) traverses the paracellular pathway. In this route the tight junctions form probably the main barrier. This pathway is also permeable to small non-electrolytes, such as mannitol and sucrose, and the permeability for divalent cations and small non-electrolytes is enhanced by cholinergic agents and pancreozymin [11,12]. The permeability increase is not a direct consequence of the stimulation of the enzyme secretion by these agents, but probably represents a separate effect of the above stimulants [12,13].

We have now further investigated the characteristics of the paracellular route for Ca^{2+} and Mg^{2+} by determining the permeability for these ions in the resting state as well as after addition of pancreozymin and carbachol at two different levels. The results indicate that the permeability for Ca^{2+} is always higher than for Mg^{2+}, which suggests that the hydrated forms of the cations are transported. Furthermore, the increase in permeability caused by carbachol and pancreozymin depends on the concentration of the stimulant, but not on the type of stimulant used, which suggests that the two stimulants exert their effect on the permeability through a common mechanism.

Materials and Methods

Chemicals. Carbachol, the carbamyl analogue of acetylcholine, is purchased from Brocades-ACF Holland and pancreozymin from the Boots Company Ltd., Nottingham, U.K. $^{45}CaCl_2$ is supplied by The Radiochemical Centre, Amersham, U.K., and $^{28}MgCl_2$ by Brookhaven National Laboratory, Associated Universities, Inc., New York, NY 11973, U.S.A. Aquasol is obtained from New England Nuclear, Dreieichenhain, F.R.G. All other substances are commercial preparations of the highest obtainable purity.

Preparation and incubation of the pancreas. The pancreas of male and female New Zealand white rabbits, weighing 2—3 kg, is prepared and mounted essentially according to the method of Rothman [14], as applied earlier by us [9,11,12].

It is incubated in a balanced Krebs-Ringer bicarbonate medium (KRB-medium), containing (in mM): Na^+, 144; K^+, 4.9; Ca^{2+}, 2.5; Mg^{2+}, 1.2; HCO_3^-, 25.0; $H_2PO_4^-$, 1.2; Cl^-, 131 and glucose, 5.5. Before incubation the pH of the solution is adjusted to 7.2 by addition of HCl. During incubation the medium is continuously aspirated with O_2/CO_2 (95 : 5, v/v) and maintained at 37°C.

Collection of pancreatic fluid

Radioactive experiments. After 1 h preincubation the incubation medium is

replaced by fresh KRB-medium supplemented with 15 μCi $^{45}Ca^{2+}$ or $^{28}Mg^{2+}$. The pancreatic fluid is collected in 10-min fractions in pre-weighed plastic counting vials. From each fraction, 10-μl samples are taken for determination of protein and of total Ca^{2+} or Mg^{2+}. The remaining volume, determined by weighing, is mixed with 10 ml Aquasol and subjected to radioactive counting. During incubation the stimulants are applied 1 or 2 h after starting the experiment.

Non-radioactive experiments. After 1 h preincubation the medium is replaced every hour (up to four times per experiment) by fresh KRB-medium with a Ca^{2+} concentration varying between 0 and 3.5 mM. Fig. 1 shows a typical experiment in which the isolated rabbit pancreas is incubated during four 1-h periods with different Ca^{2+} concentrations. Ca^{2+} in the secreted fluid appears to equilibrate with that in the incubation medium within 30 min, hence the last three 10-min fractions of every hour are used for calculations. The pancreatic fluid is collected in 10-min fractions in pre-weighed plastic vials which are then weighed again. A 5 μl sample is taken from each fraction for determination of protein and a 20 μl sample for determination of total Ca^{2+}. For Mg^{2+} the experiments are carried out in similar fashion.

Assay methods. Protein is determined according to the method of Lowry et al. [15] on a microscale with bovine albumin (Behringwerke, Marburg-Lahn, F.R.G.) serving as standard. Total Ca^{2+} and Mg^{2+} concentrations are determined with a calcium or magnesium Rapid Stat kit (Pierce Chemical Co., Rockford, IL, U.S.A.), when during the experiments a tracer has been used. The blue color of the Ca^{2+} complex of methyl-thymol or the red Mg^{2+} complex of calmagite is measured. When no tracer is used, total Ca^{2+} and Mg^{2+} are measured by atomic absorption spectrophotometry (Pye Unicam SP 1950). The

