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ROLE OF CALCIUM IN EXOCRINE PANCREAS SECRETION

VII. EFFECT OF SODIUM ON ENZYME SECRETION AND CALCIUM METABOLISM IN RABBIT PANCREATIC FRAGMENTS AND ACINAR CELLS

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Summary

1. The role of sodium in the pancreatic stimulus-secretion coupling has been studied.

2. Reduction of the extracellular sodium concentration or addition of ouabain to the medium inhibits the stimulation of enzyme secretion by carbachol.

3. Incubation in low sodium medium or in the presence of ouabain increases the exchangeable calcium content, but not the total calcium content of acinar cells.

4. Depending on preincubation time and the substance replacing sodium in the low sodium medium, the carbachol-induced $^{45}Ca^{2+}$ efflux may be blocked, but it is not blocked by addition of ouabain to a normal Na^+ medium.

5. Stimulation of enzyme secretion by the calcium ionophore A23187 in the presence of external calcium is inhibited in low sodium as well as in ouabain containing media.

6. These findings suggest that reduction of the Na^+ gradient across the plasma membrane blocks the coupling between intracellular calcium release and exocytosis, while under certain conditions it also blocks the coupling between hormone-receptor interactions and intracellular calcium release.

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Introduction

There are strong indications that the increase in the cytoplasmic calcium concentration, necessary for the initiation of the stimulation of pancreatic enzyme secretion, is caused by a release of calcium from an intracellular pool by the stimulant [1—3]. However, the calcium pool involved in this stimulus-secretion coupling has not yet been identified. Plasma membrane bound calcium has been suggested as the pool by Petersen and Ueda [4]. The mitochondrial calcium pool or yet another pool, which requires ATP for its calcium uptake, has been held responsible by Chandler and Williams [5,6]. Regardless of the location of this calcium pool, there must be a coupling between the hormone-receptor interaction and the release of calcium from its pool.

Another point of uncertainty is whether the increase in cytoplasmic calcium concentration is stimulates exocytosis directly or via one or more intermediate steps. So it is conceivable that in addition to calcium other messengers may play a role in the stimulus-secretion coupling process.

There are several indications that sodium ions are involved in pancreatic enzyme secretion. Lowering the extracellular sodium concentration inhibits stimulation of the enzyme secretion in the isolated rat pancreas [1], mouse pancreatic fragments [7], the perfused rat pancreas [8,9], rat pancreatic segments [4] and mouse pancreatic acinar cells [10]. Moreover, Case and Clausen [1] and Kanno [8] have observed that incubation in the presence of ouabain also reduces the stimulated enzyme secretion. This is, however, in contrast to the report of Petersen and Ueda [11] who do not find any effect of ouabain on amylase release.

Additional evidence for a role of sodium in the stimulus-secretion coupling is provided by electrophysiological experiments. Under normal conditions stimulation of enzyme secretion causes depolarization of the membrane potential [12—15]. Since this depolarization depends on the extracellular sodium concentration [12,13], the stimulant appears to cause an increase in the membrane permeability for sodium and a subsequent Na^+ influx [4,13].

The above findings suggest that sodium must be involved in pancreatic enzyme secretion. We have investigated in which step(s) of the stimulus-secretion coupling process sodium is involved, particularly with respect to the relation between calcium and sodium transport in the acinar cell. For this purpose, we have studied the effects of lowering extracellular sodium concentration and of addition of ouabain on the stimulation of enzyme secretion and on the calcium metabolism of the acinar cells.

Materials and Methods

Tissue preparation

Pancreatic fragments are prepared by cutting the rabbit pancreas in pieces of about 250 mg wet weight. Isolated acinar cells of rabbit pancreas are prepared according to the method described by Amsterdam and Jamieson [16].

Incubation medium

The pancreatic fragments are incubated in a Krebs-Ringer bicarbonate

medium containing: 119 mM NaCl; 3.5 mM KCl; 1.2 mM KH_2PO_4; 25 mM $NaHCO_3$; 2.5 mM $CaCl_2$; 1.2 mM $MgCl_2$; 5.8 mM glucose. The pH of the medium is adjusted to 7.4 with HCl. When incubating isolated acinar cells, trypsin inhibitor (0.2 mg/ml) and bovine serum albumin (1% w/v) are added to this medium. During (pre)incubation the medium is constantly gassed with a 95% O_2/CO_2 mixture to keep the pH at 7.4. A low sodium medium is obtained by substituting sodium chloride by an isosmotic concentration of tetraethylammonium chloride or sucrose, as indicated in the text.

Protein secretion

Enzyme secretion from acinar cells is determined by measuring the release of trichloroacetic acid-insoluble ^3H-labeled proteins, as previously described [3]. When amylase secretion from pancreatic fragments is measured, the fragments are preincubated for 30 min and then individual fragments are transferred to vials containing 5 ml fresh medium. After fixed time periods the fragments are transferred to vials with medium containing the agents indicated in the text. At the end of this incubation period the fragments are homogenized in a Potter-Elvehjem tube. Amylase activity is measured in each vial and in the homogenate, and the amylase release is expressed as percent of total amylase activity present in the fragment at the start of incubation.

Determination of ion contents of isolated acinar cells

An aliquot of the acinar cell suspension is centrifuged and the content of the desired ion is measured in the total pellet. This value is corrected for the content of the ion in the inulin space, yielding the cellular content of the ion, expressed as nmol/mg protein. The detailed procedure has previously been described for calcium [3]. The procedure has been modified by adding 10 mM EDTA before centrifugation of the sample in order to remove calcium adhering to the outside of the cells. The $^{45}Ca^{2+}$ content is measured in the same way after the cells have been incubated for 90 min in Krebs-Ringer bicarbonate medium containing 2.5 μCi/ml $^{45}Ca^{2+}$. The radioactivity is determined in a liquid scintillation counter after addition of 4 ml Picofluor TM.

