ROLE OF CALCIUM IN EXOCRINE PANCREATIC SECRETION

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VICTOR VINCENTIUS ANTONIUS MARIA SCHREURS
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This day is the first day of the rest of your live

Aan mijn ouders

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CONTENTS

Various cellular processes can be triggered by a hormone or a neurotransmitter, the "first messenger", which does not enter its target cell. In that case, the interaction of the first messenger with its receptor at the cell surface generates an intracellular substance, the "second messenger", which is directly responsible for the cellular response. The effect of glucagon on the glycogenolysis in the liver is mediated by an increase in the intracellular level of cyclic AMP and the acetylcholine stimulated release of catecholamines from the chromaffin cells in the adrenal medulla is intracellularly induced by an increase of the cytoplasmic calcium concentration. Such processes appear also to be involved in exocrine pancreatic secretion.

The exocrine pancreas secretes a fluid, which consists of water, electrolytes and digestive enzymes and which plays an important role in the digestion of the food in the intestine. The secretion of water and electrolytes on the one hand, and that of the digestive enzymes on the other hand, are under separate hormonal and neural control.

Previous studies from several laboratories, including our own, have provided considerable evidence for a role of cyclic AMP as second messenger in pancreatic fluid and electrolyte secretion, whereas such a role is less certain in the case of pancreatic enzyme secretion.

The inconclusive evidence for a role of cyclic AMP as second messenger in pancreatic enzyme secretion has led us to study whether calcium ions may act as an intracellular trigger in this case. Such a role for calcium ions, through an increase in their cytoplasmic concentration, has been firmly established in the case of the catecholamine secretion by the adrenal medulla.

In order to investigate this matter, it is necessary to have a detailed knowledge of the calcium movements in the resting and the stimulated pancreas. The results of our studies, reported in chapter 3, indicate the existence of three distinct calcium movements upon stimulation: an extracellular, a secretory and a stimulatory calcium flux. Further experiments have thrown more light on the extracellular calcium flux (chapter 4). Experiments directed toward elucidation of the secretory calcium flux, consisting of calcium secreted in association with the digestive enzymes, are reported in chapter 5. Since we do not find any exchange between the calcium secreted in association with the digestive enzymes and that in the medium, we have investigated during which phase of the intracellular pathway of the digestive enzymes this calcium fraction loses the ability to exchange (chapter 6). The stimulatory calcium flux is discussed in more detail in chapter 7, where it is shown with the aid of the divalent cation ionophore A-23187 that this flux is due to a specific release of calcium ions from an intracellular store, and that an increase in the free cytoplasmic calcium concentration can lead to enzyme secretion.

Thus the results of these studies are that of the three cal-

cium movements observed upon stimulation of pancreatic enzyme secretion the extracellular and secretory calcium fluxes are not involved in the triggering of this process. The properties of the stimulatory calcium flux indicate that stimulation causes a release of calcium ions from an intracellular store, which in an as yet unknown way may cause the secretion of digestive enzymes from the pancreatic acinar cells.

CHAPTER 1

INTRODUCTION

1.1. Physiology of the exocrine pancreas

1.1.1. Function and structure

The mammalian pancreas serves an important endocrine as well as an exocrine function. The so-called islets of Langerhans, accounting for only a minor part of the tissue, are involved in the endocrine function, the formation and excretion of the hormones insulin and glucagon which regulate the glucose level in the blood.

The exocrine pancreas plays an important role in the digestion of macro-molecular components of the food in the intestine. It secretes a fluid - consisting of water, electrolytes and digestive enzymes - which is drained to the duodenum through the main pancreatic duct. This main duct is the continuation of a branch-work of smaller ducts and ductules of which the smallest ones originate in the lumen of an acinus. Each acinus consists of a cluster of mainly acinar cells which surround a common lumen to which the digestive enzymes are primarily secreted.

The acinar cell is morphologically characterized by a highly developed endoplasmic reticulum at the serosal side and contains in the resting state many zymogen granules at the luminal side. The acinar cells synthesize the digestive enzymes on their rough endoplasmic reticulum and store the bulk of the enzymes in the

zymogen granules until the moment of secretion.

A few cells in the acinus, the centro-acinar cells, are morphologically different from the acinar cells, but are very similar to the ductular cells which border the ducts. These cells have a normally developed endoplasmic reticulum, while zymogen granules are completely absent. This indicates that the ductular and centro-acinar cells are not involved in the synthesis and secretion of digestive enzymes. The ultrastructural morphology of the exocrine pancreas has been established by several microscopic studies (Ekholm and Edlund, 1959; Ekholm et al, 1962a, 1962b; Palade et al, 1962; Sjöstrand, 1962; Kern and Ferner, 1971).

1.1.2. Control of secretory mechanisms

The volume flow and the composition of the pancreatic fluid are separately regulated by both neural and hormonal control mechanisms. The autonomic nervous system influences exocrine pancreatic secretion by the vagus and splanchnic nerves. The hormones involved in exocrine pancreatic secretion are the polypeptides secretin and pancreozymin. The former mainly controls the volume flow, while the latter mainly regulates the enzyme secretion (see Harper, 1967, 1972; Thomas, 1967).

Exocrine pancreatic secretion is controlled at different levels: brain, stomach and intestine. Pancreatic secretion can be induced by the sight, smell and taste of food by means of nervous stimulation. Atropin blocks induced enzyme secretion, but not water and electrolyte secretion (Hickson, 1970; Lenninger and Ohlin, 1971). This indicates that the stimulation of the enzyme

secretion is most probably mediated by cholinergic fibers. Pancreatic secretion is further induced by the mechanical influence of the food on the stomach. Distention of both the fundus and the antrum of the stomach increases pancreatic enzyme secretion in the cat. After vagal section, distention of the fundus had no longer effect on the pancreas but the response to antral distention persisted. This suggests that a vago-vagal reflex pathway is involved in the response to stimulation of the fundus of the stomach. The response to a mechanical stimulation of the antrum is blocked by atropin, indicating that a local cholinergic pathway is involved in this stimulation. These two modes of induction probably serve to mobilize the pancreas for the main mode of stimulation, which comes from the intestine. The entry of the acid food mass from the stomach into the small intestine lowers the pH. This leads to a release of the hormone secretin from the intestinal wall to the blood. It stimulates pancreatic fluid and electrolyte secretion. The bicarbonate present in the pancreatic fluid neutralizes the gastric chyme, which leads to a more optimal pH for the enzymatic digestion in the intestine. The hormone pancreozymin is released upon the passage of already partly digested food through the small intestine. It stimulates secretion of the digestive enzymes from the pancreas, which are responsible for complete digestion of the food components.

1.1.3. Secretion of fluid and electrolytes

The pancreatic fluid is always isosmotic with the extracellular fluid, regardless of the rate of flow. Two classical

experiments have indicated which cells are responsible for fluid secretion and which for enzyme secretion. Selective destruction of the ductular cells reduces the response of the fluid secretion to secretin (Grossman and Ivy, 1946). Administration of ethionine leads to selective destruction of the acinar cells with loss of enzyme secretion, but the volume flow and bicarbonate content are not affected (Kalser and Grossman, 1954). This suggests that the acinar cells are responsible for enzyme secretion, and the ductular cells for fluid secretion. The morphology of these two cell types (section 1.1.1.) supports this conclusion. The function of the ductular cells in the fluid secretion is further supported by the observation that their intracellular spaces distend, when fluid secretion in dog pancreas is induced by secretin (Ridderstap, 1969, p. 101). While it is morphologically quite unlikely that ductular cells contribute to the enzyme secretion, it cannot be completely excluded that acinar cells contribute to the fluid and electrolyte secretion.

The major monovalent cations in the pancreatic fluid are sodium and potassium. Their concentrations are about equal to their concentrations in the extracellular fluid regardless of the flow rate (Ball and Johnston, 1930; Dreiling and Janowitz, 1956; Rothman and Brooks, 1965; Ridderstap, 1969, p. 42, 62; Rutten, 1974, pp. 22-41). The behaviour of the divalent cations calcium and magnesium will be discussed in section 1.1.6.

The major anions in the pancreatic fluid are bicarbonate and chloride. The sum of their concentrations remains constant, but their relative concentrations are strongly dependent on the

flow rate. The bicarbonate concentration is high at high flow rates, while the chloride concentration is high at low flow rates. This suggests that the primary secretion is rich in bicarbonate, which may exchange with chloride during its passage through the ducts (Case et al, 1968).

The fluid secretion appears to be primarily controlled by a Na-K activated ATPase pump system, which transports Na^+ ions into the lumen of the pancreatic duct (Ridderstap and Bonting, 1969 a, 1969 b). An anion activated ATPase system has also been demonstrated in the pancreas (Simon et al, 1972), but its significance for the pancreatic fluid secretion has not yet been elucidated. Recently, Kempen (1976, pp 3-14) has discussed the available evidence about this subject. Increasing evidence for a role of cAMP as second messenger in the secretin stimulated fluid and electrolyte secretion has accumulated (Case and Scratcherd, 1972; Bonting et al, 1974; Smith and Case, 1975; Kempen, 1975).

1.1.4. Secretion of digestive enzymes

The macro-molecular part of the secretory products consists mainly of a mixture of hydrolytic enzymes or their zymogens (Greene et al, 1963; Tartakoff et al, 1974). As previously mentioned, secretion of digestive enzymes takes place from the acinar cells, where the enzymes have been temporarily sequestered within zymogen granules. This sequestration is the result of an intracellular transport of the enzyme molecules following their synthesis. The intracellular aspects of the process of protein synthesis in the pancreas has been extensively studied by Palade,

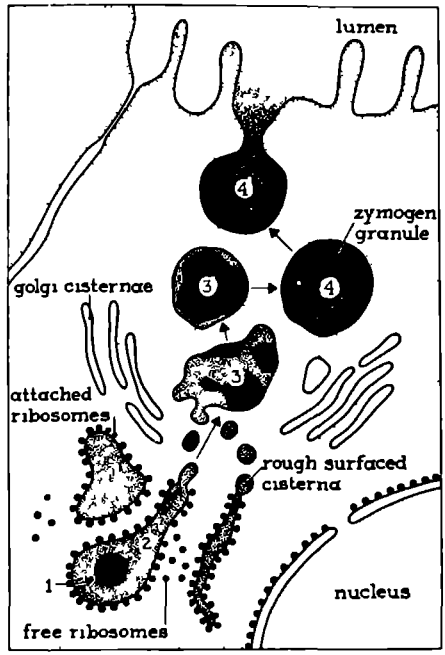

Fig. 1.1. Intracellular pathway, from the site of synthesis to the site of discharge, of digestive enzymes in the pancreatic acinar cell. 1. Synthesis, 2. Segregation, 3. Transport, 3'. Concentration, 4. Storage, 4'. Discharge (After Palade et al, 1962).

Jamieson and co-workers. Recently, Palade has summarized this extensive work in his "Nobel lecture" (Palade, 1975). The intracellular pathway of the digestive enzymes is schematically represented in fig. 1.1., the various phases being indicated by the numbers 1 - 4.

The digestive enzymes are synthesized on the polysomes of the rough endoplasmic reticulum of the acinar cell (phase 1), while the free polysomes merely synthesize the proteins for intracellular use (Siekevitz and Palade, 1960). In the subsequent phase the newly synthesized polypeptides are irreversibly segregated from

the cytoplasm by vectorial transport through the endoplasmic reticulum in the cisternal space (phase 2). About 30 min after the moment of synthesis the enzyme molecules reach the so-called condensing vacuoles at the trans side of the Golgi complex (Caro and Palade, 1964; Jamieson and Palade, 1967).

In the condensing vacuoles, which initially contain the digestive enzymes in a rather dilute solution, a process of concentration takes place (phase 3'), which finally results in the conversion of these vacuoles into mature zymogen granules (Jamieson and Palade, 1967). The extrusion of water from the condensing vacuoles is an irreversible process, which does not depend on a continuous energy supply, indicating that the concentration is not achieved by an energy consuming pump system (Jamieson and Palade, 1971). The process may be mediated by sulfated-polyanions, which form osmotically less active aggregates with the digestive enzymes with a consequent outflow of water (Berg and Young, 1971; Tartakoff et al, 1974).

The mature granules remain stored in the apical region of the acinar cell between the Golgi region and the lumen (phase 4). In the final phase the granular content is discharged to the acinar lumen by "exocytosis" (phase 4'). This process is morphologically characterized by a fusion of the granular membrane with the apical membrane with subsequent fission. This fission permits the outflow of the granular content, while the cytoplasmic content is retained by the granular membrane.

Whereas this intracellular pathway of the enzymes does not seem to depend on energy in most aspects, there are two sites

at which the process is halted when ATP production is insufficient: 1. the enzymes cannot leave the endoplasmic reticulum and 2. the enzymes are not discharged into the acinar lumen (Jamieson and Palade, 1968; 1971).

1.1.5. Stimulus-secretion coupling

As previously mentioned, the physiological condition of the exocrine pancreas is adjusted to the needs of the organism by neural and hormonal control mechanisms. This means that upon increased food supply the resting pancreas will receive a stimulus, which usually results in an immediate stimulation of secretion. The sequence of intracellular events, occurring after receipt of a stimulus and leading to secretion, is usually called stimulus-secretion coupling.

It is a common problem in the study of various types of exitable cells, how external stimulants produce the final response at the molecular level. Peptide hormones and neurotransmitters, usually indicated as first messengers, do not seem to be able to enter the cell, but must rather interact with receptors at the cell surface. This hormone-receptor interaction in turn generates an intracellular substance (second messenger), which is directly responsible for the cellular response. Cyclic nucleotides (cAMP and cGMP) and calcium ions have uptill now received most attention as second messengers, and in some cases their action is supposed to be interrelated (Robison et al, 1971; Goldberg et al, 1973; Rasmussen, 1970).

Among the hormones which utilize cAMP as second messenger

are: glucagon, adrenocorticotropic hormone, vasopressin and parathyroid hormone (see Robison et al, 1971). In these cases, the hormone-receptor interaction leads to activation of an adenylate cyclase, which converts ATP to cAMP. The resulting cAMP interacts with a cAMP-dependent protein kinase, which is able to activate by phosphorylation an enzyme system, which then produces the physiological response. The quenching of the cAMP signal is carried out by a phosphodiesterase, which hydrolyzes cAMP to 5'AMP.

The involvement of cAMP in pancreatic enzyme secretion, for which Ridderstap and Bonting (1969) have provided some evidence, has been studied extensively in our laboratory. Rutten et al (1972) demonstrated the presence of an adenylate cyclase in rat pancreas, which can be stimulated by pancreozymin, but also by secretin and not by acetylcholine. Its activation by secretin has led to further work by Kempen (1976) as well as others (Case and Scratcherd, 1972; Smith and Case, 1975), which has supplied considerable evidence for a role of cAMP as second messenger in the secretin stimulated fluid and electrolyte secretion. However, the evidence for a role of cAMP in pancreatic enzyme secretion in accordance to the criteria of Sutherland (Sutherland et al, 1968) is less convincing. The cAMP levels in isolated pancreatic acinar cells, responsible for enzyme secretion, can be increased by pancreozymin-octapeptide and secretin in a manner which is very similar to that in rat pancreatic slices. There is evidence that a large part of the hormone sensitive adenylate cyclase of the pancreas is present in the acinar cells (Kempen, 1976, pp 103-115).

Stimulation of pancreatic enzyme secretion by exogenous cAMP or its lipophilic dibutyryl derivative, or by cAMP phosphodiesterase inhibitors has been demonstrated for mouse (Kulka and Sternlicht, 1968), rabbit (Ridderstap and Bonting, 1969) and rat (Heisler et al, 1972; Bauduin et al, 1971; Kempen, 1976, pp 21-32). On the other hand, cholera toxin, which activates the pancreatic adenylate cyclase, stimulates fluid and electrolyte secretion, but not pancreatic enzyme secretion, when it is applied topically to the rat pancreas in situ (Kempen et al, 1975). Another unexplained fact is that the adenylate cyclase of the rat acinar cell is quite sensitive to secretin, but this hormone has little effect on enzyme secretion.

The still somewhat inconclusive evidence for a role of cAMP in the stimulus-secretion coupling of pancreatic enzyme secretion has led us to study the possible role of calcium ions in this process. The role of calcium in the stimulus-secretion coupling of secretory processes in the adrenal medulla has been extensively studied by Douglas and Rubin (1961). There is also evidence for such a role of calcium in the submaxillary gland and the neurohypophysis (Thorn and Petersen, 1974). In the adrenal medulla the interaction of the stimulus with its receptor at the cell surface causes a conformational change of certain membrane proteins, which allows a passive influx of sodium and calcium ions resulting in membrane depolarization. The increased cytoplasmic calcium concentration would - in an as yet unknown way - initiate the process of exocytosis. Since exocytosis is the common mechanism by which several glands secrete a wide variety of macro-molecular products,

these authors suggest that the stimulus-secretion coupling would involve in all cases an increase in the cytoplasmic calcium concentration.

The control of cellular activity by the intracellular calcium level will require a close regulation of this level. In several animal cells a considerable electrochemical gradient for calcium is maintained in the steady-state. The free calcium concentration of the cytoplasm is supposed to be in the range of 10^{-7} to 10^{-6} M as in muscle and nerve (Hodgkin and Keynes, 1957; Baker, 1972). This means that a large concentration gradient as well as the negative (inside) membrane potential provide a considerable driving force for a passive calcium influx. The low steady-state cytoplasmic calcium concentration can only be maintained by the action of an active extrusion mechanism, while mitochondria may act as an ion buffer system and as a store for intracellular calcium (Borle, 1973). The very low cytoplasmic calcium level implies that a minor disturbance of this steady-state situation may be sufficient to achieve a drastic change in the free cytoplasmic calcium concentration.

This increase in the free cytoplasmic calcium concentration could in principle be brought about in either one of three ways: inhibition of the extrusion mechanism, increase of the passive influx, or release from the intracellular store. The first mechanism is not very likely, since it would only result in a rather slow change in the cytoplasmic calcium concentration. The passive calcium influx is supposed to occur through calcium channels, formed by certain membrane proteins. A change in the conformation

of these proteins by hormone-receptor interaction could increase the permeability for calcium. This permeability increase may either be the cause or the result of a decrease in membrane potential. A permeability increase as the cause of depolarization is supposed to occur in the action of adrenocorticotropic hormone on the adrenal cortex, and of serotonin on insect salivary gland (see Berridge, 1975). These tissues can still be stimulated by their normal stimulants, when they are completely depolarized by a high potassium medium, indicating that depolarization per se does not induce secretion in these systems. Permeability increase as the result of depolarization is supposed to occur in the release of neurohormones from the neurohypophysis and in the secretion of fluid by the mammalian salivary gland (see Berridge, 1975).

Little is known about the molecular mechanisms which increase the calcium permeability of the cell membrane. The calcium channels appear to be different from those for sodium and potassium, which are blocked by tetrodotoxin and by tetraethylammonium, respectively. The calcium influx is not blocked by these substances, but is blocked by polyvalent cations like Mg^{2+} and La^{3+}, as well as by certain organic compounds like verapamil and D-600.