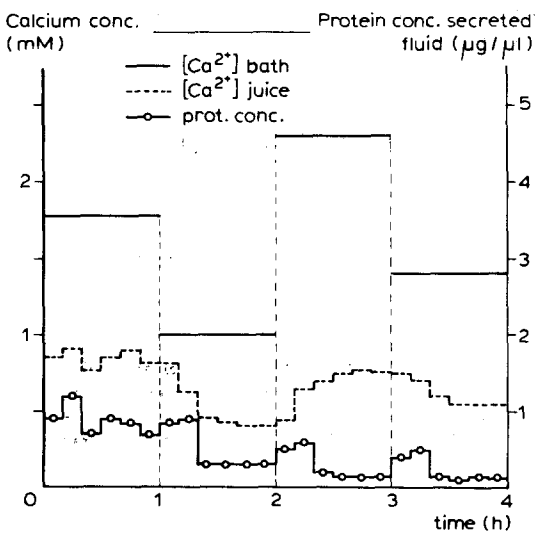

Fig. 1. Ca^{2+} secretion by the resting isolated rabbit pancreas. A typical experiment, in which during four successive 1-h periods the Ca^{2+} concentration in the incubation KRB-medium has been changed every hour. Ca^{2+} has been measured in bathing medium (———) and secreted fluid (- - - - -), protein only in the secreted fluid (o———o).

radioactivity present in the collected fractions is measured in a liquid scintillation analyzer (Philips PW 4510).

Results

Resting pancreas

The permeability of the paracellular route in the isolated rabbit pancreas for Ca^{2+} and Mg^{2+} can be determined in two ways. Firstly, a direct measurement can be obtained by adding either $^{45}Ca^{2+}$ or $^{28}Mg^{2+}$ to the bathing medium and determining the ratio of the isotope concentrations in secreted fluid and bathing medium. This is permissible, since Ca^{2+} and Mg^{2+} secreted in association with the proteins do not exchange with their radioactive tracers during the time course of such an experiment [16]. This method gives a value for Ca^{2+} of 26 ± 1.4% (n = 12) and for Mg^{2+} of 18 ± 1.5% (n = 12).

Alternatively, the Ca^{2+} and Mg^{2+} concentrations of the secreted fluid of the unstimulated rabbit pancreas can be measured directly and related to their concentrations in the bathing medium. The values obtained in this case are 29 ± 1.7% (n = 12) for Ca^{2+} and 33 ± 3.0% (n = 12) for Mg^{2+}, expressed as the percentage of their respective concentrations in the bathing medium. These two values must be corrected for the divalent ions associated with the small amount of secreted proteins (21 nmol Ca^{2+} and 24 nmol Mg^{2+} per mg protein; Ref. 9). The corrected values are shown in Table I. The correction is much larger for Mg^{2+} than for Ca^{2+}, due to the fact that the Mg^{2+} concentration in the bathing medium (1.2 mM) is lower than the Ca^{2+} concentration (2.5 mM). The resulting values for Ca^{2+} and Mg^{2+} are 27 ± 1.8 and 21 ± 3.8%, respectively, which do not differ significantly from the values obtained with the tracer method (26 ± 1.4 and 18 ± 1.5%).

The experiments described in the previous paragraph have been carried out in a normal Krebs-Ringer bicarbonate medium. In order to see whether the magnitude of the extracellular flux depends on the extracellular Ca^{2+} and Mg^{2+} concentrations, we have varied these concentrations between 0.1 and 4 mM and have measured the resulting concentrations in the secreted fluid after equilibrium has been established. The bathing concentrations are varied in random

TABLE I

DETERMINATION OF THE PARACELLULAR PERMEABILITY FOR Ca^{2+} AND Mg^{2+} BY THE CHEMICAL METHOD AND THE TRACER METHOD

Values for 12 experiments are given with S.E.

	Fluid secretion (μl/30-min)	Protein secretion (mg/30-min)	Medium concn. of cation (mM)	Protein-bound cation (nmol/mg protein)	Concentration in secreted fluid (in % of medium concentration)		
					Total	Chemical method (corrected for protein)	Tracer method
Ca^{2+}	332 ± 26	1.12 ± 0.18	2.58 ± 0.07	21 ± 2.0 *	29.3 ± 1.7	26.5 ± 1.8	26.0 ± 1.4
Mg^{2+}	320 ± 29	1.91 ± 0.27	1.29 ± 0.10	24 ± 2.8 *	32.8 ± 3.0	20.7 ± 3.8	18.0 ± 1.5

* From Schreurs et al. [9].

sequence. For each rabbit pancreas samples are taken at four different times. The Ca^{2+} and Mg^{2+} concentrations measured in the secreted fluid are corrected for the protein-associated ions as described above, using protein concentration determined in the same sample. The resulting values are plotted against the extracellular concentrations of Ca^{2+} (Fig. 2) and Mg^{2+} (Fig. 3). In both figures there is a positive linear correlation between the concentrations in secreted fluid and bathing medium. The linearity of the relationship between the concentrations of the two divalent cations in the secreted fluid and the bathing medium indicates that the paracellular permeability is independent of the extracellular concentration.