To check whether the cells take up tetraethylammonium ions, they are incubated in a medium containing 25 mM Na^+, 119 mM tetraethylammonium chloride, and 0.2 μCi/ml tetra[^{14}C]ethylammonium bromide. By measuring the radioactivity in the samples and correction for the isotope content in the inulin space, the cellular concentration of tetraethylammonium in the cell is calculated.

Sodium and potassium are determined by means of flame photometry, calcium by means of atomic absorption photometry.

Efflux of radioactive calcium

The $^{45}Ca^{2+}$ efflux from acinar cells is measured according to the method of Peikin et al. [17]. The cells of a single pancreas are suspended in 1 ml Krebs-Ringer bicarbonate medium containing 2.5 μCi $^{45}Ca^{2+}$ and are then incubated for 90 min at 37°C. The cells are then washed twice in Krebs-Ringer bicarbonate medium without isotope and are resuspended in 6 ml medium. One-ml aliquots of the suspension are incubated for 5 min with or without carbachol.

At the beginning and end of incubation, duplicate 175-μl samples are removed and centrifuged in 1 ml Eppendorf caps (5 s, 18 000 $\times g$) through 200 μl maize oil/phthalate mixture (d = 1.02). The radioactivity in a 100 μl of the supernatant is counted. The efflux is expressed as percent of total radioactivity present in the cells before incubation.

The $^{45}Ca^{2+}$ efflux from pancreatic fragments is determined by the method described by Schreurs et al. [18]: the pancreas is loaded with $^{45}Ca^{2+}$ by incubation for 2 h in 1.5 ml Krebs-Ringer bicarbonate medium containing 10 μCi $^{45}Ca^{2+}$. It is then cut in fragments, which are washed for 15 min in excess medium without isotope to remove adhering radioactivity. At fixed time intervals the fragments are transferred to vials containing 5 ml frsh medium, and the radioactivity in each vial is determined by liquid scintillation counting after addition of 10 ml Instagel. The radioactivity left in the fragment is determined after destruction in Hyamine hydroxide 10-X. The efflux rate of each fraction is calculated as: $k(\text{min}^{-1})$ = (dpm · min^{-1} in sample)/(mean dpm in tissue).

Other determinations

Protein concentration is measured according to Lowry et al. [19], using bovine serum albumin as a standard.

Amylase activity is measured in appropriate dilutions of the samples by means of the Phadebas test, modified for assay on a microscale. Since some agents present in the samples, like ouabain, tetraethylammonium and A23187, appear to interfere with the assay reaction, the results are corrected by using standards containing these agents.

Radioactivity is counted in a Philips liquid scintillation analyzer. In double-labeling experiments the radioactivity of each isotope is calculated by means of the external standard ratio method.

Materials

$^{45}CaCl_2$ (800 Ci/mol), [^{3}H]leucine (1 Ci/mmol) and [^{3}H]inulin (900 Ci/mmol) are purchased from the Radiochemical Centre (Amersham, U.K.) and tetra[^{14}C]ethylammonium bromide (4.4 Ci/mmol) from New England Nuclear (Boston, MA, U.S.A.). Hyamine hydroxide 10-X, Picofluor TM and Instagel are obtained from Packard Instruments (Brussels, Belgium) and the Phadebas amylase test from Pharmacia (Uppsala, Sweden). A23187 is a gift from the Eli Lilly Company (Indianapolis, IN, U.S.A.).

Results

Effects of low extracellular Na^+ concentration on enzyme secretion by isolated acinar cells

To check whether sodium is involved in the enzyme secretion by the rabbit pancreas, isolated acinar cells are incubated in Krebs-Ringer bicarbonate medium containing either 25 mM Na^+ (the rest of the sodium being replaced by tetraethylammonium) or 10^{-5} M ouabain, or a combination of both, before and during exposure to carbachol. Fig. 1 shows a typical experiment and Table I the mean secretory response per 20 min during the initial 20-min period and during the 60-min period of exposure to carbachol. Basal secretion is not

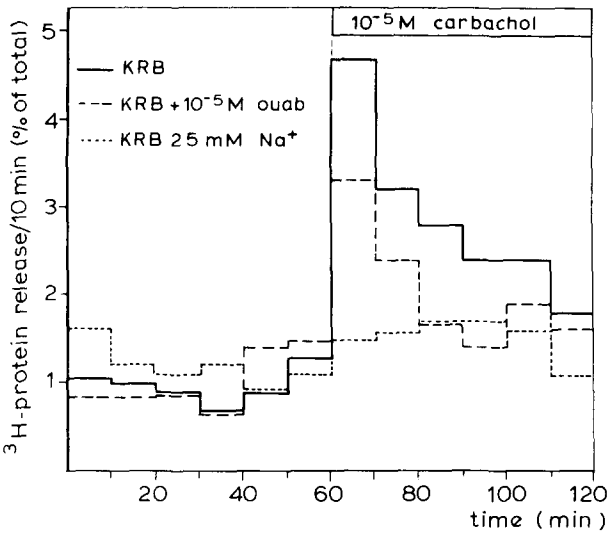


Fig. 1. Effect of 10^{-5} M carbachol on enzyme release from isolated acinar cells in Krebs-Ringer bicarbonate (KRB) medium of normal composition, containing 25 mM Na^+ (sodium replaced by tetraethylammonium) and containing 10^{-5} M ouabain. Means of eight, three and five experiments, respectively.

affected by these changes in incubation conditions. The response to carbachol is nearly abolished by incubation in 25 mM Na^+, but is only partially reduced by ouabain. The inhibition due to ouabain increases with time. The combination of 25 mM Na^+ and ouabain gives the same effect as 25 mM Na^+ alone. We find that maximal inhibition is obtained at ouabain concentrations of 10^{-5} M and higher. In all further experiments 10^{-4} M ouabain has been used.