Increasing the cytoplasmic calcium level by release of calcium from an intracellular store takes place in muscle, where calcium is released from the sarcoplasmic reticulum upon stimulation. The release of calcium from an intracellular store may also be important for secretory processes (Thorn and Petersen, 1974), and has been suggested for pancreatic enzyme secretion (Case and Clausen, 1973).

1.1.6. Role of calcium in exocrine pancreatic secretion

In view of the considerations mentioned in the previous section a study of the role of calcium in exocrine pancreatic secretion is in order. At the beginning of our study in 1973 there was already some evidence, but it was also clear that the involvement of calcium in exocrine pancreatic secretion was very complex and certainly not restricted to the stimulus-secretion coupling (see review Case, 1973).

Electrophysiological studies have shown that pancreatic nerve stimulation, parasympathetic agents and pancreozymin, all of which induce pancreatic enzyme secretion, also depolarize the acinar cell membrane (Dean and Matthews, 1972). The depolarizing current evoked by stimulation is mainly carried by sodium ions (Matthews and Petersen, 1973), and there is little or no calcium influx evoked by the stimulation (Nishiyama and Petersen, 1973). An increase of the extracellular potassium concentration, which depolarizes the acinar cell, does not induce enzyme secretion in the presence of atropin (Benz et al, 1972; Petersen and Matthews, 1972; Matthews and Petersen, 1973; Argent et al, 1973). This indicates that depolarization per se does not induce enzyme secretion.

Omission of calcium from the bathing medium or the perfusion fluid abolishes stimulated enzyme secretion in slices of pigeon pancreas (Hokin, 1966), rat pancreas (Robberecht and Christophe, 1971; Heisler et al, 1972; Case and Clausen, 1973 guinea pig pancreas (Benz et al, 1972) and in the perfused cat pancreas

(Argent et al, 1973). In the latter case there is also a reduction in basal fluid and electrolyte secretion. The effects of calcium omission develop only slowly, indicating that they may not be due to abolishing a stimulation-induced calcium influx, but rather to a more general calcium requirement of the tissue.

Increasing the magnesium concentration in the extracellular medium has little or no inhibitory effect on pancreatic enzyme secretion (Robberecht and Christophe, 1971; Benz et al, 1972; Argent et al, 1973), while barium in equal concentration can not substitute for calcium in rat pancreas (Robberecht and Christophe, 1971).

An increased $^{45}Ca^{2+}$ uptake is not detectable in rat pancreas upon stimulation with acetylcholine or pancreozymin (Case and Clausen, 1973). The kinetic behaviour of the $^{45}Ca^{2+}$ efflux rate, studied on rat pancreas tissue pre-loaded with this tracer, indicates that calcium is retained in different compartments. The $^{45}Ca^{2+}$ efflux rate as well as that of amylase secretion are increased by acetylcholine and pancreozymin in dose-dependent fashion. In the presence of 0.5 mM EGTA, the increased ^{45}Ca efflux is maintained, but the amylase release is abolished. The amylase release is, however, quite normal in the presence of only 0.1 mM external calcium. This suggests that these stimulants alter the intracellular calcium distribution, and that this can only lead to enzyme secretion when the plasma membrane is not depleted of calcium (Case and Clausen, 1973).

Substitution of sodium by lithium gradually increases the $^{45}Ca^{2+}$ efflux and amylase release, but only in the presence of

extracellular calcium. In addition, under these circumstances the $^{45}Ca^{2+}$ uptake is increased, while the effects of acetylcholine and pancreozymin are suppressed. This suggests that an influx of sodium may trigger the release of $^{45}Ca^{2+}$ upon stimulation by these stimulants.

Secretin, which is the main stimulant of fluid and electrolyte secretion, does not depolarize the acinar cell membrane (Dean and Matthews, 1972), neither does it stimulate the $^{45}Ca^{2+}$ efflux from pre-loaded pancreas tissue (Case and Clausen, 1973).

The pancreatic fluid contains calcium, but its concentration strongly depends on the physiological condition of the gland. The basal calcium concentration is always dependent on and generally lower than that of the extracellular fluid (Zimmerman et al, 1967; Goebell et al, 1972; Argent et al, 1973; Rutten, 1974, pp 137 - 154). Increase in enzyme secretion, caused by various stimuli of the exocrine pancreas, are always accompanied by increases in the calcium level in the secreted fluid. In none of these studies, however, is a constant ratio between calcium and secreted enzymes observed. This suggests that the calcium in the secreted fluid originates from different calcium compartments. Goebell et al (1972) and Argent et al (1973) have suggested that calcium is at least secreted in two fractions, one associated with the digestive enzymes and the other with the electrolytes of the fluid. More recent evidence, reported by other investigators during our study, will be discussed in the appropriate chapters and in the general discussion of this thesis.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

The materials used in this study are summed up below with their sources:

A-23187	Eli Lilly and Company Indianapolis, U.S.A.
Aquasol	New England Nuclear Boston, Massachusetts, U.S.A.
Bovin Serum Albumin	Behringwerke AG Marburg Lahn, Germany
$^{45}CaCl_2$	The Radiochemical Centre Amersham, England
Calcium Rapid Stat Kit	Pierce Chemical Company Rockford, Illinois, U.S.A.
Carbachol	Brocacef Maarssen, The Netherlands
D-600	Knoll AG Ludwigshafen, Germany
Hyamine hydroxide 10-X	Packard Instrument International S.A. Zürich, Switserland
^{3}H-Inulin	The Radiochemical Centre Amersham, England
Insta-gel	Packard-Becker B.V. Groningen, The Netherlands
L - [4,5-^{3}H] Leucine	The Radiochemical Centre Amersham, England

$^{28}MgCl_2$	Brookhaven National Laboratory Upton, New York, U.S.A.
D - [1-^{3}H] Mannitol	New England Nuclear Boston, Massachusetts, U.S.A.
Pancreozymin	The Boots Company LTD Nottingham, England
Selectron cellulose nitrate membrane filters (BA 83)	Schleicher & Schüll Dassel, West-Germany
Sephadex G-25 (coarse)	Pharmacia Uppsala, Sweden
Verapamil (Isoptin)	Knoll AG Ludwigshafen, Germany

All other substances are commercial preparations of the highest obtainable purity.

2.2. Preparation of the rabbit pancreas

The rabbit pancreas has a very thin sheetlike structure, which is mainly localized within the mesentery of the first duodenal loop. The pancreatic fluid reaches the duodenum via the main pancreatic duct which is completely separated from the common bile duct.

Male and female New Zealand white rabbits, weighing 2-3 kg are fasted 24 hrs before the start of each experiment, with water given ad libitum. The animals are killed by a sharp blow on the neck, immediately followed by carotic exsanguination. The pancreas is then removed as described below.

2.2.1. Isolated rabbit pancreas

The isolated rabbit pancreas is quite suitable for in vitro

incubation, since its sheetlike structure permits easy access of the incubation medium to the pancreatic cells. This permits adequate nutrition and oxygenation without recourse to organ perfusion. Moreover, additions made to the incubation medium maintain a relatively constant level and cannot cause secondary effects on the isolated organ.

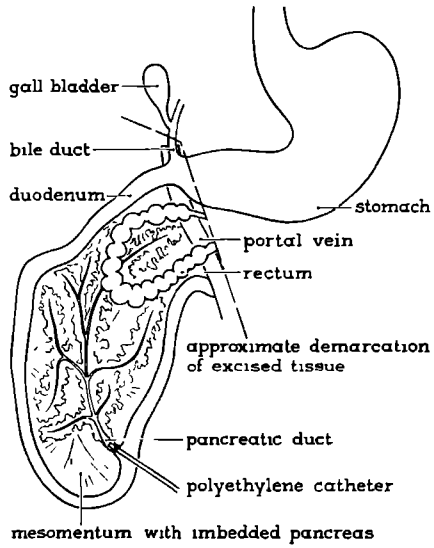

Fig. 2.1. Schematic presentation of the anatomy of the rabbit pancreas (After Ridderstap, 1969).

The isolated rabbit pancreas is prepared according to Rothman (1964) with only slight modifications. The first duodenal loop, including the attached parts of the rectum, is carefully separated from the rest of the intestine and the stomach. The open ends of the duodenum and rectum are ligated and the entire preparation is mounted on a frame (19 x 10 cm) and incubated in a lucite tank of 18.5 x 12 x 2 cm (l, h, w). The volume of the bathing medium is 300 ml. The main pancreatic duct is cannulated close to its junc-

tion with the duodenum (fig. 2.1.). For this purpose a polyethylene tubing (1.0 mm outer diameter) is used, which passes through the bottom of the tank. The preparation continues to secrete at a more or less constant level for at least 5 - 6 hrs.

2.2.2. Rabbit pancreas fragments

Pancreas fragments, pre-loaded with radioactive tracers, permit the study of the tracer efflux across the serosal membrane, though in combination with that across the luminal membrane, which is not possible with the isolated pancreas. For this purpose fragments of about 250 mg (wet weight) are cut from the pancreas streched between the spleen and the rectum.

2.3. Incubation medium

The preparations of the rabbit pancreas are normally incubated in a balanced Krebs-Ringer bicarbonate (KRB) salt solution. Its chemical composition is shown in table 2.1.

Before incubation the pH of the medium is adjusted to 7.2 by addition of hydrochloric acid. During incubation the medium is continuously gassed with carbogene, a mixture of 95% O_2 and 5% CO_2. It is maintained at 37°C. Changes in the composition of the incubation medium are described at the appropriate places in the text and in the tables and figures.

TABLE 2.1.

COMPOSITION OF THE INCUBATION MEDIUM.

Na^+	143.3
K^+	4.7
Ca^{2+}	2.5
Mg^{2+}	1.3
HCO_3^-	24.9
$H_2PO_4^-$	1.2
Cl^-	129.7
Glucose	5.5

All concentrations are expressed in mM.

2.4. Incubation and sample collection

2.4.1. Isolated rabbit pancreas

The isolated rabbit pancreas is preincubated for 1 hr after mounting in a bath, containing 300 ml incubation medium, in order to achieve a steady-state condition. After this period the medium is replaced by fresh medium, the composition of which can be changed according to need. The secreted fluid is collected from the cannulated duct in suitable 5 - 15 min fractions in pre-weighed plastic counting vials. From each fraction samples are taken for the appropriate spectrofotometric assays. The remaining volume is determined by weighing and usually subjected to radio-active counting.

2.4.2. Rabbit pancreas fragments

The fragments are pre-loaded for 2 hrs in 10 ml incubation medium containing $^{45}CaCl_2$ or $^{28}MgCl_2$ and subsequently washed for 15 min in 300 ml fresh incubation medium to remove adhering radio-

activity. The washed fragments are then transferred after fixed periods from one plastic counting vial, containing 5 ml incubation medium, to another. In the initial (control) phase of the experiment five 15-min efflux periods have been used, while in the later experimental phase twenty-four 5-min efflux periods have been applied. From each vial 300 µl samples are taken for the amylase assay. The radioactivity left in each vial is measured after mixing the medium with 10 ml Insta-gel. The experimental changes of the efflux medium are indicated in the figures. The radioactivity left in the tissue is determined by means of the internal standard method after solubilization of the tissue in Hyamine hydroxide 10-X. The tracer efflux rate (k) of each fraction is calculated as:

$$k(min^{-1}) = \frac{\text{dpm } min^{-1} \text{ in medium sample}}{\text{mean dpm in tissue}}$$

2.5. Gel filtration of the secreted fluid

The secreted fluid collected in the first hour after stimulation 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 removed 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 column (80 x 5 mm, Pasteur capillary pipette), which is equilibrated with 0.9 % NaCl by applying 150 µl aliquots to the column. Equal fractions are collected and analyzed for protein (15 µl), divalent cations (100µl) and radioactivity (15 µl). The

recovery of each fraction is calculated and plotted in the figures.

2.6. Assay methods

2.6.1. Protein determination

Protein concentration in the secreted fluid of the isolated rabbit pancreas, serving as a parameter for enzyme secretion, is determined according to Lowry et al (1951) on a micro scale, using bovine serum albumin as a standard.

Ten µl samples are diluted to 400 µl and mixed with 2 ml of an alkaline copper reagent (a mixture of 50 ml of 2% Na_2CO_3 in 0.10 N NaOH and 1 ml of 0.5% $CuSO_4.5\ H_2O$ in 1 % Na-K Tartrate). After 10 min 200 µl of a 1 N Folin-Ciocalteu phenol reagent is added and mixed rapidly. After 30 min the optical density of the solution was measured at 500 nm in a Zeiss spectrophotometer (PMQ II).

2.6.2. Volume flow determination

The volume flow of the secreted fluid of the isolated rabbit pancreas is determined by the weight increase of the collecting vial. The rapid weight determinations required for the collected samples are carried out on a fully-automatic and self-registring Mettler electronic balance, type HE 20 MV. The density of the secreted fluid is assumed to be 1.0 for all samples.

2.6.3. Calcium and Magnesium determination

Total calcium and magnesium concentrations are determined on a micro scale with a calcium or magnesium Rapid Stat Kit, measuring either the blue color (612 nm) of the calcium complex of

methylthymol blue or the red color (532 nm) of the magnesium complex of calmagite.

2.6.4. Amylase determination

The amylase activity in the efflux media of rabbit pancreas fragments, serving as a parameter for enzyme secretion, is determined according to Bernfeld (1955). Samples of 300 µl are incubated for 3 min at 30° C with 1 ml of a 1 % starch solution (w/v). The enzyme reaction is stopped by the addition of 2 ml of 1 % 3,5-dinitrosalicylic acid, which reacts with the free reducing groups liberated from the starch. The mixture is heated for 5 min in boiling water and then cooled in running tap water. After addition of 5 ml water, the optical density of the solution is measured at 570 nm. Blanks are prepared in the same manner with incubation medium. A calibration curve is established with maltose. Amylase activity is defined as 1 mg maltose liberated in 3 min at 30°C.

2.6.5. Determination of TCA-insoluble tritium-radioactivity

In experiments in which the secretion of newly synthesized protein is followed after the addition of L-[4,5-^{3}H] Leucine, the TCA-insoluble ^{3}H-radioactivity is used as a parameter. To this purpose samples of the secreted fluid are mixed with 6 ml cold 10 % TCA and the precipitate is collected on a Selectron cellulose acetate filter (0.2 µm) and washed with 30 ml cold 10 % TCA. The precipitate is dissolved in 1 ml 0.05 N NaOH, mixed with 10 ml Insta-gel and subjected to radioactive counting.

2.6.6. Radioactive counting

The samples of the secreted fluid and the efflux media to be counted are first mixed with 10 ml Aquasol and Insta-gel, respectively. The radioactivity is then measured in a Philips liquid scintillation analyzer. The radioactivity in dual label experiments is calculated by means of the external standard ratio method.

CHAPTER 3

CALCIUM MOVEMENTS IN THE RABBIT PANCREAS

3.1. Introduction

Calcium ions are closely involved in exocrine pancreatic secretion. They do not only occur in the secreted fluid, but it is also suggested that they play a role in the stimulus-secretion coupling of pancreatic enzyme secretion (section 1.1.6.). Since the pattern of calcium movements, before and after stimulation, may yield information about the role of calcium in exocrine pancreatic secretion, we have studied these movements with the aid of radioactive $^{45}Ca^{2+}$.

In these experiments we have used both the intact, isolated rabbit pancreas and fragments of this pancreas. In the former system the stimulatory (blood) and secretory (duct) compartments are separated. Thus $^{45}Ca^{2+}$ can be added to the first compartment, and can then be collected in the pancreatic fluid exclusively from the second compartment. The fragments, pre-loaded with $^{45}Ca^{2+}$, permit the study of the tracer efflux to the secretory compartment, although in combination with that to the stimulatory compartment (fig. 3.6.). The conclusions, which can be drawn about the calcium movements in the rabbit pancreas, are discussed in relation to the possible role of calcium in the stimulus-secretion coupling of pancreatic enzyme secretion.

3.2. Materials and Methods

The materials, the preparation of the rabbit pancreas, the incubation medium, the incubation and sample collection procedures and the assay methods are described in chapter 2.

3.3. Results

3.3.1. Basal secretion of the isolated rabbit pancreas

The pancreatic fluid is collected from the cannulated duct of the isolated rabbit pancreas in 5-10 min fractions. There is some variation in the flow rate and the composition of the basal secretion: volume flow 200-800 µl/h; total calcium and $^{45}Ca^{2+}$ 20-40 % of the medium concentrations; protein 1-3 mg/h. In order to permit adequate comparison between the secretion of the two calcium isotopes, their concentrations are expressed as the percentage of their concentrations in the medium.

After addition of the tracer ion to the bathing medium, $^{45}Ca^{2+}$ immediately begins to be secreted into the pancreatic fluid. After about 30 min the specific activity of calcium in the fluid becomes constant and is then about equal to that of the bathing medium, while the total calcium concentration amounts to only 30 % of the medium concentration (fig. 3.1.). The two levels remain constant for at least 4 h in the absence of stimulation. Only when the volume flow decreases below 250 µl/h, the two calcium concentrations begin to increase. The mean values of these parameters for the basal secretion are presented in the first part of table 3.1.

TABLE 3.1.

EFFECTS OF CARBACHOL ON THE COMPOSITION OF THE SECRETED FLUID COLLECTED FROM THE ISOLATED RABBIT PANCREAS

Half hour period	Flow µl/½ hr	Protein mg/½ hr	Total Ca^{2+} % bath level	$^{45}Ca^{2+}$ % bath level
1	291 ± 22	1.7 ± 0.22	32 ± 4.8	21 ± 2.4
2	323 ± 20	1.1 ± 0.21	30 ± 4.6	34 ± 3.6
3	348 ± 19	1.0 ± 0.13	31 ± 5.1	35 ± 3.2
4	358 ± 29	0.9 ± 0.12	30 ± 5.9	35 ± 3.1
5	271 ± 26	12.3 ± 1.69	92 ± 6.9	58 ± 4.2
6	291 ± 25	5.6 ± 0.76	82 ± 7.9	68 ± 5.8
7	269 ± 25	2.7 ± 0.36	64 ± 5.2	60 ± 5.0
8	239 ± 31	1.5 ± 0.22	54 ± 6.0	55 ± 4.2

Mean values with standard errors of the mean for eight experiments. Carbachol (10^{-5} M) is added to the $^{45}Ca^{2+}$ containing medium at the end of the second hour (period 4).