The slope of the regression line for Ca^{2+} is 31 ± 4.0% and for Mg^{2+} 18 ± 1.4%. These slopes could serve as an additional estimate of the paracellular permeability, if the lines would pass through the origin. This is the case for Mg^{2+} (Fig. 3), where the slope, 18 ± 1.4%, is in good agreement with the permeability values of 18 ± 1.5% and 21 ± 3.8% obtained with the tracer and the chemical methods, respectively. In the case of Ca^{2+} the intercept of 0.21 ± 0.08 mM is significantly above the origin, so that here no valid comparison is possible. The positive intercept of the Ca^{2+} line indicates that in addition to the protein-associated Ca^{2+} and the Ca^{2+} originating from the extracellular medium an amount of Ca^{2+} from another source, possibly the pancreatic cells, appears in the secreted fluid.

Stimulated pancreas

As we have shown before [9,11,12], addition of carbachol or pancreozymin to the bathing medium of the isolated rabbit pancreas does not only lead to enhanced enzyme secretion, but also to increased permeability of the para-

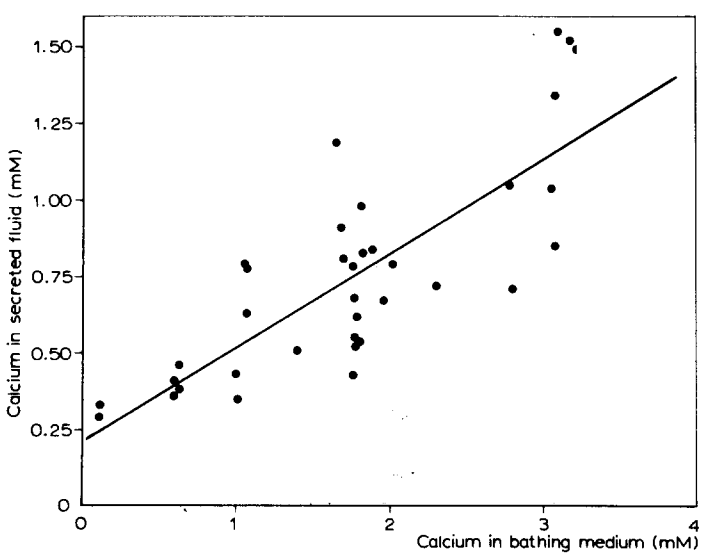

Fig. 2. Relation between free Ca^{2+} concentration in the secreted fluid and the Ca^{2+} concentration in the bathing medium under steady-state conditions. Free Ca^{2+} concentration in the secreted fluid is total minus protein-bound Ca^{2+}. The points are derived from 10 experiments. Correlation coefficient 0.802.

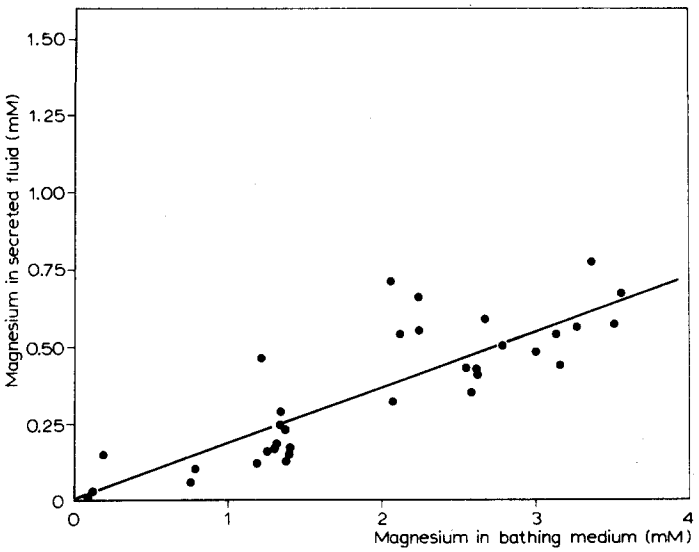

Fig. 3. Relation between the free Mg^{2+} concentration in the secreted fluid and the Mg^{2+} concentration in the bathing medium under steady-state conditions. Free Mg^{2+} concentration in the secreted fluid is total minus protein-bound Mg^{2+}. The points are derived from 11 experiments. Correlation coefficient 0.843.

cellular route for divalent cations and small neutral molecules.