Effects of low extracellular Na^+ concentration and ouabain on the cellular sodium and potassium content

Cellular sodium and potassium contents are measured after 60 min incuba-

TABLE I

EFFECTS OF EXTRACELLULAR SODIUM CONCENTRATION AND OUABAIN ON ENZYME SECRETION FROM RABBIT ACINAR CELLS

Carbachol is added after incubation for 60 min in the various media. Enzyme release per 20 min is expressed as percent of total initial enzyme content. In the 25 mM Na^+ media sodium is replaced by tetraethylammonium. Mean values with standard error of the mean are given, with the number of separate experiments (n) in parentheses. KRB, Krebs-Ringer bicarbonate medium.

	Amount of enzyme released per 20-min period after addition of carbachol		
	Basal enzyme release per 20 min	during initial 20 min	during 60-min period
KRB	2.0 ± 0.3 (10)	8.8 ± 0.9 (10)	5.8 ± 0.6 (8)
KRB, 25 mM Na^+	2.5 ± 0.2 (3)	3.1 ± 0.4 * (3)	3.1 ± 0.2 * (3)
KRB, 10^{-5} M ouabain	2.1 ± 0.3 (7)	5.8 ± 0.8 * (7)	4.1 ± 0.4 * (5)
KRB, 25 mM Na^+ 10^{-5} M ouabain	1.9 ± 0.2 (3)	3.9 ± 0.7 * (3)	1.9 ± 0.5 * (3)

* These values are significantly lower than the control value ($P < 0.05$ for paired values).

tion in each medium (Table II). Under normal conditions the cellular potassium content is about 120 mM and the sodium content about 35 mM. Incubation in the presence of ouabain (10^{-4} M) causes an increase in the sodium content and a decrease in the potassium content of the cells, the sum of the two ion concentrations remaining constant. However, incubation in low sodium medium, with or without ouabain, reduces both the sodium and potassium contents of the cells. Presumably tetraethylammonium enters the cells to maintain isotonicity. Experiments with radioactive tetraethylammonium show that after 60 min incubation in a medium with 25 mM Na^+ (containing 118 mM tetraethylammonium), 199 nmol/mg protein tetraethylammonium is present in the cells (mean of two experiments). In that case the sum of the contents of potassium, sodium and tetraethylammonium is 696 nmol/mg protein, which is equal to the sum of the sodium and potassium contents after incubation in normal Krebs-Ringer bicarbonate medium.

Effects of low extracellular sodium concentration and ouabain on the exchangeable and total cellular calcium contents of acinar cells

The $^{45}Ca^{2+}$ and total calcium contents of the cells are determined after 90 min incubation in the various media, all of which contain 2.5 mM Ca^{2+} (Table III). The total cellular calcium content after incubation in normal medium is somewhat less than previously found [3], probably due to the addition of 10 mM EDTA before centrifugation. Lowering the sodium concentration or adding ouabain to the incubation medium seems to have no effect on the total calcium content of the cells, while the $^{45}Ca^{2+}$ content of the cells increase under these conditions. This increase is further enhanced by incubation in a medium containing both 25 mM Na^+ and 10^{-4} M ouabain, which also seems to cause a small rise of the total cellular calcium content.

Effect of low extracellular sodium concentration and ouabain on the carbachol-induced $^{45}Ca^{2+}$ efflux from isolated acinar cells

In normal Krebs-Ringer bicarbonate medium carbachol causes a 4-fold increase of the $^{45}Ca^{2+}$ efflux (Table IV). Upon incubation in a medium contain-

TABLE II

EFFECTS OF EXTRACELLULAR SODIUM CONCENTRATION AND OUABAIN ON THE CELLULAR SODIUM AND POTASSIUM CONTENTS OF ACINAR CELLS

The Na^+ and K^+ contents are measured after 60 min incubation in the indicated media. In the 25 mM Na^+ media sodium is replaced by tetraethylammonium. Mean values are given with standard error of the mean (*n* is the number of separate experiments). KRB, Krebs-Ringer bicarbonate medium.

	Na^+ content (nmol/mg protein)	K^+ content (nmol/mg protein)	Sum of Na^+ and K^+ contents	*n*
KRB	174 ± 42 *	528 ± 23 *	702 ± 48	4
KRB, 25 mM Na^+	101 ± 24	396 ± 58	497 ± 93	4
KRB, 10^{-4} M ouabain	596 ± 37	164 ± 20	760 ± 42	3
KRB, 25 mM Na^+ 10^{-4} M ouabain	234 ± 68	194 ± 24	428 ± 72	3

* These values for the sodium and potassium contents correspond to 35 and 120 mM, respectively, assuming that 50% of the wet weight of the tissue is intracellular fluid and 11% of the wet weight is protein.

TABLE III

EFFECTS OF EXTRACELLULAR SODIUM CONCENTRATION AND OUABAIN AND THE EXCHANGEABLE AND TOTAL CELLULAR CALCIUM CONTENTS OF ACINAR CELLS

Calcium contents are measured after 90 min incubation in the various media. In the 25 mM media sodium is replaced by tetraethylammonium. Mean values with standard error of the mean are given, with the number of separate experiments (n) in parentheses. KRB, Krebs-Ringer bicarbonate medium.

	Exchangeable Ca content (nmol/mg protein)	Total Ca content (nmol/mg protein)
KRB	3.9 ± 0.7 (14)	12.2 ± 0.9 (21)
KRB, 25 mM Na^+	6.9 ± 1.2 * (10)	12.4 ± 2.1 (13)
KRB, 10^{-4} M ouabain	7.0 ± 1.8 * (5)	12.4 ± 1.7 (5)
KRB, 25 mM Na^+ 10^{-4} M ouabain	9.3 ± 1.1 * (5)	14.5 ± 1.3 (5)

* These values are significantly higher than the control value ($P < 0.05$ for paired values).

ing 25 mM Na^+, with or without ouabain, there is no influence of carbachol on the $^{45}Ca^{2+}$ efflux. Upon incubation in normal Krebs-Ringer bicarbonate medium containing ouabain, carbachol causes an increase of the $^{45}Ca^{2+}$ efflux, but significantly less than in the control experiment.