3.3.2. Effects of stimulation

After a 2-h pre-incubation period the enzyme secretion is stimulated by addition of 10^{-5} M carbachol, which is the carbamyl analogue of acetylcholine, to the $^{45}Ca^{2+}$ containing medium. This analogue is less susceptible to hydrolysis by acetylcholinesterase than acetylcholine (Wilson et al, 1960). After addition of carbachol, the protein concentration in the secreted fluid increases usually within 5 min and reaches its maximum in about 10 min (fig. 3.1.). The same is true for the total calcium concentration

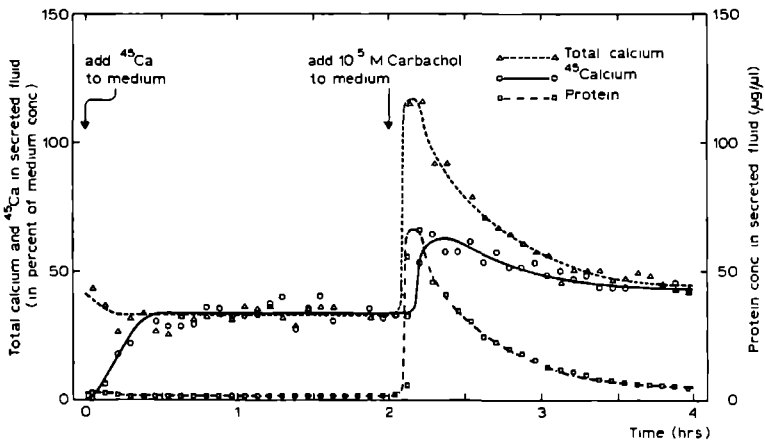

Fig. 3.1. Effects of 10^{-5} M carbachol on the composition of the pancreatic fluid. $^{45}Ca^{2+}$ is present in the bathing medium during the entire experimental period, which includes a 2-h pre-incubation period before stimulation. Typical for eight experiments.

in the secreted fluid. However, the $^{45}Ca^{2+}$ concentration begins to rise only after about 8 min, while its maximum is reached after about 20 min. Another difference is that the $^{45}Ca^{2+}$ concentration

in the pancreatic fluid increases only 2-fold, which is considerably less than the increase of the total calcium concentration. Two hours after stimulation the protein concentration has nearly returned to its basal level, while the calcium levels become equal again, but remain higher than they were before stimulation. The mean values of these parameters after stimulation with 10^{-5} M carbachol are presented in the last part of table 3.1. There is a slight decrease in the rate of fluid secretion after addition of carbachol.

When the same stimulus is applied after a pre-incubation period of only 30 min, exactly the same secretion patterns are observed (fig. 3.2.). This indicates that a pre-incubation period of

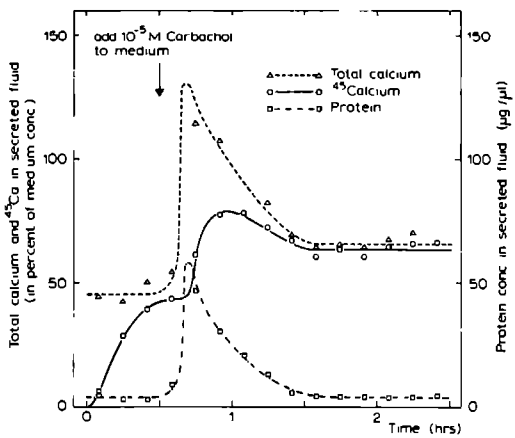

Fig. 3.2. Effects of 10^{-5} M carbachol on the composition of the pancreatic fluid. $^{45}Ca^{2+}$ is present in the bathing medium during the entire experimental period, which now includes only a 30-min pre-incubation period before stimulation. Typical for eight experiments.

only 30 min is sufficient to obtain the same increased $^{45}Ca^{2+}$

secretion as observed after a pre-incubation period of 2-h.

3.3.3. Effects of repeated stimulation

In order to check whether the delayed increase in $^{45}Ca^{2+}$ secretion is due to an exchange of calcium ions induced by stimulation, we have studied the effects of repeated stimulation. In these experiments acetylcholine has been used. The effects after the first stimulation with acetylcholine are comparable to those after carbachol stimulation (cf. figs. 3.1. and 3.3.), indicating

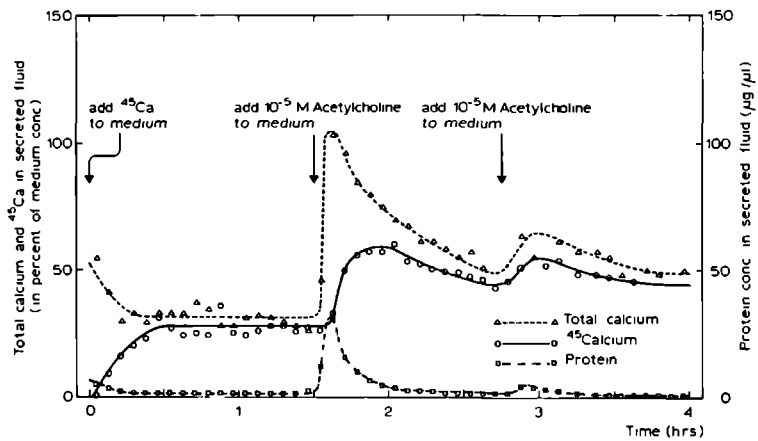

Fig. 3.3. Effects of repeated stimulation with 10^{-5} M acetylcholine on the composition of the pancreatic fluid. $^{45}Ca^{2+}$ is present in the bathing medium during the entire experimental period. Typical for two experiments.

that the hydrolysis of acetylcholine by acetylcholinesterase present in the tissue occurs only to a limited extent. Addition of a second dose of acetylcholine 80 min later gives only a minor increase in protein secretion. However, the secretion of $^{45}Ca^{2+}$ increases to about the same level as after the first stimulation.

In this case the maximal increase in total calcium concentration coincides with that of $^{45}Ca^{2+}$, both now being delayed with respect to the protein peak.

3.3.4. Effects of stimulation after removal of $^{45}Ca^{2+}$ from the medium

Partial removal of $^{45}Ca^{2+}$, down to 25 % of its original level, from the bathing medium after 90 min of pre-incubation in the presence of this isotope causes the specific activity of the pancreatic fluid to drop proportionally in about 30 min (fig. 3.4.).

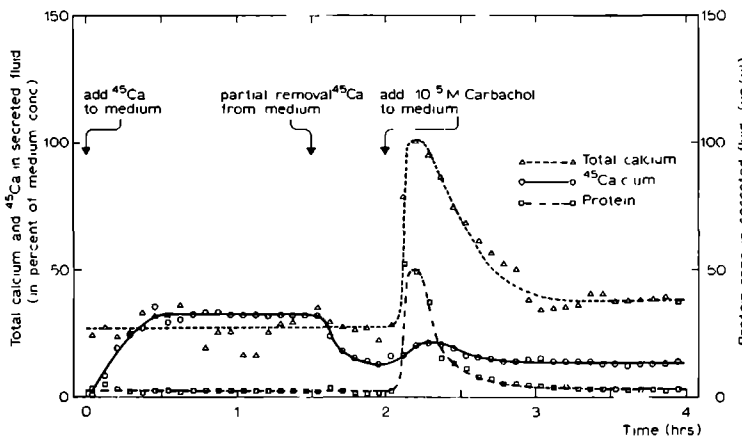

Fig. 3.4. Effects of 10^{-5} M carbachol on the composition of the pancreatic fluid. $^{45}Ca^{2+}$, present in the medium, is partly removed before stimulation. Typical for two experiments.

Stimulation at that time shows the normal responses for protein and total calcium. However, the increase in the $^{45}Ca^{2+}$ secretion is reduced, although its peak value is again about double the level just before stimulation.

Complete removal of the tracer from the medium has also been

attempted. It was, however, impossible to keep the medium completely tracer-free, probably due to leakage of the tracer from the tissue. The level decreased, however, to less than 5 %. In four such experiments the $^{45}Ca^{2+}$ level in the secreted fluid decreased to 4.2 % (SE 0.6) of the original medium concentration. The $^{45}Ca^{2+}$ peak after stimulation reached a maximum of 7.8 % (SE 0.5), which is again double the level just before stimulation.

3.3.5. $^{45}Ca^{2+}$ - efflux studies with rabbit pancreas fragments

The pancreas fragments are incubated for 2-h in the presence of $^{45}Ca^{2+}$ and are then transferred through a series of plastic counting vials, containing tracer-free medium, in order to determine the $^{45}Ca^{2+}$ efflux rate. The experiments of fig. 3.5. show a

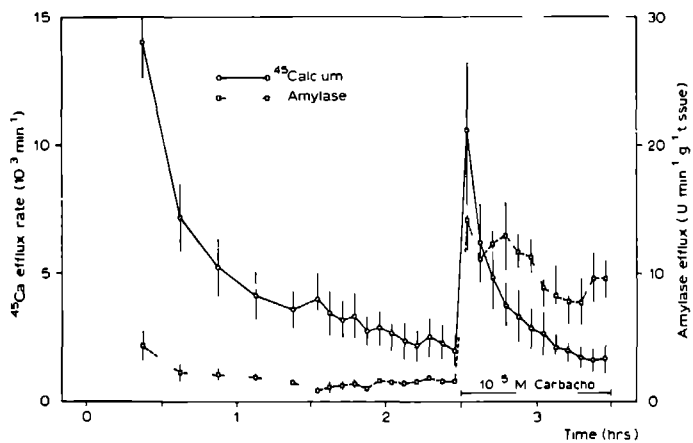

Fig. 3.5. Effects of 10^{-5} M carbachol on the efflux of $^{45}Ca^{2+}$ and amylase from rabbit pancreas fragments pre-loaded with $^{45}Ca^{2+}$. Mean values with standard errors of the mean for four experiments.

monotone $^{45}Ca^{2+}$ efflux in the absence of carbachol, while the amylase release remains relatively constant. Upon addition of 10^{-5} M

carbachol to the efflux media, there is an immediate, large increase in $^{45}Ca^{2+}$ efflux. The original efflux rate is restored within 30 min after addition of the stimulus. The increased tracer efflux is accompanied by a large increase of the amylase release. The amylase release remains, however, relatively high during the rest of the experiment.

3.4. Discussion

In order to increase our insight into the role of calcium in exocrine pancreatic secretion, we have tried to obtain detailed information about the calcium movements in this gland, before as well as after stimulation. The calcium movements before stimulation will be considered first.

At the basal secretion rate, when enzyme secretion is very low, the calcium concentration of the pancreatic fluid is normally about 30 % of that in the bathing medium. Only when the volume flow decreases below 250 µl/h, the calcium concentration in the pancreatic fluid begins to approach that of the medium. These findings, which agree with those of Argent et al (1973) in cat and Goebell et al (1972) in dog pancreas, suggest a diffusion of calcium from the stimulatory to the secretory compartment of the gland. Upon addition of $^{45}Ca^{2+}$ to the bathing medium, the isotope immediately appears in the secretory fluid and its specific activity reaches that of the medium in about 30 min (figs. 3.1-4). The $^{45}Ca^{2+}$ concentration of the pancreatic fluid changes proportionally with its level in the medium (fig. 3.4.). This suggests the existence of an extracellular route for calcium, probably by the so-called tight-

junctions between adjacent cells, which is followed by the $^{45}Ca^{2+}$ added to the bathing medium. This hypothesis is confirmed by the fact that the $^{45}Ca^{2+}$ secretion to the pancreatic fluid behaves very similarly to that of the extracellular marker mannitol (see section 4.3.1.). The low calcium concentration in the pancreatic fluid, relative to that in the bathing medium, may be due to a combination of two effects: a low diffusion constant for calcium in the extracellular pathway and the relatively high flow rate of the in vitro preparation (Rothman, 1964). The increase in calcium concentration in the pancreatic fluid at decreasing flow rates pleads against a re-uptake of calcium from the primary secreted fluid by the ductular cells as an explanation for the low calcium concentration in the pancreatic fluid. Hence, in the resting state the calcium secretion into the fluid is mainly due to an extracellular calcium flux.

Now we shall consider the calcium movements after stimulation. When the pancreas is stimulated with 10^{-5} M carbachol (figs. 3.1. and 3.2.) or with 10^{-5} M acetylcholine (fig. 3.3.), the resulting increased protein secretion is accompanied by a simultaneous increase in the secretion of calcium. This has previously been reported for several pancreatic preparations (Argent et al, 1973; Zimmerman et al, 1967; Goebell et al, 1972; Rutten, 1974). There is always a positive correlation between the increase in calcium and the magnitude of the enzyme secretion, but their ratio is not constant. Although in our experiments the calcium and protein peaks coincide in time, the protein concentration returns to its original level faster than the calcium concentration does (figs.

3.1-3). This indicates that only a part of the total calcium secretion after stimulation represents calcium secreted together with the enzymes from the acinar cells. We call this calcium flux the secretory flux.

Unexpected are the findings that the increase in $^{45}Ca^{2+}$ secretion is always less than that of total calcium and especially that the $^{45}Ca^{2+}$ peak is delayed by about 10 min with respect to the total calcium peak. Only when after a second stimulation there is virtually no enzyme secretion, does the $^{45}Ca^{2+}$ secretion increase in parallel with the increase of non-labeled calcium (fig. 3.3.). In addition, the increase of $^{45}Ca^{2+}$ secretion is directly related to the specific activity of calcium in the medium at the moment of stimulation. These observations suggest that the $^{45}Ca^{2+}$ secretion again represents the extracellular Ca flux from the stimulatory to the secretory compartment. This flux is normally masked by the large amount of calcium secreted in conjunction with the enzymes upon stimulation. The presence of a distinct flux is also indicated by the fact that the total calcium decreases somewhat slower and to a lesser degree than the protein. Most likely this calcium movement and thus the delayed $^{45}Ca^{2+}$ secretion are due to an increase in the permeability of the extracellular route, since the secretion of $^{45}Ca^{2+}$ increases simultaneously with that of the extracellular marker mannitol upon stimulation with carbachol (fig. 4.1.). It is not clear whether such an increase in the extracellular pathway has any physiological significance in itself, but it may explain why no constant correlation between the increase in calcium and protein secretion is found. It may be an artifact of the isolated

rabbit pancreas, which may also be the case for the fact that the original calcium concentration in the pancreatic fluid is not restored after stimulation (fig. 3.1-3). So after stimulation there is, in addition to the calcium secreted in conjunction with the digestive enzymes, a second distinct calcium flux due to an increase in permeability of the extracellular route.

The calcium secreted in conjunction with the digestive enzymes must originate from an endogenous calcium pool, which does not exchange with its tracer in the medium during a 2-h incubation period before stimulation. It probably represents calcium sequestered by the zymogen granules, as occurs in the parotid (Wallach and Schramm, 1971). The calcium may be bound to the enzymes or to the granule membranes, which have a high calcium content (Clemente and Meldolesi, 1975). In any case, it is an intracellular pool which does not freely exchange with $^{45}Ca^{2+}$ in the incubation medium. These results strongly support the suggestion of Argent et al (1973) and Goebell et al (1972) that the calcium content of the pancreatic fluid is produced by at least two distinct calcium movements: one associated with the digestive enzymes and the other with the electrolytes of the pancreatic fluid. In addition it is shown that both are influenced by cholinergic agents.

The absence of a $^{45}Ca^{2+}$ peak in the pancreatic fluid, collected from the isolated rabbit pancreas upon stimulation after prior removal of the tracer from the medium, seems at first sight to conflict with earlier findings with mouse and rat pancreas fragments. Efflux studies with these fragments, pre-loaded with $^{45}Ca^{2+}$, show an acetylcholine-stimulated $^{45}Ca^{2+}$ efflux in an isotope-free

medium (Case and Clausen, 1973; Heisler, 1974; Matthews et al, 1973). In repeating such experiments with rabbit pancreas fragments we have made essentially the same observation (fig. 3.5.). Hence, there is no species difference involved. Rather, the difference between the isolated rabbit pancreas and the pancreas fragments must be attributed to the fact that in the latter system secretion to the stimulatory and secretory compartments (i.e. across the basal and apical sides of the acinar cells) is measured together. This suggests that the $^{45}Ca^{2+}$ efflux, observed only in pancreas fragments, is mainly due to an increased efflux across the basal membrane. This can be due either to an increases in the permeability of this membrane or to a release of calcium from an intracellular store or both. Both possibilities would account for the fact that the calcium released in the stimulatory compartment is easily exchangeable. We shall call this flux the stimulatory calcium flux, to distinguish it from the secretory calcium flux, which represents poorly exchangeable calcium and which is released to the secretory compartment in conjunction with the enzymes.

Our experiments support an important role of calcium in exocrine pancreatic secretion. In rabbit pancreas cholinergic agents seem to influence calcium fluxes at different levels (fig. 3.6.): 1. it increases the permeability of the extracellular route for several components including calcium; 2. it causes a release of calcium over the apical membrane in conjunction with the enzyme proteins; 3. it causes an increase in the calcium flux across the basal membrane, either by an increase in the permeability of this membrane or by a release of calcium from an intracellular store.

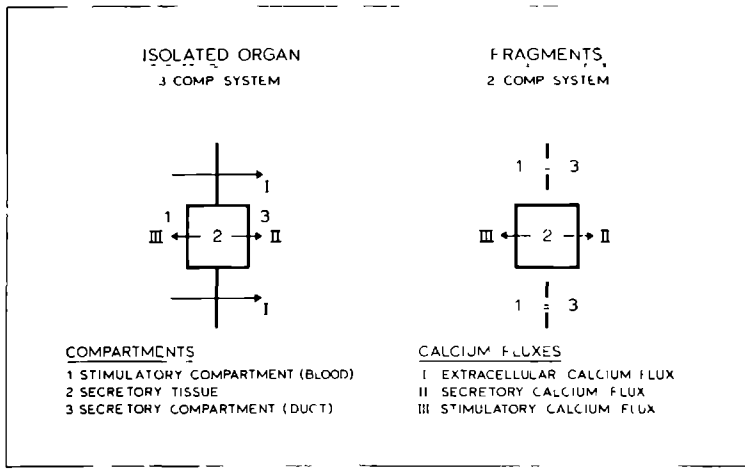

Fig. 3.6. Schematic presentation of the stimulated calcium fluxes in two preparations of the rabbit pancreas.

Regardless of the mechanism involved in the third flux, its occurrence indicates that the cytoplasmic calcium concentration increases upon stimulation. Our $^{45}Ca^{2+}$ - efflux studies with rabbit pancreas fragments, in the absence of calcium in the efflux medium (chapter 7), suggest that the second explanation for the calcium flux across the basal membrane is the most likely one.

The movements of calcium in the resting and stimulated pancreas can now be summarized as follows. In the resting pancreas there are at least two endogenous calcium pools in the acinar cells, the zymogen granules and another, possibly the mitochondria. Calcium from the bathing medium reaches the secreted fluid mainly through the extracellular spaces between the ductular cells. Only a very small amount of calcium may pass through the pancreatic

cells. Upon stimulation three calcium fluxes operate: the extracellular route is increased, calcium is released to the lumen from the zymogen granules in conjunction with the digestive enzymes, calcium is released from the other calcium store to the cytoplasm and can escape across the basal membrane. These calcium movements are discussed separately and in detail in the following chapters.

CHAPTER 4

THE EXTRACELLULAR CALCIUM FLUX

4.1. Introduction

Previously, it has been suggested that the calcium present in the pancreatic fluid derives from at least two distinct calcium movements, one associated with the digestive enzymes and the other with the electrolytes of the secreted fluid (Goebell et al, 1972; Argent et al, 1973).

In chapter 3 we have established these calcium movements in the isolated rabbit pancreas and have provisionally called them the secretory and extracellular calcium fluxes (see fig. 3.6.). When radioactive calcium is added to the bathing medium of the isolated rabbit pancreas, it rapidly equilibrates with the calcium in the pancreatic fluid in the resting state, but upon stimulation a tracer peak appears which is delayed relative to the protein and total calcium peaks. Even after 2-h pre-incubation the separate tracer peak remains, suggesting that up to that moment the secretion of the tracer represents exclusively the extracellular calcium flux. The experiments of chapter 3, where 10^{-5} M carbachol is used as the stimulus, show that after the transient increase in the tracer secretion the latter returns to a constant level, which is however higher than the pre-stimulation level (see fig. 3.1.).