The permeability increase depends on the concentration of the stimulant. When a high concentration of carbachol ($1 \cdot 10^{-5}$ M) or pancreozymin (600 units/l) is used, the concentrations of $^{45}Ca^{2+}$ and $^{28}Mg^{2+}$ in the secreted fluid increase after stimulation, reach a maximum after 20—40 min, whereupon a

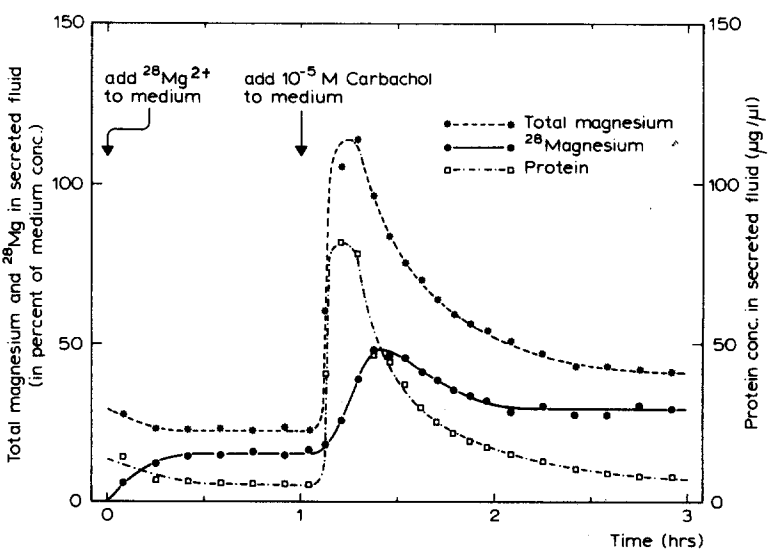

Fig. 4. Mg^{2+} secretion in the pancreatic fluid before and after stimulation with $1 \cdot 10^{-5}$ M carbachol. During the entire experiment $^{28}Mg^{2+}$ is present in the medium, which also contains 1.2 mM non-radioactive Mg^{2+}. Typical for three experiments.

TABLE II

EFFECTS OF CARBACHOL AND PANCREOZYMIN ON THE PARACELLULAR PERMEABILITY OF $^{45}Ca^{2+}$ AND $^{28}Mg^{2+}$ IN THE ISOLATED RABBIT PANCREAS

$^{45}Ca^{2+}$ and $^{28}Mg^{2+}$ are added in trace amounts to the bathing medium of the isolated rabbit pancreas. After 60 min incubation the stimulants are added in the concentrations indicated. Period I, 30—60 min (control period); period II, 80—100 min (peak period); period III, 120—150 min (steady-state period). Results for three experiments are given with S.E.

Stimulant	$^{45}Ca^{2+}$ concentration ratio		$^{28}Mg^{2+}$ concentration ratio	
	Period II/I	Period III/I	Period II/I	Period III/I
Control	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
Carbachol				
$1 \cdot 10^{-6}$ M	1.3 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.1
$1 \cdot 10^{-5}$ M	2.6 ± 0.5	2.1 ± 0.5	2.3 ± 0.4	1.9 ± 0.1
Pancreozymin				
60 units/l	1.6 ± 0.2	1.5 ± 0.2	1.2 ± 0.2	1.3 ± 0.2
6000 units/l	2.5 ± 0.1	2.1 ± 0.4	3.3 ± 0.1	2.8 ± 0.1

slight decrease occurs (e.g., see Fig. 4). The steady-state concentration which is reached 60 min after addition of the stimulant is always higher than before stimulation. When a lower concentration of carbachol ($1 \cdot 10^{-6}$ M) or pancreozymin (60 units/l) is used, the concentrations of the radioactive divalent cations also increase after addition of the stimulant, but in this case a steady level is reached after 30 min and is not preceded by a transient peak.

Table II summarizes the results of these experiments. The isotope concentrations in the 30-min period before addition of the stimulant are set at unity and the concentrations in the 80—100 min period (period II) and the 120–150 min period (period III) are related to that in the control period. This table shows that the pattern of increase depends much more on the stimulant concentration than on the type of stimulant used. The relative increases in permeability are approximately the same for the two divalent cations.

Discussion

In a previous study [9] we have shown that the divalent cations in the pancreatic fluid mainly represent two fractions: a protein-dependent fraction and a fraction which is independent of the amount of secreted protein. The latter appears to be of extracellular origin, passing through the paracellular pathway between the cells. In the present study we have investigated the properties of this pathway in more detail.