Short-term effects of ouabain on enzyme secretion and calcium metabolism in pancreatic fragments

In all experiments described so far carbachol has been added after incubation for 1 h or more. This means that when carbachol is added the intracellular ionic concentrations as well as the ion fluxes across the plasma membrane have been changed. To determine which of these two phenomena is responsible for the observed effects, we have performed experiments in which the Krebs-Ringer bicarbonate medium is replaced by the other media only 5 min before addition of carbachol. It is unlikely that the cellular ion concentrations have changed appreciably after 5 min, so that only the ion fluxes will have changed when carbachol is added. Pancreatic fragments are used in these experiments, since they allow a quick change of medium. Comparison of Figs. 1 and 2 shows that

TABLE IV

EFFECTS OF EXTRACELLULAR SODIUM CONCENTRATION AND OUABAIN ON THE CARBACHOL-INDUCED $^{45}Ca^{2+}$ EFFLUX FROM ACINAR CELLS

Values represent the amount of $^{45}Ca^{2+}$ released to the medium during a 5 min period, expressed as percent of total initial cellular $^{45}Ca^{2+}$ content. Carbachol is added after 90 min incubation in the indicated media. In the 25 mM Na^+ media sodium is replaced by tetraethylammonium. Mean values with standard error of the mean for three separate experiments. KRB, Krebs-Ringer bicarbonate medium.

	$^{45}Ca^{2+}$ efflux	
	Control	10^{-5} M carbachol
KRB	6.2 ± 1.1	23 ± 7.7 *
KRB, 25 mM Na^+	4.9 ± 2.6	5.0 ± 3.0
KRB, 10^{-4} M ouabain	4.8 ± 0.4	11.2 ± 2.2 *
KRB, 25 mM Na^+ 10^{-4} M ouabain	4.9 ± 2.4	4.8 ± 2.2

* These values are significantly higher than the control value ($P < 0.05$ for paired values).

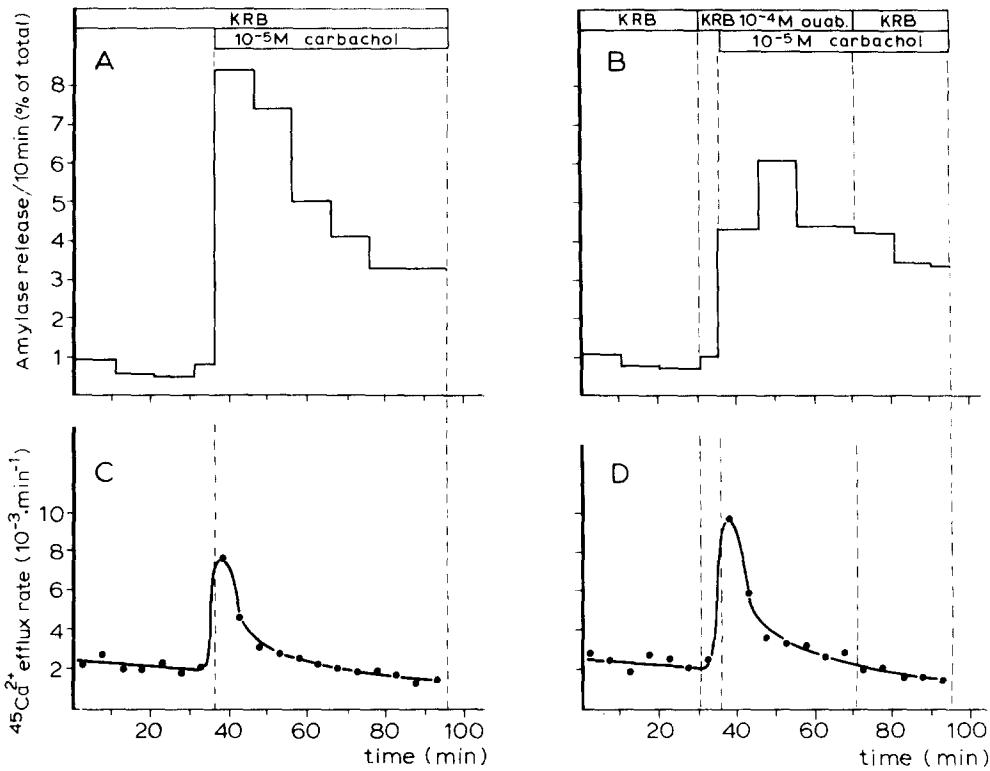


Fig. 2. Effect of 10^{-5} M carbachol on amylase release and $^{45}Ca^{2+}$ efflux from pancreatic fragments in Krebs-Ringer bicarbonate (KRB) medium of normal composition (A,C) and containing 10^{-4} M ouabain (B,D). The efflux during the first 90-min period after removing extracellular $^{45}Ca^{2+}$ is not shown. Means of four (A,B), five (C) and three (D) experiments.

in normal Krebs-Ringer bicarbonate medium the pattern of enzyme release from pancreatic fragments and from isolated acinar cells is similar. Only the stimulation of the enzyme secretion is somewhat higher in pancreatic fragments, which may be due to partial damage of the receptors during cell isolation.

Fig. 2A and B shows the short-term effects of ouabain on the carbachol-induced enzyme secretion. In the presence of ouabain the amount of enzyme released in the first 20 min following addition of carbachol (9.6%, S.E. 2.3, $n = 4$) is significantly lower ($P = 0.017$ for paired values) than in normal Krebs-Ringer bicarbonate medium (15.9%, S.E. 2.7, $n = 4$). Removal of ouabain does not cause an additional peak of enzyme release: either the effect of ouabain is irreversible or ouabain remains bound to the cells, or both. Fig. 2C and D shows that the ability of carbachol to increase the $^{45}Ca^{2+}$ efflux is not inhibited by the presence of ouabain. These results are similar to those found for isolated acinar cells after prolonged incubation with ouabain.