More definitive proof for equating the delayed tracer peak

with a stimulation-induced increase in the extracellular calcium flux has been obtained in this chapter by a comparison of the $^{45}Ca^{2+}$ secretion with that of ^{3}H-mannitol. This substance is known not to penetrate cells and thus can be used as a marker for the extracellular pathway (Wright and Pietras, 1974). Information about the specificity of the extracellular calcium flux has been obtained by studying the secretion of magnesium, the only other divalent cation present in the bathing medium.

The secretory flux is thought to represent enzyme-associated calcium, which has led other investigators to study the quantitative relation between the secretion of calcium and of digestive enzymes into the pancreatic fluid (Sullivan et al, 1974; Rutten, 1974). In their studies they have not taken into account that the contribution of the extracellular flux does not remain constant upon stimulation, since they have simply plotted the total calcium concentration in the secreted fluid vs. the digestive enzyme concentration. The amount of calcium associated with the digestive enzymes is then calculated from the slope of the regression line, while the calcium intercept is assumed to represent a constant flux component, which is independent of the amount of digestive enzymes secreted. This procedure will lead to erroneous results, when the extracellular calcium flux does not remain constant upon stimulation. Therefore, we have studied the effects of stimulation on the extracellular calcium flux in closer detail. With respect to the variability of the extracellular flux we have also studied the effects of a 10-fold lower dose of carbachol. In addition we have used pancreozymin in about equipotent concentrations, in order to

check whether or not the effects on the extracellular flux are restricted to cholinergic stimulation.

Our results indicate that the secretion of calcium and magnesium is qualitatively comparable, before and after stimulation. The effects of carbachol and pancreozymin on each are roughly the same, and their effects on the extracellular fluxes are markedly dose-dependent.

4.2. Materials and Methods

The materials, the preparation of the rabbit pancreas, the incubation medium, the incubation and sample collection procedures and the assay methods are described in chapter 2.

4.3. Results

4.3.1. Studies with extracellular markers

In these experiments $^{45}Ca^{2+}$ and ^{3}H-mannitol (mol.wt. 182) are added simultaneously at time zero to the bathing medium, which in addition contains 2 mM non-labelled mannitol. There is a striking similarity between the concentration curves for $^{45}Ca^{2+}$ and ^{3}H-mannitol in the pancreatic fluid (fig. 4.1.). The equilibrium between the stimulatory and secretory compartment is reached within 30 min for both substances, while they both show a maximal value about 20 min after stimulation with 10^{-5} M carbachol. The two curves differ only in their absolute levels. The level of mannitol is about twice that of calcium before stimulation. Upon stimulation the mannitol concentration increases less than that of $^{45}Ca^{2+}$ and then returns to its original level, while the $^{45}Ca^{2+}$

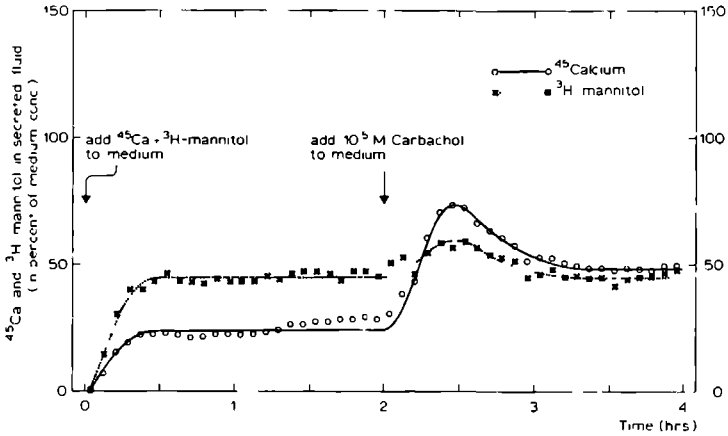

Fig. 4.1. Comparison of the $^{45}Ca^{2+}$ and ^{3}H-mannitol secretion into the pancreatic fluid, before and after stimulation with 10^{-5} M carbachol. During the entire experimental period tracers are present in the medium, which also contains non-radioactive calcium (2.5 mM) and mannitol (2 mM). Typical for three experiments.

concentration remains increased relative to its original level. The volume flow and the levels of total calcium and protein secretion, before and after stimulation, are not affected by the addition of 2 mM mannitol, suggesting that this substance has no delaterious effect on the tissue under these conditions.

When in similar studies the macro-molecular substance ^{3}H-inulin (mol.wt. 5000) is used as an extracellular marker, hardly any ^{3}H-radioactivity can be detected in the secreted fluid both before and after stimulation.

4.3.2. Basal secretion of magnesium

The volume flow and the total calcium, $^{45}Ca^{2+}$ and protein levels in the pancreatic fluid during basal secretion have already

been described in section 3.3.1. The basal secretion of total magnesium and radioactive $^{28}Mg^{2+}$ after addition of this isotope to the bathing medium, behaves very similarly to that of calcium and its tracer. Equilibrium between the levels of $^{28}Mg^{2+}$ in the bathing medium and the secreted fluid is also reached within half an hour after addition of the tracer and is retained for several hours. The mean steady-state concentrations for magnesium, relative to those in the bathing medium are: 32 (SE 3.0, n = 12) % for total magnesium and 18 (SE 1.5, n = 12) for $^{28}Mg^{2+}$, at a volume flow of 320 (SE 29, n = 12) $\mu l/\frac{1}{2}h$ and a protein output of 1.9 (SE 0.27, n = 12) $mg/\frac{1}{2}h$. In contrast to the concentrations for calcium: 30 (SE 4.6, n = 8) % for total calcium and 34 (Se 3.6, n = 8) % for $^{45}Ca^{2+}$ (see table 3.1.), the values for total magnesium and $^{28}Mg^{2+}$ are significantly different from each other.

4.3.3. Effects of 10^{-5} M carbachol on the secretion of magnesium

Stimulation of pancreatic enzyme secretion by pancreozymin as well as by cholinergic agents produces a transiently increased output of digestive enzymes, which is accompanied by an increased secretion of calcium and magnesium ions (Sullivan et al, 1974; Rutten, 1974).

In chapter 3 we have established that the secretion of radioactive calcium present in the bathing medium also shows a transient increase upon stimulation with 10^{-5} M carbachol. This increase does not, however, coincide with the increase of the total calcium secretion (see fig. 3.1.). In addition the increased secretion of the tracer, which represents the extracellular flux, is independent

of the pre-incubation period (chapter 3) and is due to an increased permeability of the extracellular route (section 4.3.1.). We have now extended the information about the extracellular calcium flux by a study of the secretion of magnesium ions under the same conditions.

The effects of stimulation with 10^{-5} M carbachol on the secretion of magnesium are shown in fig. 4.2. The $^{28}Mg^{2+}$ is added to the

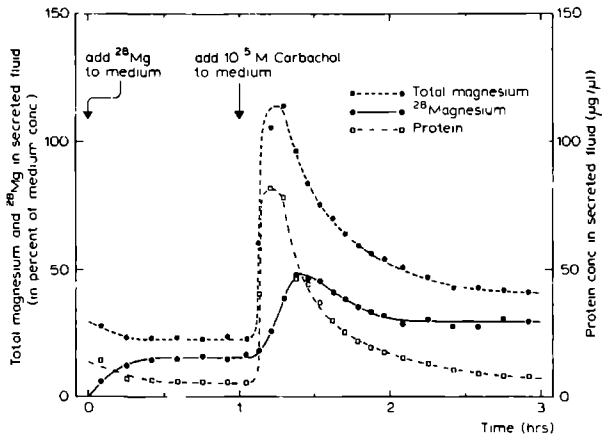

Fig. 4.2. Magnesium secretion into the pancreatic fluid, before and after stimulation with 10^{-5} M carbachol. During the entire experimental period the tracer is present in the medium, which also contains 1.3 mM non-radioactive magnesium. Typical for three experiments.

bathing medium 1 h prior to stimulation. Upon stimulation the total magnesium concentration increases simultaneously with the protein content of the pancreatic fluid. Both concentrations reach their maximal value in about 10 min and decrease continuously thereafter. The $^{28}Mg^{2+}$ secretion shows a delayed increase, reaching a maximal value about 20 min after stimulation. Another difference is that the $^{28}Mg^{2+}$ concentration in the secreted fluid increases only

3-fold, which is considerably less than the increase of the total magnesium concentration. Two hours after stimulation the protein concentration has nearly returned to its basal level, while the magnesium concentrations decrease to levels which remain higher than they were before stimulation. Exactly the same behaviour has been observed for the secretion of total calcium and radioactive $^{45}Ca^{2+}$ upon stimulation with 10^{-5} M carbachol (section 3.3.2.).

The similarity in the secretion patterns of calcium and magnesium suggests that the secretion of both cations is provided by an extracellular and secretory flux.

4.3.4. Dose-response relationship between the extracellular fluxes and carbachol

Since the amounts of protein-associated divalent cations can only be derived directly from their total content in the pancreatic fluid, if the contribution of the extracellular flux remains constant, we have tried to find conditions under which the extracellular fluxes are less variable than after stimulation with 10^{-5} M carbachol. This turns out to be the case at a 10-fold lower dose of carbachol. The different effects of carbachol at these two dose levels on the extracellular calcium flux are seen by comparing figs. 4.3A. (10^{-5} M) and 4.3B. (10^{-6} M). Carbachol at both levels increases the $^{45}Ca^{2+}$ level of the secreted fluid. After application of the higher dose there is a high transient increase followed by a permanently raised increase. After the lower dose the transient peak is absent and there is only a permanently raised increase in the $^{45}Ca^{2+}$ secretion. Similar behaviour is found for the $^{28}Mg^{2+}$

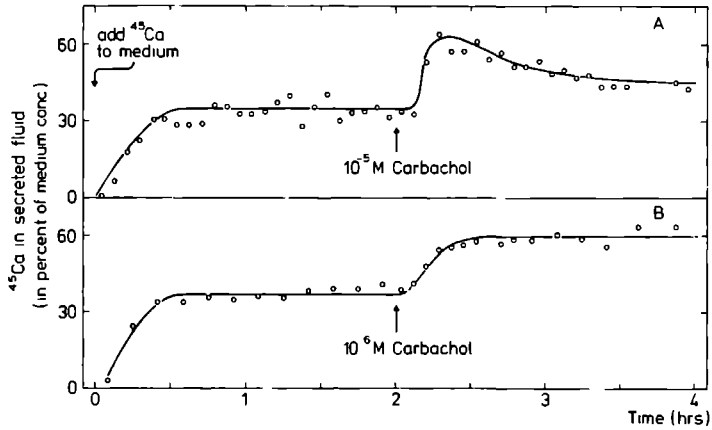

Fig. 4.3. Comparison of the $^{45}Ca^{2+}$ secretion into the pancreatic fluid, before and after stimulation with 10^{-5} M (A) and 10^{-6} M (B) carbachol. During the entire experimental period the tracer is present in the medium, which also contains 2.5 mM non-labeled calcium. Typical for eight and three experiments, respectively.

secretion after stimulation with 10^{-5} M (fig. 4.2.) and 10^{-6} M (fig. 4.4.) carbachol, respectively. The protein and total divalent cation secretion show a less sharp increase upon stimulation with 10^{-6} M as compared with 10^{-5} M carbachol. Quantitative data for the extracellular fluxes are before and after the two carbachol levels presented in tables 4.1. and 4.2.

4.3.5. Receptor specificity of the effects on the extracellular fluxes

By means of pancreozymin, applied in about equipotent doses, we have checked whether or not the effects on the extracellular fluxes are restricted to cholinergic stimulation. We have studied

TABLE 4.1.

RELATIVE CONCENTRATIONS OF $^{45}Ca^{2+}$ IN THE SECRETED FLUID BEFORE AND AFTER STIMULATION

Half hour period	10^{-6} M carbachol	10^{-5} M carbachol	20 U pancreozymin	200 U pancreozymin
1	14 ± 1.9	14 ± 1.7	15 ± 0.8	16 ± 3.0
2	21 ± 2.3	27 ± 2.8	28 ± 2.0	28 ± 3.2
3	27 ± 4.8	52 ± 3.2	37 ± 1.1	43 ± 1.5
4	29 ± 5.7	63 ± 4.4	43 ± 2.9	65 ± 8.4
5	30 ± 6.7	57 ± 3.9	42 ± 3.0	56 ± 8.3
6	31 ± 6.7	50 ± 3.3	42 ± 2.4	51 ± 6.7

Mean values (in percent of medium conc.) with standard errors of the mean (n=3). The stimulant is added to the $^{45}Ca^{2+}$ containing medium at the end of the second half hour period. The calcium concentration in the medium is 2.5 mM.

TABLE 4.2.

RELATIVE CONCENTRATIONS OF $^{28}Mg^{2+}$ IN THE SECRETED FLUID BEFORE AND AFTER STIMULATION

Half hour period	10^{-6} M carbachol	10^{-5} M carbachol	20 U pancreozymin	200 U pancreozymin
1	9 ± 0.1	17 ± 5.8	12 ± 4.4	9 ± 1.1
2	17 ± 2.1	21 ± 3.6	19 ± 4.5	16 ± 2.1
3	21 ± 2.1	37 ± 4.7	21 ± 4.8	32 ± 4.5
4	23 ± 1.3	42 ± 6.6	22 ± 3.1	50 ± 4.8
5	22 ± 1.3	39 ± 5.7	23 ± 1.3	44 ± 4.7
6	23 ± 1.2	49 ± 9.7	24 ± 1.5	47 ± 9.6

Mean values (in percent of medium conc.) with standard errors of the mean (n=3). The stimulant is added to the $^{28}Mg^{2+}$ containing medium at the end of the second half hour period. The magnesium concentration in the medium is 1.3 mM.

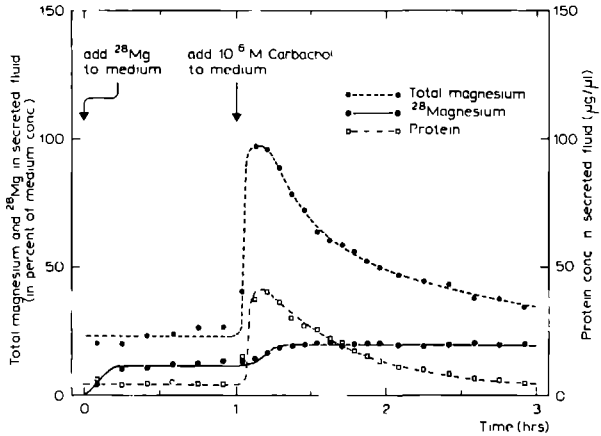

Fig. 4.4. Magnesium secretion into the pancreatic fluid, before and after stimulation with 10^{-6} M carbachol. During the entire experimental period the tracer is present in the medium, which also contains 1.3 mM non-radioactive magnesium. Typical for three experiments.

pancreozymin at two dose levels and studied the effects on the secretion of $^{45}Ca^{2+}$ and $^{28}Mg^{2+}$. For both tracers the effects of the higher dose (200 U pancreozymin) are qualitatively the same as observed after stimulation with the higher dose of carbachol (cf. figs. 3.1. and 5.1.), while the effects of the lower dose (20 U pancreozymin) are comparable to those of the lower dose of carbachol).

These results show that the effects of carbachol and pancreozymin are comparable in a qualitative way, and that the effects of these stimulants on the extracellular fluxes of calcium and magnesium are strongly dose-dependent. Quantitative data for the extracellular fluxes before and after the two pancreozymin levels are also presented in the tables 4.1. and 4.2.

4.4. Discussion

The results presented in this chapter indicate that the comparison between the secretion of ^{3}H-mannitol (mol.wt. 182) and that of $^{45}Ca^{2+}$ may be extended to the secretion of $^{28}Mg^{2+}$. These tracers, added to the medium, equilibrate all within half an hour with the secreted fluid and their secretion remains constant for at least 4 hrs. On the other hand, the macromolecular substance ^{3}H-inulin (mol.wt. 5000) does not pass from the medium to the secreted fluid at all. This suggests that at the basal secretion rate the extracellular marker mannitol as well as the divalent cations permeate through an extracellular route, which is permeable to the two cations and to small molecules. The permeability characteristics of this pathway appear to depend on size, shape and charge of the permeant, which may account also for the fact that the absolute magnesium concentration is only half that of calcium at the basal secretion rate. The presence of the extracellular route pleads against the alternative possibility that the basal divalent cation secretion would derive from a rapidly equilibrating intracellular cation pool.

The equilibrium levels, reached by the tracer-ions at the basal secretion rate, are maintained for several hours. For purposes of convenience we have expressed all cation concentrations in the pancreatic fluid as the percentage of those in the medium. For the resting pancreas the value of the relative $^{45}Ca^{2+}$ level is not significantly different from that for total calcium (table

3.1.), while the $^{28}Mg^{2+}$ level is considerably less than that for total magnesium (section 4.3.2.). These data indicate that the divalent cations in the pancreatic fluid mainly originate from the bathing medium during basal secretion, while only a minor fraction is secreted in association with the enzyme proteins. The size of this fraction can be calculated from the data presented in section 4.3.2. and our finding in the next chapter that about 25 nmol calcium and magnesium are associated with 1 mg protein. This calculation shows that the contribution of the protein associated cations to the total divalent cation concentrations amounts to 0.15 mM during basal secretion. Expressed relative to their concentrations in the medium this value corresponds to about 12 % for magnesium but only 6 % for calcium. This difference may explain why the relative $^{45}Ca^{2+}$ level (34 %) is found not to be significantly different from that for total calcium (30 %), while the relative $^{28}Mg^{2+}$ level (18 %) is considerably less than that for total magnesium (32 %).

Upon stimulation, the extracellular pathway becomes transiently more permeable to the two cations and mannitol, but remains impermeable to inulin. At the moment of peak secretion there is always a drastic fall in the specific activity of calcium and magnesium in the pancreatic fluid. This must be due to the secretion of non-radioactive divalent cations from a non-labeled endogenous cation pool. After the transient peak secretion the isotope levels of the pancreatic fluid decrease but remain higher than they were before stimulation. The specific activities of the cations reach again their pre-stimulation levels, when the protein secretion has

returned to its basal level. This indicates that the increase of the tracer secretion is not due to an exchange with the protein associated cations, but rather to a partly irreversible increase in the extracellular flux.

The effects of stimulation on the extracellular cation fluxes are not restricted to those of cholinergic substances, since qualitatively the same effects are observed after stimulation with pancreozymin. This confirms previous findings that hormonal and neural stimulation have the same effects on all aspects of pancreatic secretion, except that atropin inhibits only the effects of neural stimulation (Case and Clausen, 1973). The effects of both carbachol and pancreozymin are strongly dose-dependent in similar fashion. Upon application of a high dose of carbachol (10^{-5} M) or pancreozymin (200 U) the permanent increase is preceded by a transiently higher increase, which is absent after a 10-fold lower dose of each of these stimulants. The corresponding curves for protein and total calcium and magnesium secretion are much less dose-dependent. These observations again indicate that the tracers in the medium do not exchange with the divalent cations secreted in association with the digestive enzymes, but are exclusively secreted by the extracellular pathway.