The magnitude of the paracellular flux can be determined either directly by using tracers of the divalent cations (tracer method), or indirectly by determining the ratio of the divalent cation concentrations in the pancreatic fluid and the bathing medium (chemical method). In the latter case, a correction for the contribution of protein-bound divalent cations in the secreted fluid is necessary. This correction is larger for Mg^{2+}, which is present in a relatively lower concentration in the bathing medium than Ca^{2+}. After this correction,

the resulting values are the same for both methods (Table I).

In the previous study [9] we have shown that the magnitude of the extracellular flux is much smaller when Ca^{2+} and Mg^{2+} are omitted from the bathing medium. By varying the extracellular divalent cation concentrations in the bathing medium between 0.1 and 4 mM, we have now demonstrated that the magnitude of the paracellular flux is linearly determined by the extracellular concentration of each cation. This indicates that the paracellular flux of the two cations represents a passive process of permeation. From the slope of the line obtained by plotting the paracellular flux against the extracellular Mg^{2+} concentration, a third value for the permeability of the paracellular pathway has been obtained. This value is not significantly different from these derived from the tracer method and the chemical method used with constant Mg^{2+} concentrations.

The permeability of the resting pancreas for Ca^{2+} is larger than for Mg^{2+}. Since the permeability of substances through the paracellular pathway depends on their molecular size [12], and the hydrated Mg^{2+}, in contrast to the unhydrated ion, is larger than the hydrated Ca^{2+}, we conclude that most likely the hydrated ions are the permeant entities.

We have also investigated the properties of the paracellular permeability for divalent cations after stimulation with carbachol and pancreozymin. For the sake of accuracy we have used the isotope method. This method does not require correction for the protein-bound cations in the secreted fluid, and this correction is rather large in the case of the stimulated pancreas.

These stimulants increase the permeability of the two divalent cations by approximately the same percentage. The magnitude and time course of the permeability increase does not depend on the type of stimulant, when they are compared at either a high or a 10-fold lower level of stimulant (Table II). There is, however, a similar difference between the effects at the two levels for the two stimulants. At high stimulant level the permeability first increases by a factor of 2—3, then decreases slightly. At low stimulant level the increase is much less (1.2—1.6 times) and a steady state is reached without passing through a peak. The same concentration dependence has also been found for the permeability of non-electrolytes like sucrose [12].

These findings indicate that stimulants of the enzyme secretion process lead to a rather unspecific, but dose-dependent increase in the permeability of the paracellular pathway for a number of substances like divalent cations and small non-electrolytes. The parallelism between the effects of pancreozymin and carbachol may suggest that both stimulants act through a common mechanism. Nevertheless, they appear to act on different receptors, since atropine [12] and 2,4,6-triaminopyrimidine [13] block the acetylcholine induced but not the pancreozymin-induced increase in tight-junction permeability.

The increase in permeability of this pathway is not a direct consequence of the increased enzyme secretion, since addition of atropine 5 min after carbachol does not affect the stimulation of the enzyme secretion, but strongly inhibits the increase in solute permeability [12]. In addition, 2,4,6-triaminopyrimidine does not inhibit the enzyme secretion process.

What is the physiological significance of the paracellular pathway, and in particular of its increased permeability after stimulation with the stimulants of

the enzyme secretion? A relatively large transepithelial permeability is a property of many epithelial tissues. We have found strong indications that Na^+ and K^+ also permeate by the paracellular pathway (Jansen, J.W.C.M., de Pont, J.J.H.H.M. and Bonting, S.L., unpublished observations). Assuming that anions are primarily secreted by the epithelial cells, then the paracellular pathway could be of help in the passive transport of monovalent cations required for electroneutrality. The permeability increase is not useful for monovalent cations since their permeability is already maximal in the resting state. However, it could be that the secreted proteins, once they have been released from the zymogen granules into the secretory fluid, need additional divalent cations for their activity, which are made available by the increased permeability of the paracellular route. Ca^{2+} plays a role in maintaining the molecular integrity of amylase [17], trypsinogen and chymotrypsinogen [18] and lipase [19]. Ca^{2+} is also required for the conversion of trypsinogen into trypsin [20] as well as for the activity of trypsin, lipase and phospholipase A_2 [21—23]. Although a role for Mg^{2+} is not yet known, Mg^{2+} may also be needed for the activity of certain pancreatic enzymes. Another possible role for the permeability increase could be that is favours the transport from blood to secretory fluid of other small molecules which play a role in the intestinal digestion process. Further studies to establish the physiological role of this increase in permeability are obviously needed.

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