Short-term effect of low extracellular sodium concentration on enzyme secretion and calcium metabolism in pancreatic fragments

Lowering the sodium concentration of the medium to 25 mM (by isotonic

replacement with tetraethylammonium) 5 min before addition of carbachol nearly completely abolishes the carbachol-induced enzyme secretion (Fig. 3A). When the low sodium medium is then replaced by normal Krebs-Ringer bicarbonate medium containing carbachol, there is a marked increase in enzyme release. This suggests that the effect of low sodium is reversible. However, since pancreatic fluid secretion is inhibited by low sodium medium [20], it is possible that inhibition of enzyme secretion from pancreatic fragments is secondary to inhibition of fluid secretion and that the subsequent enhanced enzyme secretion represents a wash-out phenomenon. Since stimulation of enzyme secretion from isolated cells is also inhibited during incubation in a low sodium medium (Fig. 1), this possibility seems unlikely, but we have further investigated it by replacing carbachol with atropine 10 min before changing to the normal medium. We do not find an additional release of enzyme, although the fluid is then restored to normal values [20]. This indicates that the inhibition of enzyme secretion is not due to inhibition of the fluid secretion.

Fig. 3 C and D shows that carbachol does not cause an increased $^{45}Ca^{2+}$ efflux, when the sodium concentration in the medium is lowered to 25 mM 5 min before addition of carbachol. Replacing the medium again by normal

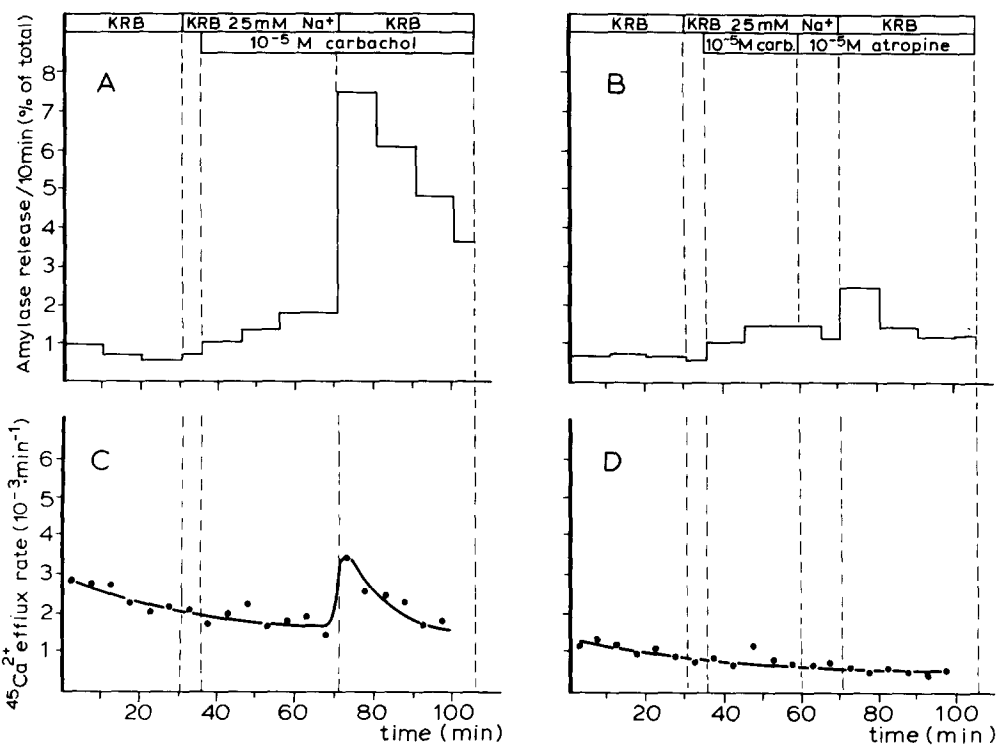
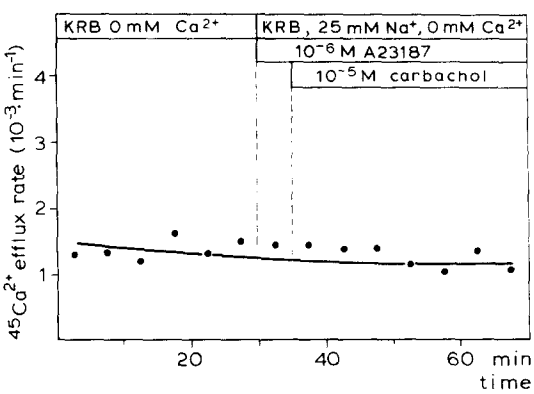


Fig. 3. Effect of 10^{-5} M carbachol on amylase release and $^{45}Ca^{2+}$ efflux from pancreatic fragments in Krebs-Ringer bicarbinate (KRB) medium containing 25 mM Na^+ (sodium replaced by tetraethylammonium) and effect of replacing this medium by normal Krebs-Ringer bicarbonate medium, carbachol still being present (A,C) or carbachol being replaced by atropine (B,D). The efflux during the first 90-min period after removing extracellular $^{45}Ca^{2+}$ is not shown. Means of five (A,C), three (B) and two (D) experiments.

Krebs-Ringer bicarbonate medium results in a slight increase in $^{45}Ca^{2+}$ efflux in the presence of carbachol. Prolonged incubation in low medium before addition of carbachol gives the same results as shown in Fig. 3.