In chapter 3 it has been noted that the calcium concentration in the pancreatic fluid increases at decreasing flow rates. This can, however, not explain the transiently increased $^{45}Ca^{2+}$ secretion, since stimulation with 10^{-5} M carbachol does not cause a transient decrease of the flow rate (table 3.1.). Another possible explanation would be that the increase of the extracellular cation

fluxes is caused by a change in transepithelial potential difference. This is also unlikely, since the pancreatic duct is a leaky epithelium, which shows a high conductance through the so-called "tight-junctions" of adjacent cells (Frömter and Diamond, 1972) and has a low transepithelial potential difference. The transepithelial potential difference in all duct segments of the rabbit pancreas ranges between 2 and 6 mV, lumen negative (Schulz et al, 1969). It is therefore most likely that the increase of the extracellular fluxes is due to a general increase in the solute permeability of the extracellular route for small molecules and ions.

The physiological significance of the latter phenomenon is not clear. The extra amount of calcium secreted upon stimulation of pancreatic enzyme secretion could be important for the biological activity of the digestive enzymes. Calcium ions play a role in maintaining the molecular integrity of amylase (Stein et al, 1964), trysinogen and chymotrypsinogen (Delaage and Lazdunski, 1967) and lipase (Benzonana, 1968). They are also involved in the activation of trypsinogen (Desnuelle and Gabeloteau, 1957) and in the activity of trypsin, lipase and phospolipase (Sipos and Merkel, 1970; Sarad et al, 1957; De Haas et al, 1971). About the importance of magnesium ions for the biological activity of the digestive enzymes little is known.

The continuing elevated secretion of divalent cations into the pancreatic fluid after the transient peaks is quite possibly an artifact of the isolated pancreas preparation. In any case, the increase of the extracellular flux must be taken into account, when the relation between divalent cation secretion and protein

secretion is studied quantitatively, since the extracellular flux is surely not linearly related to the amount of digestive enzymes secreted.

This study indicates that the total calcium and magnesium content of the pancreatic fluid is composed of cations supplied from two distinct sources: 1. cations directly originating from the medium through the so-called "tight-junctions" between adjacent cells (extracellular flux); 2. cations from an endogenous pool which has lost the ability to exchange with the medium (secretory flux). Both fluxes increase in similar way upon hormonal and neural stimulation. The effects on the extracellular flux depend rather strongly on the applied dose of the stimulant. The secretory calcium flux will be studied in the next chapter.

CHAPTER 5

THE SECRETORY CALCIUM FLUX

5.1. Introduction

The increased enzyme secretion after stimulation of the exocrine pancreas is always paralleled by an increased secretion of calcium and magnesium ions (chapter 4). This similarity in the secretion pattern of calcium and magnesium suggests that magnesium shares the two secretory pathways elucidated for calcium (fig. 3.6.): an extracellular flux directly originating from the bathing medium through the "tight junctions" of adjacent cells, and a secretory flux originating from an endogenous divalent cation pool and associated with the secreted enzymes.

Radioactive tracers, added to the bathing medium, rapidly equilibrate with the extracellular fluxes, but they do not equilibrate with the secretory flux (chapter 4). This indicates that in these experiments the total divalent cation content of the pancreatic fluid is composed of a labeled and a non-labeled component, representing the contributions of the extracellular and secretory fluxes, respectively.

Both fluxes increase upon pancreatic stimulation, but the increase of the extracellular flux is not linearly related to the amount of digestive enzymes secreted. Therefore we have examined whether such a relation may exist for the secretory fluxes.

Quantitative support for this relation can be obtained by correlating the divalent cation secretion with the enzyme secretion after correction for the contribution of the extracellular flux by subtraction. This correction can be omitted when the extracellular flux after stimulation is constant. This is shown to be the case at certain dose levels of carbachol (10^{-6} M) and pancreozymin (20 U). The quantitative relation between the divalent cations of the secretory flux and the digestive enzymes is determined by each of these two methods in this chapter. In addition to these quantitative studies we have compared the exchangeability of the protein-associated cations before and after their secretion.

It is concluded that the calcium and magnesium ions in the secretory fluxes are each secreted in a constant ratio of about 25 nmol per mg enzyme protein. The loss of the ability to exchange of the protein-associated cations before their secretion is not due to the fact that they are tightly bound to the enzyme proteins, because they regain this ability after their secretion.

5.2. Materials and Methods

The materials, the preparation of the rabbit pancreas, the incubation medium, the incubation and sample collection procedures and the assay methods are described in chapter 2.

5.3. Results

5.3.1. Correlation of divalent cation and protein secretion after correction for the extracellular flux.

Stimulation of pancreatic enzyme secretion with 200 U pancreozymin (fig. 5.1A.) has quite similar effects on the secretion of

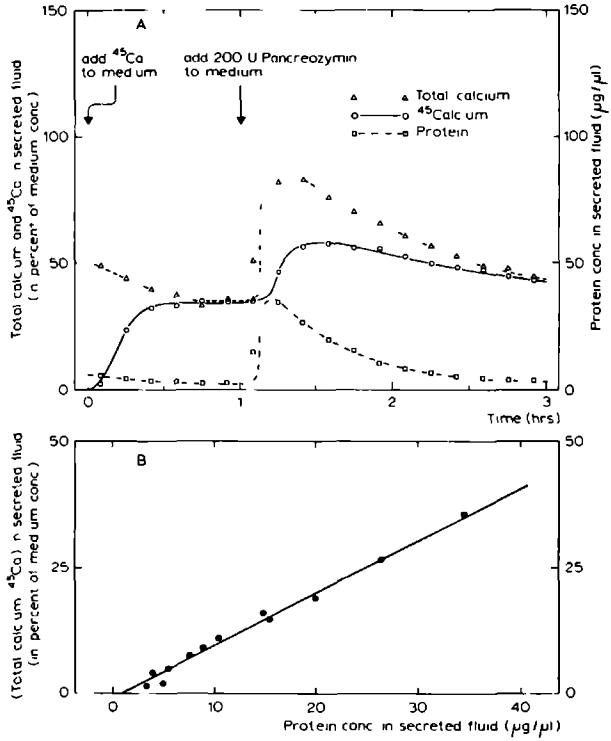

Fig. 5.1. (A) Effects of 200 U pancreozymin on the composition of the pancreatic fluid. During the entire experimental period $^{45}Ca^{2+}$ is present in the medium, which also contains 2.5 mM non-radioactive calcium. (B) Calculated regression line for the relation between the total divalent cation secretion after correction for the extracellular flux and the protein secretion after stimulation. Typical for three experiments.

protein, total calcium and $^{45}Ca^{2+}$ as stimulation with 10^{-5} M carbachol. As already discussed in section 3.4. for carbachol, this means that in both cases the total calcium content of the pancreatic fluid is composed of a labeled and a non-labeled component, representing the extracellular and secretory fluxes, respectively.

In order to examine the correlation between the calcium ions of the secretory flux and the digestive enzymes in the secreted fluid, the total calcium secretion is corrected for the variable contribution of the extracellular flux by subtraction of the latter. Upon plotting the corrected values (after stimulation) vs. the enzyme protein levels in the secreted fluid a nearly linear relation is obtained (fig. 5.1B.). The parameters of the resulting regression lines have been calculated for calcium and magnesium after stimulation by carbachol (10^{-6} and 10^{-5}M) and pancreozymin (20 and 200 U) and are presented in table 5.1.

It will be clear that subtraction of the two cation components may lead to rather large errors in the corrected cation concentrations. Nevertheless, the intercepts (B) are negligible in most cases, indicating that the secretory cation secretion is nearly proportional to the protein secretion. The amounts (A) of protein-associated calcium and magnesium are equal within the experimental error and these amounts are proximately the same for both stimulants at each of the two dose levels. These findings lend further support to our conclusion that the secretory cation fluxes represent enzyme-associated calcium and magnesium exclusively. The average value of A (20 nmol/mg protein) is equivalent to one mol cation per mol enzyme protein at an average assumed protein molecular weight of 50.000.

5.3.2. Correlation of divalent cation and protein secretion under conditions of constant extracellular flux

When the extracellular cation flux after stimulation is con-

TABLE 5.1.

PARAMETERS RELATING DIVALENT CATION AND PROTEIN SECRETION AFTER CORRECTION FOR THE EXTRACELLULAR FLUX

Stimulus	A	B	r	n
		CALCIUM		
carbachol, 10^{-6} M	21 ± 0.9	-0.02 ± 0.03	0.98 ± 0.01	3
carbachol, 10^{-5} M	20 ± 2.0	-0.04 ± 0.04	0.97 ± 0.01	3
pancreozymin, 20 U	19 ± 1.5	-0.01 ± 0.02	0.86 ± 0.04	3
pancreozymin, 200 U	22 ± 2.3	-0.04 ± 0.02	0.98 ± 0.01	3
		MAGNESIUM		
carbachol, 10^{-6} M	20 ± 4.0	0.15 ± 0.08	0.97 ± 0.02	3
carbachol, 10^{-5} M	21 ± 3.6	0.07 ± 0.04	0.98 ± 0.02	3
pancreozymin, 20 U	17 ± 3.7	0.25 ± 0.04	0.92 ± 0.08	2
pancreozymin, 200 U	23 ± 2.1	0.02 ± 0.08	0.91 ± 0.01	3

Parameters of the regression lines calculated according to the least-squares method. Mean values with standard errors of the mean. A, the amount of protein-associated cations (nmol per mg protein); B, the concentration of non-radioactive cations in the 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.

stant, correction for this flux can be omitted. This is the case when 10^{-6} M carbachol is used as the stimulus (see figs. 4.3. and 4.4.). As a consequence the use of the radioactive isotopes of calcium and magnesium can be omitted in these experiments.

Fig. 5.2A. shows the results of a typical experiment in which

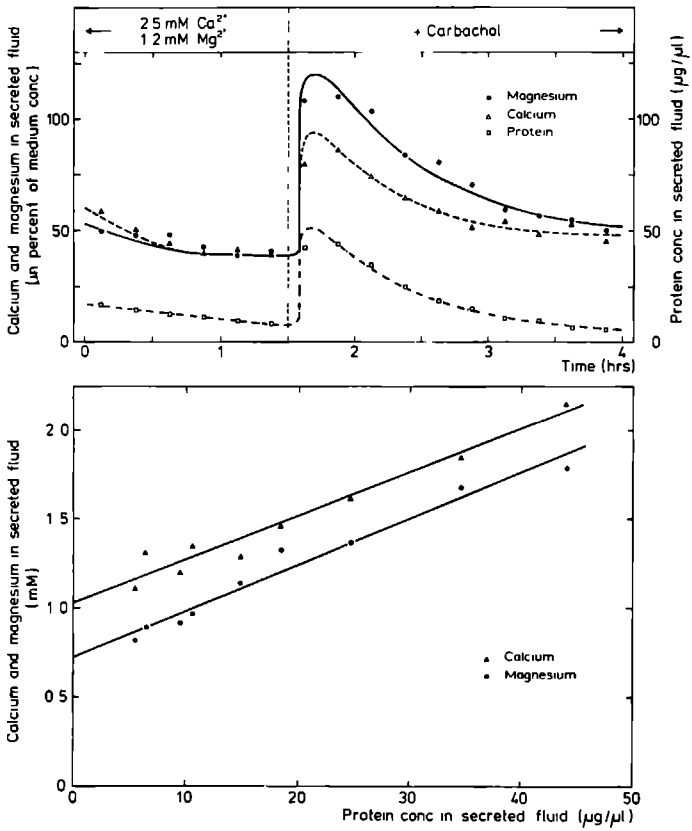

Fig. 5.2. (A) Effects of 10^{-6} M carbachol on the composition of the pancreatic fluid, after stimulation in normal medium. (B) Calculated regression lines for the relation between divalent cation and protein secretion after stimulation. Typical for six experiments.

stimulation is induced by 10^{-6} M carbachol in a normal medium. 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, the relative stimulation of the protein secretion is much larger than that of calcium and magnesium. In fig. 5.2B. the uncorrected concentrations of the divalent cations (y) are plotted vs. the protein concentration (x) for the period beginning 20 min after stimulation, during which the extracellular fluxes remain constant (see figs. 4.3. and 4.4.). A linear relation of the type $y = Ax + B$ is obtained in individual experiments. The intercept B represents the cation concentration of the pancreatic fluid in the absence of any protein and the slope A the amount of protein-associated cations. The calculated parameters relating divalent cation and protein secretion are shown in table 5.2.

When the normal medium is replaced by a calcium and magnesium free medium, there is a marked decrease in the secretion of these divalent cations, while the protein secretion is only slightly affected (fig. 5.3A.) When 10^{-6} M carbachol is then added to the divalent cation-free medium, there is still an increase in the calcium and magnesium secretion simultaneously with the increase in the protein secretion. This indicates that these cations do not originate directly from the medium, but rather from endogenous cation pools. Plotting the concentrations of the divalent cations vs. the protein concentration, as described above, again yields a linear relation for individual experiments (fig. 5.3B.). The calculated

TABLE 5.2.

PARAMETERS RELATING DIVALENT CATION AND PROTEIN SECRETION AT CONSTANT EXTRACELLULAR FLUX

Cation	A	B	r	n
		NORMAL MEDIUM		
calcium	21 ± 2.0	1.11 ± 0.06	0.96 ± 0.02	6
magnesium	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

Parameters of the regression lines calculated according to the least-square method. Mean values with standard errors of the mean. A, the amount of protein-associated cations (nmol per mg protein); B, the cation concentration in the 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.

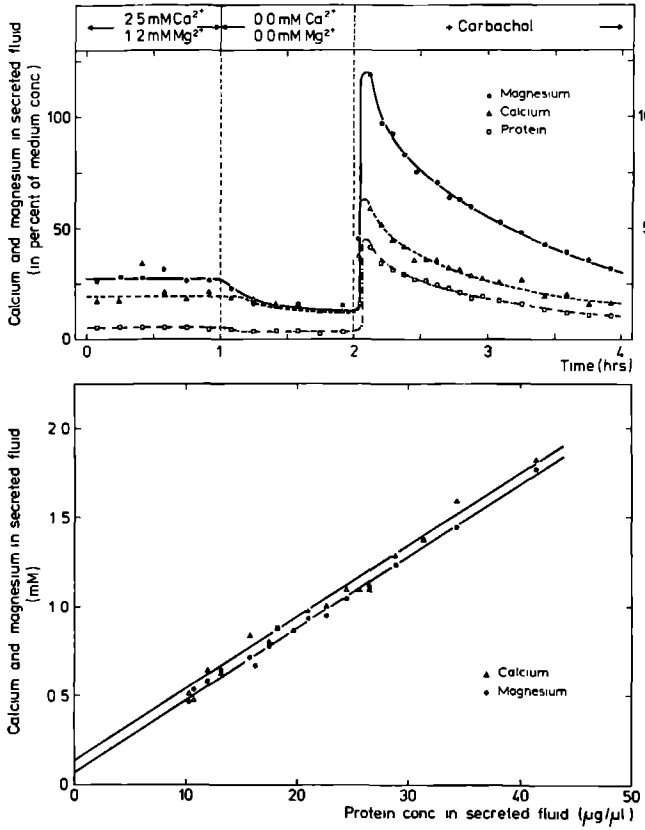

Fig. 5.3. (A) Effects of 10^{-6} M carbachol on the composition of the pancreatic fluid, after stimulation in a divalent cation-free medium. (B) Calculated regression lines for the relation between divalent cation and protein secretion after stimulation. Typical for five experiments.

parameters relating divalent cation and protein secretion are shown in table 5.2. The intercept B representing the cation concentration of the pancreatic fluid in the absence of any protein is now close to zero, while the amounts of protein-associated cations (A) are slightly higher than those found when calcium and magnesium are

present in the medium.

These findings indicate again that the total divalent cation content of the pancreatic fluid is composed of a protein-dependent and a protein-independent component. The value of the protein-independent component strongly depends on the cation concentration in the medium. The protein-dependent component is hardly influenced by the cation concentrations in the medium and amounts to about the same values as determined by means of the correction method described in the previous section.

5.3.3. Gel filtration of the pancreatic fluid

The foregoing experiments clearly show the existence of a protein-associated component in the secretion of divalent cations, originating from an endogenous cation pool. In chapter 4 we have shown that this cation pool poorly exchanges with the cations 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 enzyme proteins but trapped within the zymogen granules.

In order to distinguish between these two possibilities, we have subjected samples of the pancreatic fluid to gel filtration. This procedure separates freely diffusible and protein-bound cations. The elution patterns (fig. 5.4.) show that the divalent cations are virtually completely separated from the bulk protein. Only a small fraction of the calcium (about 20 % of the protein-associated calcium) remains associated with the protein, while magnesium is completely separated. The fraction of protein-asso-

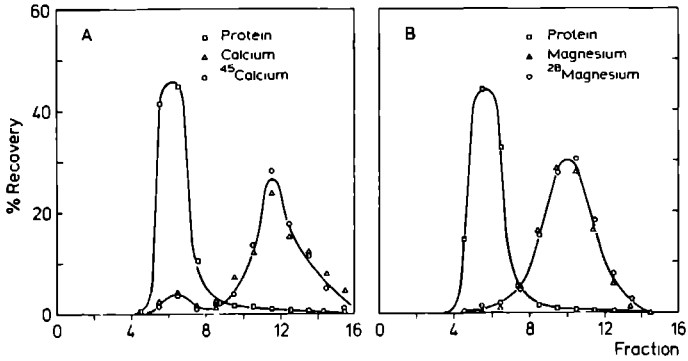

Fig. 5.4. Gel filtration on Sephadex G-25 (coarse) of samples of the pancreatic fluid. Radioactive tracers are added to the samples just before their application to the column. (A) Elution pattern of samples to which $^{45}Ca^{2+}$ is added. (B) Elution pattern of samples to which $^{28}Mg^{2+}$ is added. Mean values of three experiments.

ciated calcium can be reduced by using longer columns. The easy exchangeability of the cations in both fractions is further illustrated by the fact that both calcium and magnesium are completely equilibrated with their tracers, added to the samples just before elution.

5.4. Discussion

In this chapter we have further examined the quantitative aspects of the calcium and magnesium secretion in relation to the secretion of digestive enzymes. The qualitative studies, presented in chapters 3 and 4, have shown that the divalent cations in the secreted fluid are derived from two distinct sources: from the incubation medium via the extracellular flux and from an endogenous cation pool via the secretory flux. In addition it has been

shown that radioactive tracers, present in the medium, rapidly exchange with the extracellular flux, while such an exchange is not observed for the secretory flux. Thus when radioactive calcium and magnesium are present in the medium, the divalent cation flux into the pancreatic fluid will consist of a labeled and a non-labeled fraction. Both fluxes increase upon stimulation of the enzyme secretion (chapter 3). The increase in the extracellular flux depends rather strongly on the applied dose of the stimulant, but it is not linearly related to the secreted amount of digestive enzymes (chapter 4). In this chapter we have examined whether or not a linear relation exists for the secretory flux.

Correction of the total divalent cation flux for the variable contribution of the extracellular flux by subtraction provides the secretory flux. A linear relation is obtained when the secretory flux is plotted against the protein concentration in the pancreatic fluid (fig. 5.1B.). The absence of a marked intercept on the cation-axis suggests that the non-labeled cation fraction derives from a single intracellular pool. The slope indicates the existence of a constant ratio of 20 nmol of both calcium and magnesium associated per mg protein, independent of the stimulation conditions.