The above results can be explained in two ways: (1) carbachol is unable to release calcium from the intracellular pool in the presence of a low extracellular sodium concentration, (2) carbachol causes a release of calcium from the intracellular pool, but in a low sodium environment calcium cannot leave the cell. The latter possibility has been investigated by using the calcium ionophore A23187. This substance in 10^{-6} M concentration causes an increase of membrane permeability for calcium in pancreatic fragments [21] without other drastic effects on the metabolism of the cells [22]. Fig. 4 shows an experiment in which low sodium medium containing A23187 is added to pancreatic fragments. Calcium-free medium is used in order to prevent calcium influx. Incubation in calcium-free medium normally has no effect on the $^{45}Ca^{2+}$ efflux pattern [20]. It is clear that carbachol in the presence of A23187 causes no increase in the $^{45}Ca^{2+}$ efflux in low sodium medium. This implies that under this condition there is no increase in cytoplasmic calcium concentration by carbachol. So apparently in the low sodium medium the coupling between hormone-receptor interaction and the release of calcium from the intracellular pool is blocked. Raising the cytoplasmic calcium concentration by incubation



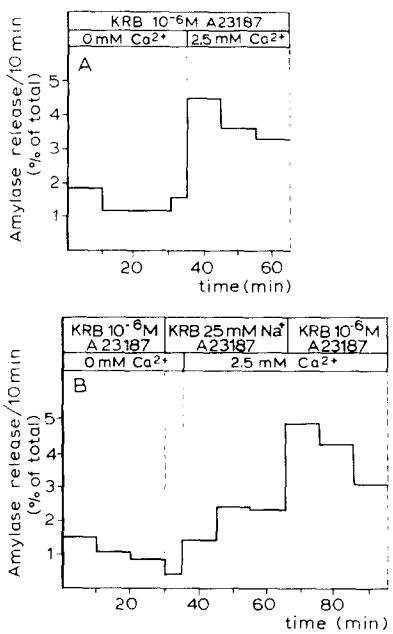


Fig. 4. Effect of 10^{-5} M carbachol on $^{45}Ca^{2+}$ efflux in calcium-free Krebs-Ringer bicarbonate (KRB) medium containing 25 mM Na^+ and 10^{-6} M A23187 (sodium replaced by tetraethylammonium). The efflux during the first 90-min period after removing extracellular $^{45}Ca^{2+}$ is not shown. Means of four experiments.

Fig. 5. Effect of 10^{-6} M A23187 in the presence of calcium on amylase release from pancreatic fragments in normal Krebs-Ringer bicarbonate medium (A) and in 25 Na^+ medium (B), sodium replaced by tetraethylammonium). Means of four experiments.

in the presence of A23187 and external calcium results in stimulation of the enzyme secretion (Fig. 5A). However, when the extracellular sodium concentration is lowered to 25 mM, stimulation is considerably inhibited (Fig. 5B). This inhibition is reversible, since replacement of the low sodium by the normal medium results in a peak of enzyme release. This indicates that in the low sodium medium the coupling between intracellular calcium release and exocytosis is also blocked.

Effect of replacing sodium chloride in the medium by sucrose on enzyme secretion and calcium metabolism in pancreatic fragments

In all previous experiments with a low sodium medium, sodium is replaced isotonically by tetraethylammonium. Since this substance penetrates the cell, it may cause additional effects. Therefore, we have also used sucrose to replace sodium chloride, although this has the disadvantage that not only the sodium but also the chloride concentration is lowered.

Incubation in the sucrose medium with 25 mM Na^+, with or without preincubation in this medium, shows a reversible inhibition of the carbachol-induced enzyme secretion (Fig. 6 A and B), just as in the case of replacing sodium by tetraethylammonium. On the other hand, the effect on the $^{45}Ca^{2+}$ efflux pattern is somewhat different. When the Krebs-Ringer bicarbonate medium is

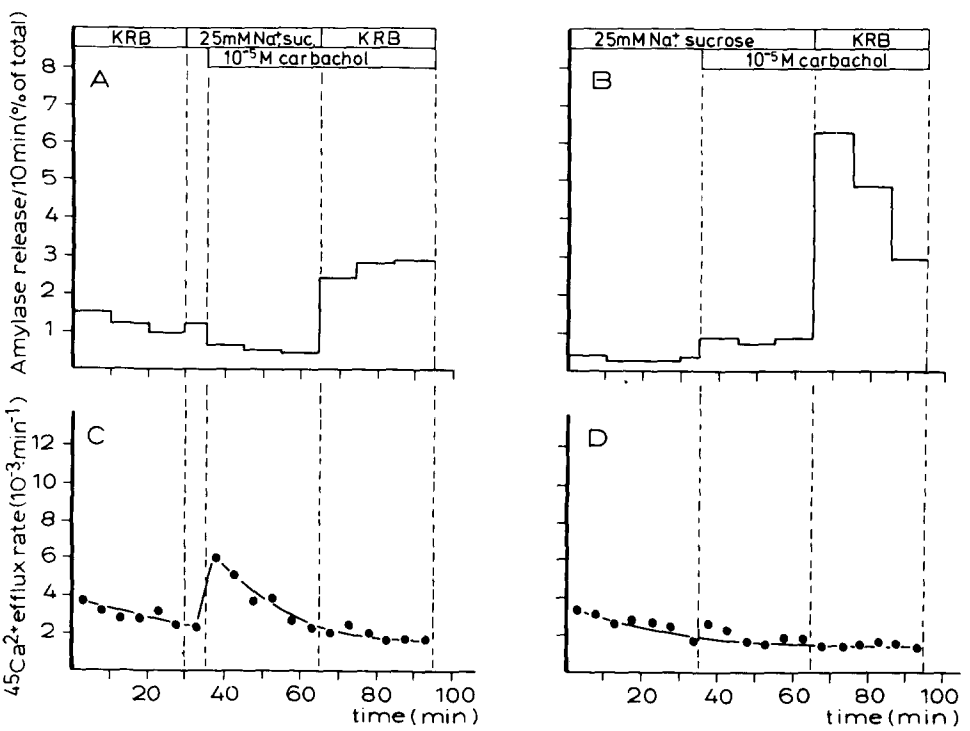


Fig. 6. Effect of 10^{-5} M carbachol on amylase release and $^{45}Ca^{2+}$ efflux from pancreatic fragments in 25 mM Na^+ medium (sodium chloride replaced by sucrose) after 5 min (A,C) and after prolonged (B,D) preincubation in this medium. The efflux during the first 90 min after removing extracellular $^{45}Ca^{2+}$ is not shown. Means of three (A,C,D) and two (B) experiments.

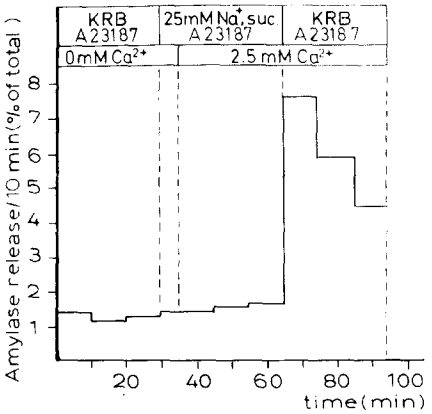


Fig. 7. Effect of 10^{-6} M A23187 in the presence of calcium on amylase release from pancreatic fragments in 25 mM Na^+ medium (sodium chloride replaced by sucrose). Means of three experiments.

replaced by the sucrose medium before addition of carbachol, this stimulant causes an increased $^{45}Ca^{2+}$ efflux, which does not occur when sodium is replaced by tetraethylammonium (Fig. 3C). This means that in the sucrose medium the inhibition of carbachol-induced enzyme secretion is caused by interruption of the stimulus-secretion coupling after the rise in cytoplasmic calcium concentration. After prolonged incubation (120 min) in the sucrose medium, the carbachol-induced $^{45}Ca^{2+}$ efflux disappears (Fig. 6D), indicating that now the coupling between hormone-receptor interaction and calcium release is blocked. Replacing the medium again by normal Krebs-Ringer bicarbonate medium does not result in an increased $^{45}Ca^{2+}$ efflux, as might be expected from the enzyme secretion pattern.

The experiment shown in Fig. 7, in which A23187 is used to increase the cytoplasmic calcium concentration, confirms that in the sucrose medium a step between calcium release and exocytosis is blocked, just as in the case of the tetraethylammonium medium.

Discussion

The aim of this study has been to determine the role of sodium ions in pancreatic enzyme secretion, it was already known that lowering the extracellular sodium concentration inhibits stimulation of enzyme secretion in the isolated uncinate part of the young rat pancreas [1], in the perfused rat pancreas [8,9, 11], in rat pancreatic segments [4], mouse pancreatic fragments [7,22] and mouse pancreatic acini [23]. In isolated mouse pancreatic acinar cells stimulation is inhibited only after prolonged incubation in Na^+-free medium (Na^+ replaced by Tris, Ref. 10). We find that in isolated acinar cells and fragments of rabbit pancreas stimulation of enzyme secretion by carbachol is inhibited in low sodium medium (sodium replaced by tetraethylammonium and in experiments with fragments also by sucrose). In pancreatic fragments this inhibition also occurs without prolonged preincubation in the low sodium medium and it is found to be reversible.

It is unlikely that the inhibition of carbachol-induced enzyme release in low sodium medium would be due to a decrease in the affinity of the carbachol receptor, since sodium does not affect the binding of carbachol to the muscarinic receptor [23]. We also conclude that the inhibition of enzyme secretion is not due to inhibition of fluid secretion in the low-sodium medium, since we observe the effect in isolated acinar cells and since replacement of the low-sodium medium by normal Krebs-Ringer bicarbonate medium without carbachol does not result in increased enzyme release. This conclusion conflicts with that of Petersen and Ueda [11], derived from experiments similar to ours but on pancreatic fragments only, and that of Williams derived from enzyme secretion studies on acinar cells [10] and from electron-microscopic observations on acini [34]. We do not know whether these conflicting results are due to species differences (rat and mouse, respectively, vs. rabbit) or to their use of Tris to replace sodium.

Ouabain also inhibits carbachol-induced stimulation of the enzyme secretion by isolated acinar cells and pancreatic fragments, both with and without preincubation, but the inhibitory effect is less pronounced than in low sodium medium. Pedersen and Ueda [11] were unable to find an inhibitory effect of ouabain (without preincubation) on the caerulein-induced enzyme release in the perfused rat pancreas, but this may be due to the low sensitivity of rat tissues for ouabain [25]. Since inhibition of carbachol-induced enzyme release already occurs after only 5 min preincubation in a medium containing 25 mM Na^+ or ouabain, lowering of the active ion fluxes across the membrane, rather than changes in the intracellular ionic concentrations, seem to be responsible for the effect.

Lowering the extracellular sodium concentration and addition of ouabain also affect the distribution of calcium in pancreatic acinar cells. The exchangeable calcium content is increased in low sodium medium and by ouabain. This is in agreement with the two-fold increase in $^{45}Ca^{2+}$-uptake by isolated acinar cells of rat pancreas in low sodium medium observed by Schulz et al. [26]. This effect could be explained by the operation of a Na^+—Ca^{2+}-exchange mechanism in the plasma membrane, as previously observed in smooth muscle [27,28]. Removal of the sodium gradient across the membrane by lowering the extracellular sodium concentration or by incubation in the presence of ouabain, reduces the passive sodium influx and consequently the Ca^{2+} efflux, resulting in a rise of the cytoplasmic calcium content. However, this would imply that the total calcium content of the cells increases by about the same amount as the exchangeable calcium content. We find no indication for such an increase.

The constancy of the total cellular calcium content upon incubation in media containing 25 mM Na^+ or ouabain, suggests that the increase in $^{45}Ca^{2+}$ content would be due to the labeling of a calcium pool, which under normal conditions does not exchange with $^{45}Ca^{2+}$. This pool might represent calcium sequestered in the zymogen granules, for this does not exchange with $^{45}Ca^{2+}$ under normal conditions [29]. However, since lowering of the extracellular sodium concentration or addition of ouabain to the medium affects the stimulated enzyme secretion within 5 min, it is not very likely that the relatively slow change in the calcium distribution in the cells can be responsible for the inhibitory effect.