The determination of the amounts of protein-associated cations is simpler when there is a constant extracellular flux. This condition exists when 10^{-6} M carbachol or 20 U pancreozymin is used as the stimulus, as described in chapter 4. In that case correction for the extracellular flux with the aid of radioactive tracers is superfluous. Plotting the uncorrected divalent cation concentra-

tions (y) vs. the protein concentrations (x) yields a linear relation of the type $y = Ax + B$ for both cations in individual experiments. This means that there is a variable fraction of protein-associated cations in addition to a constant protein-independent fraction. The contribution of the secretory flux (A) amounts to 21 (SE 2.0, n = 6) nmol calcium and 24 (SE 2.8, n = 6) nmol magnesium per mg protein. These values agree fairly well with the values found with the correction method, which amount to 21 (SE 0.9, n = 3) nmol calcium and 20 (SE 4.0, n = 3) nmol magnesium per mg protein.

The protein-independent component (B) amounts to 1.11 (SE 0.06, n = 6) mM calcium and 0.69 (SE 0.08, n = 6) mM magnesium. These B values cannot be meaningfully compared to those from the correction method, since there the contribution of the extracellular flux has been brought to zero by the applied correction. However, it is possible to compare them to corresponding values for the tracer experiments with 10^{-6} M carbachol, which can be derived from the data in tables 4.1. and 4.2. by converting the relative tracer levels to absolute values. The resulting extracellular fluxes are 0.78 (SE 0.17, n = 3) mM calcium and 0.30 (SE 0.02, n = 3) mM magnesium. The lower values in the latter case may be due to the relatively high flow rates observed in these experiments, since we have previously observed that cation concentrations in the pancreatic fluid increase at decreasing flow rates (section 3.3.1.). If we calculate the B values in these experiments by the direct regression line method, we find good agreement with the extracellular flux values just mentioned: 0.73 (SE 0.10, n = 3) mM calcium

and 0.41 (SE 0.06, n = 3) mM magnesium. This confirms once again the validity of this method for determining the two types of cation fluxes under conditions of constant extracellular flux.

When stimulation is carried out in a divalent cation-free medium, again a linear relation is found. The amounts of protein-associated calcium and magnesium in this case are 31 (SE 3.1, n = 5) and 33 (SE 3.9, n = 5) nmol per mg protein, respectively. These values are hardly different ($0.05 < P < 0.1$) from the corresponding values in the preceding experiments, indicating that the cation content of the bathing medium does not greatly influence the amount of protein-associated cations. This finding is in agreement with the observed absence of exchange between these cation pools and the corresponding radioisotopes in the medium (see figs. 3.1. and 4.1.). On the other hand, the extracellular fluxes (B) in a divalent cation-free medium are close to zero: 0.19 (SE 0.05, n = 5) mM calcium and 0.14 (SE 0.05, n = 5) mM magnesium, indicating that the extracellular flux depends directly on the concentration of the cation in the bathing medium. The very small residual protein-independent flux in a divalent cation-free medium may be due to leakage of divalent cations from the tissue. These experiments clearly indicate that the protein-independent flux strongly depends on the presence of divalent cations in the medium, while this is not the case for the protein-associated component. This again strongly supports our conclusion that the cations are derived from different cation pools.

Our values for the amounts of protein-associated divalent cations are not much different from those of Rutten (1974), who

found 42 nmol calcium and 33 nmol magnesium per mg protein after stimulation with different types and doses of drugs. Ceccarelli et al (1975) reported a value of about 50 nmol calcium per mg protein for guinea pig, while Sullivan et al (1974) found much lower values for pig: 3.3 nmol calcium and 1.3 nmol magnesium per mg protein.

Gel filtration of the pancreatic fluid shows that the divalent cations can be virtually completely separated from the proteins. In addition, there is a complete exchange of cations with their tracers, added just before elution, indicating that the protein-associated cations are rapidly exchangeable after their secretion. This indicates that the absence of any exchange before and during secretion is not due to an irreversible binding of the divalent cations to the enzyme proteins, but rather to a trapping of the cations inside the zymogen granules. Since this sequestration ceases after secretion, the question arises why exchange does not occur 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 the medium concentrations. This may be because the time for the divalent cations to equilibrate with the bathing medium (about 30 min, see fig. 3.1.) is relatively long with respect to the time that the secretory products stay within the ductular system (about 5 min, see fig. 3.1.).

The value of the protein-associated cations of 20 - 30 nmol per mg protein is equivalent to only one mol of each cation per mol enzyme protein at an average assumed protein molecular weight

of 30.000 - 50.000. The physiological significance of these rather small amounts of protein-associated cations is unclear. Calcium may have an effect on the ultimate activity of the digestive enzymes, as already discussed in section 4.4. There is also some evidence that calcium may be bound to the granule membranes (Clemente and Meldolesi, 1975). And finally both cations may play a role in packing the enzyme proteins in the granules through neutralization of surface charges of the proteins. The protein-associated cations do not exchange with cations present in the medium, which is most probably due to a sequestration within the zymogen granules. This phenomenon will be studied in detail in the next chapter.

Notwithstanding the fact that the secretory calcium flux increases in parallel with the secreted enzymes upon stimulation, it is unlikely that this flux plays a specific role in the stimulus-secretion coupling of pancreatic enzyme secretion, since the secretory magnesium flux behaves in a complete similar way.

CHAPTER 6

EXCHANGEABILITY OF THE SECRETORY CALCIUM POOL

6.1. Introduction

The secreted fluid of the exocrine pancreas contains, depending on its physiological condition, variable amounts of calcium and magnesium, which are provided by at least two distinct cation fluxes. At the basal secretion rate divalent cation secretion is mainly due to the extracellular flux directly originating from the extracellular fluid through the "tight junctions" of adjecent cells. The contribution of the secretory flux is of major importance only upon stimulation of pancreatic enzyme secretion. The amounts of cations in the latter flux are directly proportional to the amounts of enzyme proteins secreted and derive from an endogenous pool, which in our experiments does not exchange with tracer ions in the medium. The lack of exchange is, however, not due to irreversible binding of divalent cations to the enzymes, since the ability to exchange is regained after secretion. These findings have led us to assume that the endogenous cation pools lose their ability to exchange during the intracellular pathway of digestive enzymes in the pancreatic acinar cells (section 1.1.4.).

In order to investigate this matter, we have determined the TCA-insoluble ^{3}H-radioactivity in the pancreatic fluid after addition of "carrier-free" ^{3}H-Leucine to the normal incubation medium.

The isolated pancreas appears to retain the ability to synthesize digestive enzymes though at a relatively low rate. The rate of synthesis can be increased by addition of amino acids to the medium. The specific activity of newly synthesized (labeled) proteins is, however, still very low, which must be due to the large bulk of non-labeled proteins stored in the zymogen granules. In order to overcome this problem, we have first removed the stored, non-labeled material by an early stimulation. Later stimulation is then expected to cause the secretion of products with a higher specific activity. Comparison between the secretion of radioactive protein-associated calcium with that of ^{3}H-labeled enzyme proteins suggests that at 35 - 50 min after synthesis of these enzymes the calcium ions secreted along with these enzymes lose their ability to exchange with other calcium ions present in the tissue and the medium. This suggests that sequestration of the calcium ions takes place during conversion of the condensing vacuoles to mature zymogen granules.

6.2. Materials and Methods

The materials, the preparation of the rabbit pancreas, the incubation medium, the incubation and sample collection procedures and the assay methods are described in chapter 2.

6.3. Results

6.3.1. Basal secretion of newly synthesized enzyme proteins

In our previous studies with the isolated rabbit pancreas we have always incubated the organ in a medium without amino acids.

In order to obtain information about the secretion of newly synthesized proteins under these circumstances, we have added "carrier-free" ^{3}H-Leucine to the incubation medium and have analyzed the secreted fluid for TCA-insoluble ^{3}H-radioactivity. The amount of radioactive enzyme protein is calculated from the ^{3}H-Leucine specific activity in the medium, since it is known that extracellular amino acids are preferentially used for protein synthesis (Van Venrooij et al, 1972) and that leucine contributes about 6 % of the total weight of the enzymes (Rothman and Isenberg, 1974).

Detectable amounts of the tracer appear after about one hour in the TCA-precipitate and thereafter increase continuously at basal protein secretion. The secreted amount of radioactive protein, however, remains extremely low. In an attempt to increase this amount we have added a complete amino acid mixture (Eagle, 1959) to the medium. The secreted amount of radioactive protein then increased at least thousandfold, although it still amounts to only 2 % of the total protein after a 2-h incubation. The continuous increase of the specific activity at the basal secretion rate indicates that both the synthesis and the intracellular transport of enzyme proteins are maintained in the isolated rabbit pancreas.

6.3.2. Effects of stimulation on the secretion of newly synthesized enzyme proteins

Stimulation of pancreatic enzyme secretion with 10^{-6} M carbachol after 2-h incubation causes a normal increase of the protein secretion, but has no marked effect on the secretion of TCA-inso-

luble ^{3}H-radioactivity. This preferential secretion of endogenous and non-labeled enzyme proteins hinders the study of the association of $^{45}Ca^{2+}$ ions to the newly synthesized enzyme proteins. We have, therefore, first removed the bulk of this non-labeled material by an early stimulation. A second stimulation after 2 h. incubation is then meant to lead to secretion of protein with higher specific activity.

In chapter 3 we have, however, reported that a second dose of acetylcholine 80 min after the first one (which has not been removed) does not cause a marked enzyme secretion (fig. 3.3.). In order tφ improve this situation we have increased the time between the first and the second stimulus to 2 hrs and have washed out the carbachol from the first stimulation. The results of these experiments are described in the next two sections.

6.3.3. Effects of repeated stimulation on the secretion of newly synthesized enzyme proteins

The total incubation period of the isolated rabbit pancreas is divided in five distinct periods in which the composition of the incubation medium is changed. The changes indicated in the figures are made at the beginning of each period. Two different types of experiments have been carried out. In the first type normal medium is used (fig. 6.1.) and in the second type a normal medium to which an amino acid mixture (Eagle, 1959) is added (fig. 6.2.). The total protein output during half-hour periods is listed in table 6.1.

At the start of the experiments, $^{45}Ca^{2+}$ and ^{3}H-Leucine are

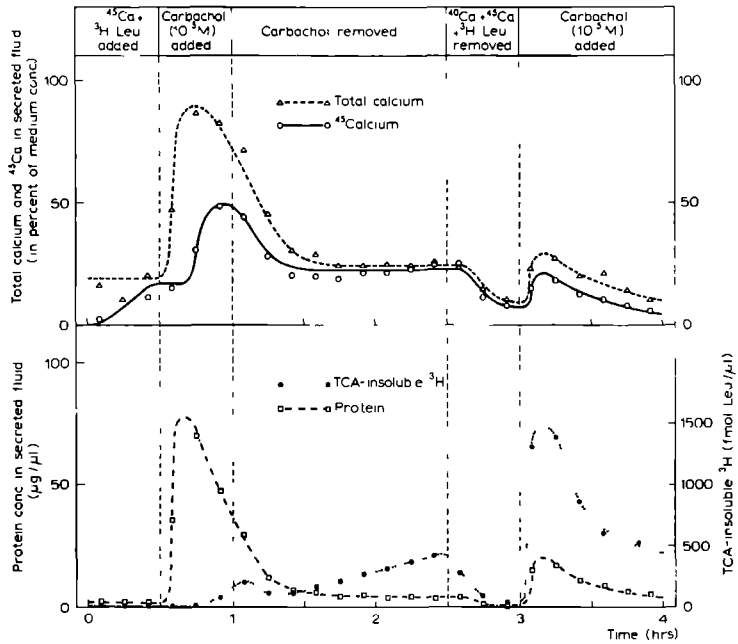

Fig. 6.1. Effects of repeated stimulation on the specific activity of secretory products in the absence of amino acids in the medium. Typical for three experiments.

added to the bathing medium. The secretion of $^{45}Ca^{2+}$ is described extensively in section 3.3.1., and will thus be summarized here. Secretion of $^{45}Ca^{2+}$ into the pancreatic fluid begins immediately and its specific activity becomes constant and equal to that of the medium in about 30 min. The total calcium concentration amounts to only 30 % of that in the bathing medium. During this period protein is secreted at the basal rate, while no ^{3}H-radioactivity is detectable in the TCA-insoluble fraction of the secreted fluid.

Stimulation of pancreatic enzyme secretion with 10^{-5} M carbachol after half an hour causes a normal response of the protein

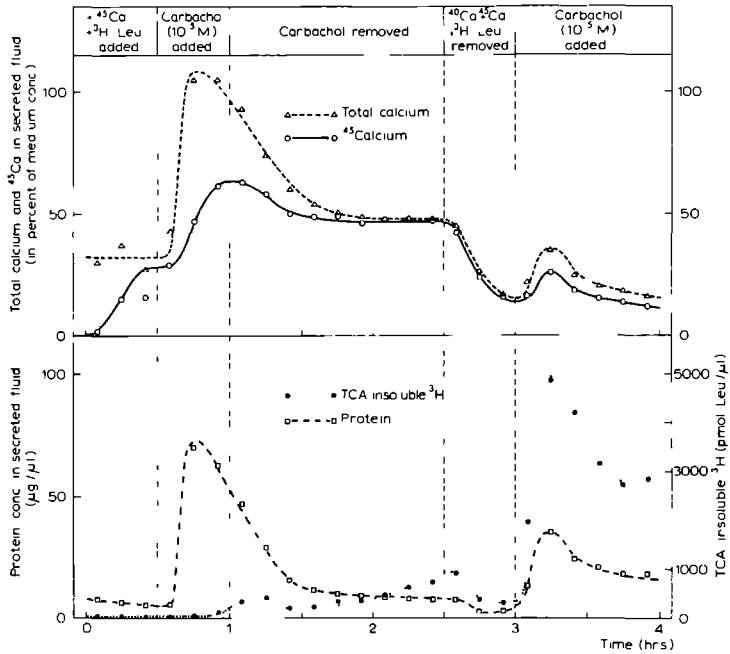

Fig. 6.2. Effects of repeated stimulation on the specific activity of secretory products in the presence of amino acids in the medium. Typical for three experiments.

secretion (cf. tables 3.1. and 6.1.), but with little effect on the secretion of TCA-insoluble ^{3}H-radioactivity, either with or without added amino acids (figs. 6.1. and 6.2.). The protein concentration in the secreted fluid reaches a maximum value in about 10 min and the same is true for the total calcium secretion. The $^{45}Ca^{2+}$ secretion also increases but behaves completely different from the total calcium secretion. Its maximum is delayed with respect to the total calcium secretion and is lower than that for the latter. We have shown in chapter 4 that this is due to the

TABLE 6.1.

PROTEIN OUTPUT OF THE ISOLATED RABBIT PANCREAS

Half-hour period	Amino acids absent	Amino acids present
1	1.7 ± 0.54	1.2 ± 0.22
2	12.3 ± 2.96	10.3 ± 0.59
3	4.8 ± 0.48	6.5 ± 1.21
4	1.5 ± 0.31	1.6 ± 0.22
5	1.0 ± 0.17	1.2 ± 0.48
6	0.5 ± 0.11	0.9 ± 0.17
7	3.9 ± 1.03	5.8 ± 1.10
8	1.4 ± 0.44	4.3 ± 0.89

Mean values (mg/½h) with standard errors of the mean for three consistent experiments.

fact that the secretion of $^{45}Ca^{2+}$ represents only the secretion of calcium via the extracellular flux. The initial decrease of the specific activity of calcium in the secreted fluid is due to the secretion of endogenous non-labeled calcium via the secretory flux (chapter 5). The secretion of TCA-insoluble ^{3}H-radioactivity becomes apparent after about 1-h incubation.

The third period is meant to achieve maximal de novo synthesis of enzyme proteins. The stimulant is washed out to enable an affective second stimulation later on. During this period the secretion patterns of $^{45}Ca^{2+}$, total calcium and protein reach relatively constant levels. The relative levels of total and

radioactive calcium become equal but are slightly higher than before stimulation. The amount of TCA-insoluble ^{3}H-radioactivity decreases transiently, probably due to the removal of the stimulant, but increases thereafter again continuously.

In the fourth period all calcium, incl. $^{45}Ca^{2+}$, and ^{3}H-Leucine are removed from the medium. The radioactive calcium is removed in order to exclude interference of radioactive protein-associated calcium with that of the extracellular flux upon the stimulation in the fifth period. ^{3}H-Leucine is removed in order to obtain the same length of incubation period for both tracers. The secretion of the calcium isotopes is reduced due to decrease of its extracellular flux, which depends directly on the calcium concentration in the medium (chapter 4). The protein secretion is slightly decreased, an effect which is also visualized by the reduction of the secreted amount of TCA-insoluble ^{3}H-radioactivity.

At the start of the last period a second dose of 10^{-5} M carbachol is applied to the incubation medium. This causes another stimulation of the pancreatic enzyme secretion, which differs rather markedly from that obtained after the first stimulation. The protein secretion is increased, but considerably less than after the first stimulation. There is, however, a large increase in the secretion of TCA-insoluble ^{3}H-radioactivity and the secretion of both calcium isotopes coincides with this increase. Qualitatively the same secretion patterns are observed during incubation in media with and without amino acids present, but the TCA-insoluble ^{3}H-radioactivity is clearly higher in the presence of amino acids. The specific activity of calcium is about equal in both

cases. The quantitative data are presented in table 6.2.

TABLE 6.2.

SPECIFIC ACTIVITY OF SECRETORY PRODUCTS

	^{3}H-protein	$^{45}Ca^{2+}$
Amino acids absent	0.02 + 0.01	66 ± 2.6
Amino acids present	29.0 ± 5.1	71 ± 2.5

Mean values (%) during the hour after the second stimulation with standard errors of the mean for three consistent experiments.

6.3.4. Specific activity of calcium in relation to the incubation period

The results reported in the previous section indicate that the protein secretion after the second stimulation coincides in time with the secretion of TCA-insoluble ^{3}H-radioactivity and of both calcium isotopes. This suggests that these parameters are now interrelated. However, the specific activity of the enzyme proteins amounts to only 29 %, while the specific activity of calcium amounts to 71 %, although the amount of protein-associated calcium is the same as previously observed (section 5.3.2.). This indicates that radioactive calcium ions are associated with non-radioactive enzyme proteins, suggesting that the sequestration of protein-associated calcium ions does not take place during the synthesis of the enzyme proteins, but rather during a later phase of their intracellular transport.