Addition of carbachol to pancreatic fragments [1,18,30] or dispersed acini [17], preloaded with $^{45}Ca^{2+}$, increases the $^{45}Ca^{2+}$-efflux rate, which is thought to be the result of a release of calcium from an intracellular pool. We have checked whether this calcium efflux is affected by incubation in a medium containing 25 mM Na^+ or ouabain. We have found that in the presence of ouabain carbachol causes an increased $^{45}Ca^{2+}$ efflux, both in isolated acinar cells and in fragments, which is in agreement with earlier findings of Case and Clausen [1]. This means that ouabain does not prevent the release of calcium from the intracellular pool by carbachol, but that the inhibitory effect takes place in a later phase of the stimulus-secretion coupling.

The effect of a low sodium medium on the carbachol-induced $^{45}Ca^{2+}$ efflux seems to depend on the substance which is used to replace sodium. When tetraethylammonium is used, carbachol is unable to increase the $^{45}Ca^{2+}$ efflux from isolated cells and pancreatic fragments, even after only 5 min preincubation in the low sodium medium. Since this is even true in the presence of A23187, this implies that in the tetraethylammonium medium carbachol is unable to release calcium from the intracellular pool. In the sucrose/low sodium medium the carbachol-induced $^{45}Ca^{2+}$-efflux from pancreatic fragments is inhibited only after prolonged preincubation. This may explain why Williams [24] observed no effect of low sodium (choline replacing sodium) on caerulein induced ^{45}Ca efflux in mouse acini.

Another difference between the tetraethylammonium/low sodium and the sucrose/low sodium media is that replacement of the former medium by normal Krebs-Ringer bicarbonate medium results in an increased enzyme release and a slight increase in $^{45}Ca^{2+}$ efflux, while replacing the latter medium by normal medium results in an increased amylase release, but not in a detectable increase in $^{45}Ca^{2+}$ efflux. It is not yet clear how this disparity can be explained, but the uptake of tetraethylammonium or changes in intracellular K^+ level may be responsible.

In both low sodium media stimulation of enzyme secretion is inhibited, when the cytoplasmic calcium concentration is raised by incubation in the presence of A23187 and Ca^{2+}. This suggests that the inhibition takes place between the increase in Ca^{2+} level and the exocytotic process. Fig. 8 represents a simplified scheme for the stimulus-secretion coupling process in the pancreas. From our results it can be concluded that incubation in a low sodium or an ouabain containing medium blocks step 2. Incubation in the tetraethylammonium/low sodium medium or prolonged incubation in the sucrose/low sodium medium

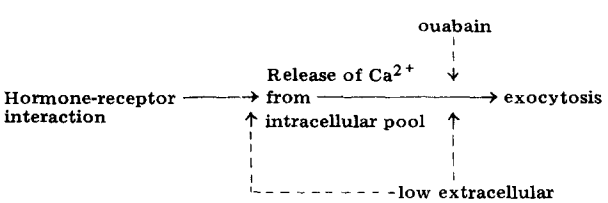


Fig. 8. Schematic presentation of the pancreatic stimulus-secretion coupling.

apparently has an additional inhibitory effect on step 1 of the stimulus-secretion coupling process.

It is not yet clear how sodium is involved in the coupling between intracellular calcium release and exocytosis. Possibly, carbachol-induced depolarization of the membrane potential, which is abolished at low extracellular sodium levels [12,13] and perhaps also by ouabain, is necessary for step 2 of stimulus-secretion coupling. On the other hand, the observation that incubation in a high potassium medium, which causes depolarization of the membrane potential, does not result in an increased amylase release [15], indicates that depolarization per se is not sufficient for enzyme secretion. So apparently both intracellular calcium release and depolarization of membrane potential are necessary for exocytosis.

Alternatively, it is possible that no depolarization as such, but the subsequent sodium influx is required for coupling of intracellular calcium release and exocytosis. Caerulein and cholinergic agents induce a dose-dependent increase of $^{22}Na^{+}$ uptake by rat pancreatic fragments [31] and acinar cells [32]. Our experiments with low sodium medium support this possibility. In a low sodium medium there should be no sodium gradient across the plasma membrane, and so the increase of the membrane permeability for sodium by carbachol would not result in a sodium influx in this situation. This may explain the inhibition of the stimulated enzyme secretion in the low sodium medium.

When ouabain is used without preincubation, there should still be a sodium gradient upon addition of carbachol, and this gradient will only gradually disappear through the inhibition of the $(Na^{+}\text{-}K^{+})$-ATPase activity. This may explain why in the presence of ouabain the stimulated enzyme secretion is only partially inhibited. We have investigated this idea by incubating pancreatic fragments in normal Krebs-Ringer bicarbonate medium in the presence of the sodium ionophores gramicidin and amphotericin B. We find no change in the basal enzyme secretion (Renckens et al., unpublished results). Assuming that these substances induce a sodium influx in pancreatic acinar cells, this would indicate that this sodium influx does not stimulate enzyme secretion. However, Na-influx via a specific transport protein might be necessary to induce enzyme secretion. When carbachol is added after 30 min incubation in a medium containing a sodium ionophore, there is normal stimulation of enzyme secretion. This suggests that a sodium gradient across the plasma membrane is not required for stimulation of enzyme secretion. Further investigation will be needed to provide more information about the mechanism of the inhibition of the carbachol-induced enzyme secretion in media containing low sodium or ouabain.

Summarizing, it can be concluded that sodium ions are involved in pancreatic enzyme secretion. Disturbance of the sodium fluxes across the acinar cell membrane seems to affect the coupling between intracellular calcium release and exocytosis. Since we do not yet know how calcium ions stimulate exocytosis, it is impossible to fully explain the involvement of sodium ions in this process.

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