In order to investigate this matter, we have carried out a

set of experiments similar to those described in the previous section, but in which $^{45}Ca^{2+}$ is added to the medium at different time intervals after the addition of ^{3}H-Leucine. The mean specific activity of the enzyme proteins during the hour after the second stimulation does not change, but the calcium specific activity decreases with increasing time intervals. The results presented in fig. 6.3. indicate that the specific activities of the enzyme proteins and the protein-associated calcium become equal, when the

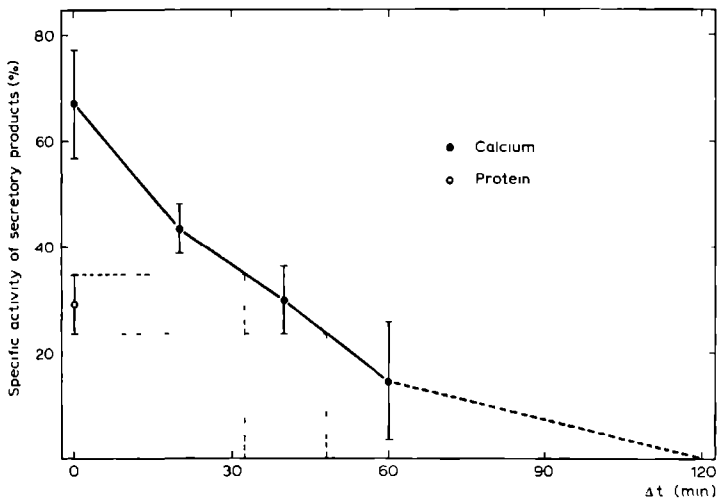

Fig. 6.3. Specific activity of calcium as a function of the time interval between addition of ^{3}H-leucine and $^{45}Ca^{2+}$. Mean values with standard errors of the mean during the hour after the second stimulation.

$^{45}Ca^{2+}$ is added to the medium 35 - 50 min after addition of the ^{3}H-Leucine. These findings suggest that at this time interval after the synthesis of the enzyme proteins the protein-associated calcium ions, secreted along with these enzymes, lose their ability to exchange with other calcium ions present in the tissue and

in the medium. This indicates that the most likely phase of the intracellular transport of digestive enzymes, during which immobilization of calcium ions takes place, is the conversion of the condensing vacuoles to mature zymogen granules.

6.4. Discussion

The enzyme proteins in the pancreatic fluid are accompanied by a constant amount of calcium and magnesium ions. These protein-associated divalent cations, constituting the secretory flux, do not normally exchange with radioactive tracers present in the incubation medium, despite the fact that the tissue accumulates both tracers. This lack of exchange is not due to irreversible binding of the divalent cations to the enzyme proteins, since the cations regain the ability to exchange after their secretion (section 5.3.3.). These findings have led us to the conclusion that the protein-associated divalent cations are sequestered during the intracellular transport of the enzyme proteins in the acinar cells. A somewhat analogous situation occurs in the glycosylation of glycoproteins during intracellular transport of secretory products in the salivary gland (Schachter, 1974). Autoradiographic and cell fractionation studies suggest that glycosylation of mucoproteins occurs predominantly in the Golgi apparatus.

In order to obtain information about the sequestration of the protein-associated calcium ions, we have compared the secretion of $^{45}Ca^{2+}$ with that of ^{3}H-labeled enzyme proteins after adding to the medium $^{45}Ca^{2+}$ and an amino acid mixture including ^{3}H-Leucine. During 2-h incubation the basal secretion of radioactive enzyme

proteins increases to 2 % of the total protein content in the pancreatic fluid, which suggests that synthesis and intracellular transport of enzyme proteins still occur in the isolated pancreas.

Stimulation with 10^{-6} M carbachol after 2-h incubation causes a normal protein response, without a marked effect on the secretion of radioactive enzyme proteins. This indicates a preferential secretion of non-labeled protein from the granules still present in the acinar cells. Since there is still synthesis of enzyme proteins occurring, the secretion of newly synthesized proteins is expected to be favoured when stimulation is preceded by depletion of the non-labeled protein stores. Such experiments have been carried out in the absence and the presence of amino acids in the medium (section 6.3.3.).

In these experiments the composition of the incubation medium is changed in order to improve protein secretion after the second stimulation and thus to permit a good comparison between the secretion of $^{45}Ca^{2+}$ and ^{3}H-labeled enzyme proteins. Table 6.1. shows that the presence of amino acids in the medium has no marked effect on the amount of enzyme proteins secreted during and after the first stimulation. The protein output after the second stimulation seems to be somewhat higher in the presence of amino acids, suggesting that the first stimulation does not completely deplete the intracellular protein stores.

The data on the specific activities of the secretory products are presented in table 6.2. Although the specific activity of the enzyme proteins is at least a 1000-fold higher in the presence of amino acids, the total protein output and the specific activi-

ty of calcium are not much different in the two cases. This indicates that the digestive enzymes are in both cases secreted from pools of about equal size, which are labeled with $^{45}Ca^{2+}$ to the same extent. This observation suggests that the first stimulation causes a mixing of "older" and "younger" secretory products in one phase of their intracellular transport, which results in a more homogenous pool with respect to the age of the secretory products. In the presence of amino acids there will then be a considerable mixing of labeled and non-labeled proteins. After stimulation the intracellular transport of secretory products will continue at the normal rate. This could explain why the total protein secretion is about equal in both cases, but the specific activity of the enzyme proteins is considerably higher in the presence than in the absence of amino acids in the medium.

The equal labeling of the secreted enzyme proteins with $^{45}Ca^{2+}$ can now be explained by the fact that this labeling takes place during a later phase of the intracellular transport. In an attempt to determine at which phase this happens we have followed the decrease in the specific activity of calcium upon later addition of $^{45}Ca^{2+}$. In the experiments, described in section 6.3.4., we have added $^{45}Ca^{2+}$ to the medium at increasing time intervals after the addition of ^{3}H-Leucine, and determined the mean specific activity of the secretory products during the hour following the second stimulation. The specific activity of the protein remains constant, but the specific activity of calcium decreases with increasing time interval. The results presented in fig. 6.3. indicate that the two specific activities do not differ significantly, when

the $^{45}Ca^{2+}$ is added between 35 and 50 min after the addition of ^{3}H-Leucine to the medium. This suggests that at 35 - 50 min after the synthesis of the enzyme proteins the calcium ions, which are later secreted in association with these enzymes, lose the ability to exchange with other calcium ions present in the tissue and in the medium. The studies on the timing of the intracellular transport in the exocrine pancreas of guinea pig (Jamieson and Palade, 1967) and frog (Slot et al, 1974) indicate that the most likely phase of the intracellular pathway of the digestive enzymes, during which sequestration of the calcium ions takes place, is the conversion of the condensing vacuoles to mature zymogen granules. Autoradiographic studies are in progress in order to confirm this conclusion.

CHAPTER 7

THE STIMULATORY CALCIUM FLUX

7.1. Introduction

In chapter 3 we have reported studies of the calcium movements, before and after stimulation, aimed at obtaining information about the role of calcium in exocrine pancreatic secretion. Three distinct calcium fluxes after cholinergic stimulation of pancreatic enzyme secretion are found. (fig. 3.6.). The involvement of calcium ions in the stimulus-secretion coupling may be reflected by one of these fluxes.

Two of the calcium fluxes, the extracellular and the secretory flux, are directed to the secretory compartment. In chapters 4 and 5 we have examined these fluxes in closer detail and have concluded that notwithstanding the fact that they are increased upon stimulation, it is unlikely that they play a specific role in the stimulus-secretion coupling, since completely similar fluxes are found to exist for magnesium.

The third flux, indicated as the stimulatory calcium flux, is only observed in pancreas fragments. When these fragments are pre-loaded with $^{45}Ca^{2+}$ and then stimulated, there is a transiently increased $^{45}Ca^{2+}$ efflux (fig. 3.5.). This increased efflux is presumably mainly localized over the basal membrane, and therefore unlikely to be associated with the enzymes secreted over the lu-

minal membrane. An increased efflux of $^{45}Ca^{2+}$ ions over the basal membrane can be explained by either of two mechanisms: an increase in the calcium permeability of the membrane or an increase in the $^{45}Ca^{2+}$ ions over the basal membrane can be explained by either of two mechanisms: an increase in the calcium permeability of the membrane or an increase in the $^{45}Ca^{2+}$ from an intracellular store. Both mechanisms would normally cause an increase in the cytoplasmic calcium concentration, which could induce pancreatic enzyme secretion.

In order to obtain more insight in the involvement of the stimulatory calcium flux in the stimulus-secretion coupling of pancreatic enzyme secretion we have first examined its specificity by a study of the effects of stimulation on the $^{28}Mg^{2+}$ efflux. A comparison of the simultaneous effects of carbachol and the ionophore A-23187 on the efflux of $^{45}Ca^{2+}$ and amylase from rabbit pancreas fragments pre-loaded with $^{45}Ca^{2+}$ has also been carried out.

It is concluded that the stimulatory calcium flux reflects an increased cytoplasmic calcium concentration due to a release of calcium from an intracellular store, which may play an essential and specific role in the stimulus-secretion coupling of pancreatic enzyme secretion.

7.2. Materials and Methods

The materials, the preparation of the rabbit pancreas, the incubation medium, the incubation and sample collection procedures and the assay methods are described in chapter 2.

7.3. Results

7.3.1. $^{45}Ca^{2+}$ and $^{28}Mg^{2+}$ efflux studies with pancreas fragments

In chapter 3 it is shown that the so-called stimulatory calcium flux is mainly localized over the basal membrane. Since this flux cannot be studied properly in the isolated rabbit pancreas, we have used rabbit pancreas fragments, which permit simultaneous measurements of the secretion to the stimulatory and the secretory compartments of the tissue.

The pancreas fragments are pre-loaded with $^{45}Ca^{2+}$ for 2-h, washed and then transferred to a series of plastic counting vials with tracer-free medium in order to determine the $^{45}Ca^{2+}$ efflux rate. Fig. 7.1. shows a continuously decreasing $^{45}Ca^{2+}$ efflux rate during the resting state, reaching a relatively constant value after about 2 h.

When 10^{-5} M carbachol is added to the efflux medium, there is an immediate and large increase in the $^{45}Ca^{2+}$ efflux rate, confirming the experiments described in section 3.3.5. 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 affected at all, but remains relatively constant during the rest of the experiment (fig. 7.1.). In both experiments there is the usual increase in the amylase release by carbachol (see fig. 3.5.). The absence of a stimulatory magnesium

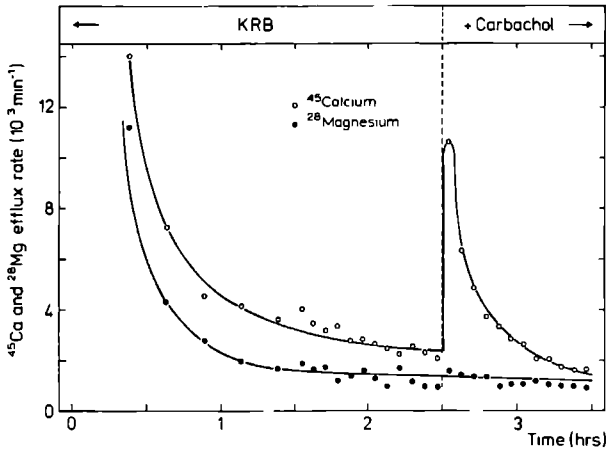

Fig. 7.1. Effects of carbachol (10^{-5} M) on the $^{45}Ca^{2+}$ and $^{28}Mg^{2+}$ efflux from pre-loaded rabbit pancreas fragments. Mean values of four experiments.

flux strongly suggests that the increase of the cytoplasmic calcium concentration is a specific event in the stimulus-secretion coupling of pancreatic enzyme secretion.

7.3.2. Calcium requirement of stimulation

In this section we have examined whether or not the presence of divalent cations in the medium is required for the stimulatory action of the divalent cation ionophore A-23187 (fig. 7.2.) and carbachol. Fig. 7.3. shows that the basal enzyme secretion of the isolated rabbit pancreas is slightly reduced upon replacing the normal KRB-medium with one lacking Ca^{2+} and Mg^{2+} and to which 10^{-4} M EGTA (ethylene glycol bis (-aminoethyl ether)-N,N'-tetraacetic acid) is added. This medium is called "EGTA-medium". The calcium secretion is strongly diminished, confirming our pre-

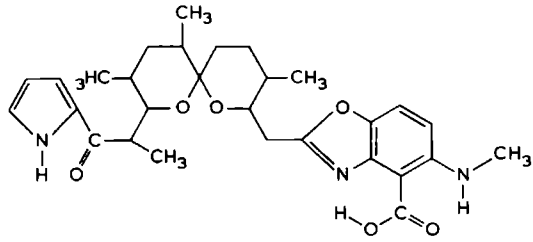

Fig. 7.2. Chemical structure of the divalent cation ionophore A-23187.

vious finding that the basal calcium secretion is mainly dependent on the calcium concentration in the bathing medium. Addition of 10^{-6}M ionophore to the EGTA-medium has only minor effects on the protein and calcium content of the pancreatic fluid. Subsequent incubation in a normal ionophore-containing medium yields an increase in enzyme secretion as well as in the total calcium secretion (fig. 7.3A.). The latter increase seems to be faster than in the control experiment (fig. 7.3B.), which can be explained by the fact that in this case there is in addition to the extracellular flux a contribution of the protein-associated secretory calcium flux.

When towards the end of the experiment magnesium is omitted from the incubation medium the stimulation of protein and calcium secretion remains unchanged. When, however, calcium instead of magnesium is omitted from the medium, no increase in protein and calcium secretion is observed.

When 10^{-5} M carbachol is added to the EGTA-medium, an immediate increase in both protein and calcium secretion is observed.

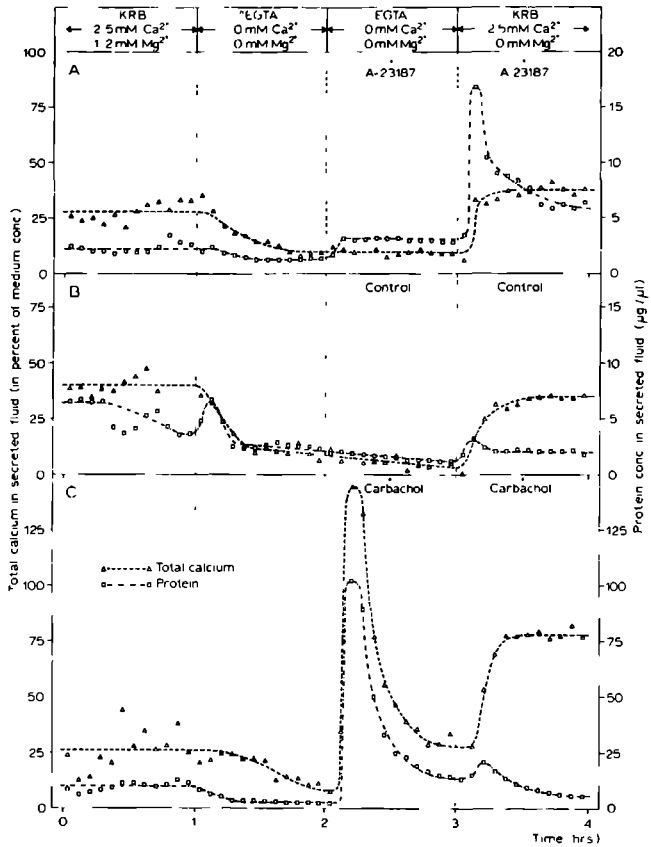

Fig. 7.3. Protein and calcium contents of the pancreatic fluid. The isolated organ is incubated in KRB-medium during the first and the fourth hour and in EGTA-medium during the second and the third hour of the experiment. During the third and the fourth hour 10^{-6} M ionophore A-23187 (A) and 10^{-5} M carbachol (C) are added, while no additions have been made in the control experiment (B).

Subsequent incubation in a normal carbachol containing medium does not give an additional increase in enzyme secretion, but the calcium concentration increases again, to a higher level even than before stimulation. This is however normal after stimulation (section 3.3.2.).

The changing of the media in the absence of a stimulus also results in changes in the protein and calcium secretion (fig. 7.3B.). The effects on the protein secretion are relatively small, while those on the calcium secretion can be attributed to the relation between the extracellular calcium flux and the calcium concentration in the medium.

These findings suggest that the need for the presence of calcium in the medium is much less pronounced in the case of carbachol than of the ionophore in order to obtain stimulation of pancreatic enzyme secretion. Control experiments show that the small amounts of acetone and ethanol added with the ionophore solution do not affect the protein and calcium secretion.

7.3.3. Comparison of the secretory effects of carbachol and A-23187

The experiments described in the previous section suggest that the ionophore stimulates pancreatic enzyme secretion by introducing extracellular calcium into the cytoplasm, as seems to be the case in several other secretory processes (Rubin, 1970). In addition it is shown that calcium cannot be replaced by magnesium in this respect. The experiments in section 7.3.1. indicate that cholinergic stimulation also causes a specific increase in the cytoplasmic calcium concentration. To determine the origin of this

calcium we have investigated whether the transiently increased $^{45}Ca^{2+}$ efflux is mainly due to an increase in the calcium permeability of the basal membrane or rather to an increase in the $^{45}Ca^{2+}$ from an intracellular store. Therefore we have tested the effects of carbachol and the ionophore A-23187 in parallel efflux experiments on rabbit pancreas fragments.

Since the effect of the ionophore on the enzyme secretion is more pronounced when it is added to a divalent cation-free medium and calcium is added thereafter (Eimerl et al, 1974), we have applied this procedure to compare the effects of carbachol and the ionophore. These experiments also supply information about the influence of extracellular calcium and magnesium on the efflux rates of $^{45}Ca^{2+}$ and amylase.

Addition of 10^{-5} M carbachol 30 min after changing from the normal medium to the medium lacking calcium and magnesium still causes a considerable increase in both effluxes (fig. 7.4.). When after another 30 min the medium is replaced again by the normal medium, an additional increase in amylase secretion is noticed, but the $^{45}Ca^{2+}$ efflux rate is slightly diminished. When 30 min after the addition of the carbachol a medium lacking only calcium, instead of a complete medium, is added, no extra increase of the amylase release is observed (fig. 7.4B.). Omission of magnesium gives essentially the same efflux patterns as obtained in a complete medium (fig. 7.3C), indicating that magnesium is not needed for maintaining the amylase response.

Addition of 10^{-6} M ionophore A-23187, 30 min after replacing the normal medium by a medium lacking calcium and magnesium, leads

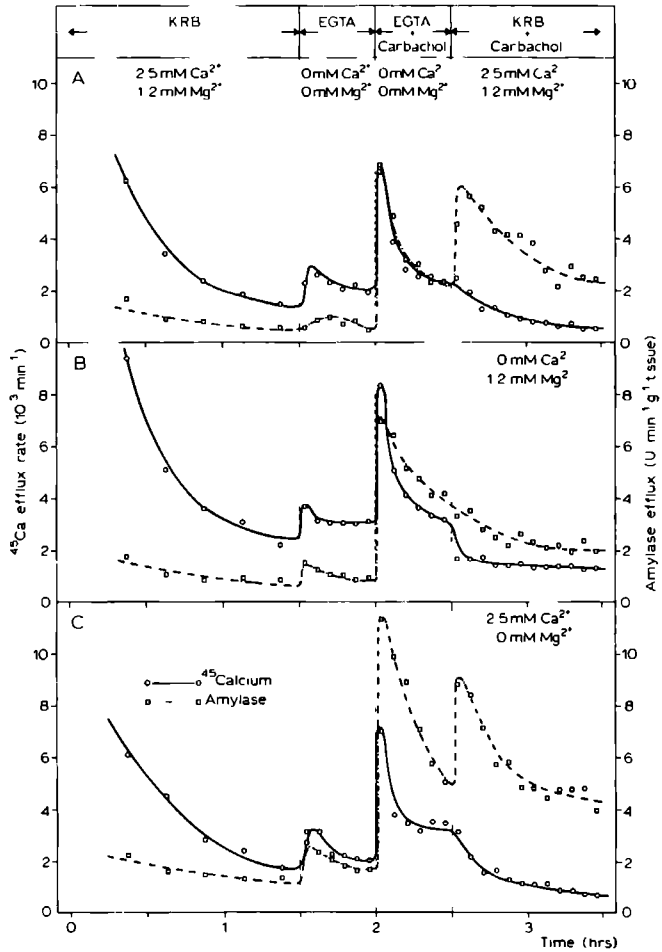

Fig. 7.4. Effects of carbachol (10^{-5} M) on the $^{45}Ca^{2+}$ and amylase efflux from pre-loaded rabbit pancreas fragments in EGTA-medium followed by divalent cation-containing media. The divalent cation contents in the media are: 2.5 mM Ca^{2+} + 1.2 mM Mg^{2+} (A), 0 mM Ca^{2+} + 1.2 mM Mg^{2+} (B) and 2.5 mM Ca^{2+} + 0 mM Mg^{2+} (C), respectively. Mean values of four experiments.

to only minor effects on the $^{45}Ca^{2+}$ and amylase efflux rates (fig. 7.5.). When 30 min after addition of the ionophore the divalent cation free medium is replaced by a normal medium (fig. 7.5A) or by a normal medium without magnesium (fig. 7.5C.), a significant increase in the amylase efflux is found, while the $^{45}Ca^{2+}$ efflux rate further decreases like in the control experiment. When calcium is omitted from the medium, no stimulation of the enzyme secretion is found (fig. 7.5B.). This shows that the stimulating effect of the ionophore A-23187 upon returning to a complete medium is due to the presence of calcium ions.

When the efflux medium is changed from a normal medium to one lacking divalent cations, or vice versa, only minor and transient effects are seen on the efflux of $^{45}Ca^{2+}$ and amylase. The presence of 10^{-6} M atropin does not block the stimulation of pancreatic enzyme secretion by the ionophore.

While the pattern of behaviour is consistent for each type of experiment, there is a considerable variability between repeated experiments. E.g. in fig. 7.4. each curve is the average of four experiments, which show a twofold variation in the $^{45}Ca^{2+}$/amylase ratio in the period immediately after addition of carbachol. Likewise, the small amylase peak observed in fig. 7.5A. after addition of the ionophore is not observed in figs. 7.5B and C, although the experimental conditions are the same.

7.3.4. Secretory effects in the presence of Verapamil and D-600

In several systems the calcium-antagonistic compounds Verapamil (α-isopropyl-α-[(N-methyl-N-homoveratryl)-γ-aminopropyl] -

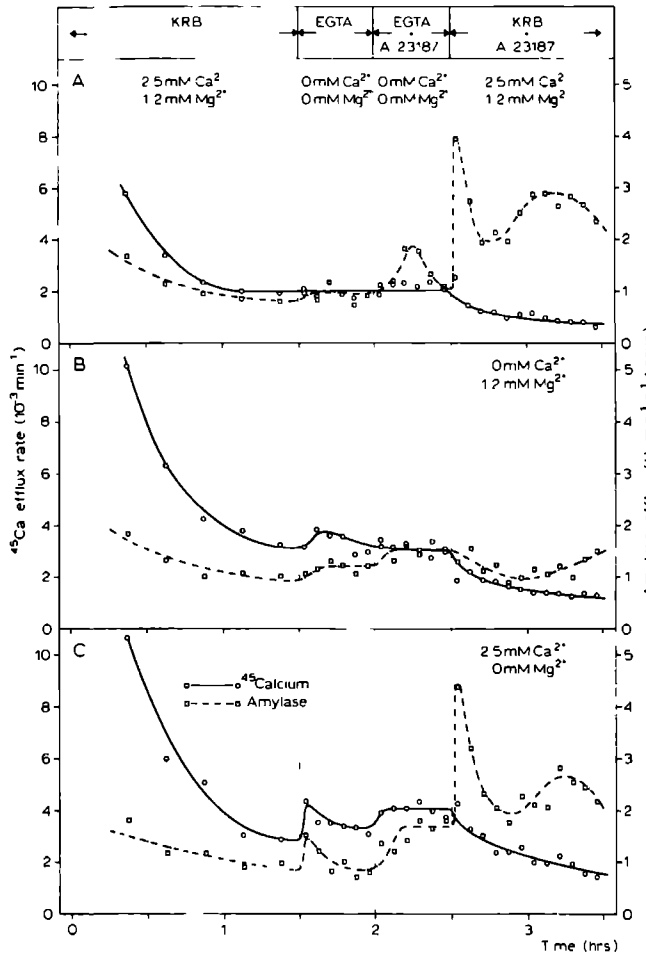

Fig. 7.5. Effects of ionophore A-23187 (10^{-6} M) on the $^{45}Ca^{2+}$ and amylase efflux from pre-loaded rabbit pancreas fragments in EGTA-medium followed by divalent cation containing media. The divalent cation contents in the media are: 2.5 mM Ca^{2+} + 1.2 mM Mg^{2+} (A), 0 mM Ca^{2+} + 1.2 mM Mg^{2+} (B) and 2.5 mM Ca^{2+} + 0 mM Mg^{2+} (C), respectively. Mean values of four experiments.

3,4-dimethoxyphenyl-acetonitril) and its methoxy derivative D-600 block calcium channels in the plasmamembrane, thereby inhibiting processes in which calcium transport through this membrane play a role (Fleckenstein et al, 1971; Russel and Thorn, 1974; Eto et al, 1974). In the isolated rabbit pancreas and in rabbit pancreas fragments pre-incubation with 5×10^{-5} M of either compound does not result in an inhibition of the carbachol effects on enzyme and $^{45}Ca^{2+}$ secretion (results not shown), although this concentration exceeds that able to inhibit Ca^{2+}-induced contractions of the isolated rat uterus (Fleckenstein et al, 1971).

7.4. Discussion

In chapter 3 we have shown that the rabbit pancreas in vitro maintains three distinct calcium movements, all of which are influenced upon cholinergic stimulation. One of these calcium movements, indicated as the stimulatory calcium flux, is reflected by an increased $^{45}Ca^{2+}$ efflux from pancreas fragments, pre-loaded with this tracer. It has also been reported for rat (Case and Clausen, 1973; Heisler, 1974) and mouse pancreas (Matthews et al, 1973). We have been able to establish that this movement of calcium occurs mainly across the serosal membrane and does not accompany the secretion of enzymes across the luminal membrane. The release of $^{45}Ca^{2+}$ can be due either to an increase in the permeability of the serosal membrane or to an increase of the $^{45}Ca^{2+}$ gradient over this membrane as a consequence of a release of the tracer from an intracellular store. Independently of the mechanism involved, this flux seems to indicate that the cytoplasmic calcium

concentration is a specific event in the stimulus-secretion coupling of pancreatic enzyme secretion.

The divalent cation ionophore A-23187, known to facilitate the transport of divalent cations across biological membranes (Reed and Lardy, 1972), has been shown to stimulate pancreatic enzyme secretion (Eimerl et al, 1974). Its action on pancreatic tissue is calcium dependent and leads to an increase in the calcium content (Williams and Lee, 1974). This suggests that a specific increase of the cytoplasmic calcium concentration may be involved in the stimulus-secretion coupling of pancreatic enzyme secretion. The effects of the ionophore on the $^{45}Ca^{2+}$ efflux have not yet been studied. In our study we have investigated, whether the ionophore also mimicks the effects of carbachol on the $^{45}Ca^{2+}$ efflux, in order to obtain more detailed information about the involvement of calcium in the stimulus-secretion coupling. Our experiments clearly show that the stimulating effect of cholinergic agents on pancreatic enzyme secretion can be mimicked by the divalent cation ionophore A-23187, provided calcium is present in the incubation medium. Calcium in this respect cannot be replaced by magnesium, in accordance with studies on rat pancreas (Eimerl et al, 1974). Carbachol, on the other hand, does not require the presence of calcium in the medium. Our observations suggest not only that an increase of the cytoplasmic calcium concentration is a common feature in the action of both stimuli, but they also suggest that these two agents increase the cytoplasmic calcium concentration in different ways.

The action of the ionophore is strongly calcium dependent,

whereas carbachol stimulates even in a medium lacking calcium and magnesium. Moreover, the $^{45}Ca^{2+}$ efflux is scarcely influenced by removal of calcium from the medium, suggesting that the $^{45}Ca^{2+}$ efflux does not involve a calcium exchange mechanism. The ionophore, which facilitates the passive transport of calcium across biological membranes, has little or no effect on the $^{45}Ca^{2+}$ efflux rate, indicating that the cytoplasmic $^{45}Ca^{2+}$ concentration is normally very low. Since at the moment of stimulation the tissue still contains about 50 % of its original $^{45}Ca^{2+}$ content, most of the calcium must be sequestered in intracellular stores.

Carbachol causes even in a medium lacking calcium and magnesium a marked increase in the $^{45}Ca^{2+}$ efflux. Since the cytoplasmic calcium concentration is very low, this effect of carbachol can hardly be explained by an increased permeability of the serosal membrane. The absence of a transiently increased $^{28}Mg^{2+}$ efflux also indicates that an increase of the permeability of the serosal membrane is very unlikely, since magnesium normally occurs in appreciable amounts in the cytoplasm (Brinley and Scarpa, 1975). This means that carbachol releases calcium from an intracellular store, which is apparently not attacked by the ionophore. This conclusion is further supported by the fact that the calcium antagonistic compounds verapamil and D-600 do not inhibit the action of carbachol.

These experiments suggest that an increase in the cytoplasmic calcium concentration is a specific and essential step in the physiological stimulus-secretion coupling and that the calcium originates from an intracellular store.

Extracellular calcium is not directly involved, but may be important for the maintainance of the secretory response. This can be derived from fig. 7.4. which shows that re-addition of calcium after stimulation with carbachol in a medium lacking calcium and magnesium increases the amylase efflux level without affecting the $^{45}Ca^{2+}$ efflux. Similar observations have also been made by Petersen and Ueda (1976). Case and Clausen (1973) have shown that stimulation of pancreatic enzyme secretion is abolished in a calcium-free medium containing 0.5 mM EGTA. Normal responses are however obtained in a medium containing only 0.1 mM calcium. Since we have observed that EGTA adsorbs to the tissue, when this is incubated in a medium containing 1 mM EGTA, it is unclear whether the abolition of the secretory effects must be attributed to depletion of extracellular calcium or rather to the presence of EGTA itself.

The involvement of intracellular calcium in the stimulus-secretion coupling is consistent with the absence of an increased $^{45}Ca^{2+}$ uptake by fragments of rat (Case and Clausen, 1973) and pig (Schreurs et al, unpublished observations) pancreas. They seem however in conflict with the increased $^{45}Ca^{2+}$ uptake observed for isolated acinar cells by Kondo and Schulz (1976).

The requirement of intracellular calcium indicates that this calcium acts as a third messenger in the stimulus-secretion coupling of pancreatic enzyme secretion. The way in which the original stimulus causes the release of calcium from the intracellular store still needs further elucidation.

CHAPTER 8

GENERAL DISCUSSION

The study described in this thesis has been undertaken in order to extend our knowledge about the role of calcium in exocrine pancreatic secretion. The evidence, available at the beginning of our study in 1973, indicated that the calcium metabolism of the pancreas is very complex and is involved at several levels of secretion depending on the physiological condition of the gland (section 1.1.6.).

Our main interest concerned the possible existence of a stimulation dependent increase of the cytoplasmic free calcium concentration, which could act as an essential and specific intracellular trigger in the stimulus-secretion coupling of pancreatic enzyme secretion. We have therefore studied the calcium movements in the resting and stimulated pancreas (chapter 3). We find three stimulation dependent calcium movements in the rabbit pancreas (see fig. 3.6.):

1. an extracellular flux from the bathing medium to the ductular fluid through the so-called "tight-junctions" of adjacent cells;
2. a secretory flux to the ductular fluid, originating from an endogenous pool in the acinar cells and associated with the secreted enzymes;
3. a stimulatory flux, which is due to a release of calcium from

an intracellular store and which is mainly localized over the basal membrane of the acinar cells.

Previously, it has been suggested that the calcium in the pancreatic fluid derives from a protein-dependent and a protein-independent calcium flux (Goebell et al, 1972; Argent et al, 1973). We have established both fluxes in the isolated rabbit pancreas and studied their properties in closer detail. Our observations indicate that these fluxes are not calcium specific, since they also exist for magnesium.

At the basal secretion rate, when only very small amounts of digestive enzymes are secreted, the calcium and magnesium content of the pancreatic fluid is exclusively due to a protein-independent component. This component we have called the extracellular flux. In the isolated rabbit pancreas the basal calcium and magnesium concentrations of the pancreatic fluid never exceed those of the bathing medium, and they decrease at increasing flow rates. These findings agree with those of Goebell et al (1972) in dog and of Argent et al (1973) in cat pancreas. The protein-independent calcium flux seems however to be absent in the human pancreas (Goebell et al, 1973).

The higher flow rate in the resting isolated pancreas, compared to that of the in situ rabbit pancreas (Rothman and Brooks, 1965), may account for the fact that the basal calcium and magnesium concentration in the secreted fluid amount to only 30 % of that in the bathing medium. In this respect divalent cation secretion behaves differently from the secretion of the monovalent cations sodium and potassium, which always occur in concentrations

about equal to those in the incubation medium (Rothmann and Brooks, 1965; Ridderstap, 1969, pp. 70-73; Rutten, 1974, pp. 22-41; Schreurs et al, unpublished results). The experiments of Ridderstap (1969) with radioactive $^{22}Na^{+}$ present in the bathing medium of the isolated rabbit pancreas indicate that this tracer appears immediately in the pancreatic fluid and increases to a relatively constant level. Subsequent addition of ouabain, known to reduce the pancreatic flow rate, also reduces the $^{22}Na^{+}$ output. When on the other hand secretin, which stimulates pancreatic fluid secretion, is added to the $^{22}Na^{+}$ containing medium, the $^{22}Na^{+}$ output increases by about 39 %, which is equal to the stimulation of the fluid secretion of about 35 %. From these observations Ridderstap has concluded that secretin stimulates the secretion of sodium by the pancreas and that the active secretion of sodium is the primary process in fluid and electrolyte secretion by this organ.

The existence of an extracellular pathway for small molecules and ions from the bathing medium to the secreted fluid has been further studied in section 4.3.1. The permeability characteristics of this pathway seem to depend on the size, shape and charge of the permeant. Although direct experimental proof is lacking, it seems very likely that monovalent as well as divalent cations can permeate through this extracellular pathway. Since we have made essentially the same observations for the secretion of $^{45}Ca^{2+}$ as Ridderstap (1969) did for the secretion of $^{22}Na^{+}$, the possibility needs to be considered that a passive flux along this extracellular pathway also contributes to the total $^{22}Na^{+}$secretion.

The existence of an extracellular pathway through which ions

and small molecules may permeate can also be derived from the experiments of Ceccarelli et al (1975) with ^{3}H-sorbitol in the in vivo guinea pig pancreas. A new observation in our study is the increase in permeability of the extracellular pathway upon stimulation of pancreatic enzyme secretion with pancreozymin and carbachol, which is strongly dose-dependent (chapter 4). The physiological significance of this increase is still unclear, but the extra amount of divalent cation secreted upon stimulation may play a role in the biological activity of the digestive enzymes as already discussed in section 4.4. It would be worthwhile to examine the stimulation dependency of the extracellular flux in other species and also whether this flux is influenced by secretin.

The contribution of the protein-dependent secretory fluxes to the total calcium and magnesium content of the pancreatic fluid assumes only significant proportions upon stimulation of pancreatic enzyme secretion (chapter 5). The amounts of divalent cations in the secretory fluxes are directly proportional to the secreted amounts of digestive enzymes. They are independent of the nature and the concentration of the stimulant used and of the divalent cation content of the incubation medium. They derive from a single endogenous pool, which in our experiments does not normally exchange with tracer ions in the medium. This lack of exchange is not due to an irreversible binding of divalent cations to the enzyme proteins, since they regain the ability to exchange after their secretion. Although the physiological significance of the protein-associated divalent cations is still unclear they seem to have a very special function, since they are immobilized during the

intracellular transport of the digestive enzymes and are present in a constant ratio to these enzymes (1 meq calcium and magnesium per mol enzyme protein of 40.000 mol. wt.). The immobilization appears to take place during the conversion of the condensing vacuole to the mature zymogen granule (chapter 6). These observations strongly suggest that the protein-associated divalent cations of the secretory fluxes originate from the zymogen granules, where they may be involved in the packing of the enzyme proteins through neutralization of surface charges (Wallach and Schramm, 1971) or they may be associated with the zymogen granule membranes (Clemente and Meldolesi, 1975).

Despite the fact that the extracellular and secretory calcium fluxes are stimulation dependent, it is unlikely that they play a specific role in the stimulus-secretion coupling of pancreatic enzyme secretion, since comparable fluxes exist for magnesium.

Direct evidence that an increase in the cytoplasmic free calcium concentration acts as an essential and specific intracellular trigger of the pancreatic enzyme secretion is obtained from those experiments in which an increased cytoplasmic calcium concentration is shown to be accompanied by stimulated enzyme secretion. The divalent cation ionophore A-23187, known to facilitate the transport of divalent cations across biological membranes, increases the $^{45}Ca^{2+}$ uptake (Williams and Lee, 1974) and also stimulates pancreatic enzyme secretion. This stimulation requires the presence of extracellular calcium, which cannot be replaced by magnesium (chapter 7). Case and Clausen (1973) have shown that the substitution of sodium by lithium, which increases the $^{45}Ca^{2+}$

uptake in rat pancreas, also stimulates its amylase release. These observations suggest that pancreatic enzyme secretion can be triggered by an increase in the cytoplasmic free calcium concentration.

Evidence that a physiological stimulation with cholinergic agents or pancreozymin is also mediated by an increase in the cytoplasmic free calcium concentration can be derived from the $^{45}Ca^{2+}$ efflux studies with pancreas fragments. Fragments, pre-loaded with $^{45}Ca^{2+}$, show a stimulation dependent $^{45}Ca^{2+}$ efflux in rat (Case and Clausen, 1973; Heisler, 1974), mouse (Matthews et al, 1973; Williams and Chandler, 1975) and guinea pig (Gardner et al, 1975). We have made essentially the same observations with rabbit pancreas fragments (section 3.3.5.). Since the increased $^{45}Ca^{2+}$ efflux is not observed in the secreted fluid of the isolated rabbit pancreas, we have concluded that this increased efflux is presumably mainly localized over the basal membrane of the acinar cells and therefore released to the stimulatory compartment. For this reason we have called this flux the stimulatory calcium flux (fig. 3.6.). The increased efflux of $^{45}Ca^{2+}$ ions over the basal membrane can be explained by either of two mechanisms (fig. 8.1.): 1. an increase in the calcium permeability of this membrane or 2. an increase in the $^{45}Ca^{2+}$ gradient over this membrane due to a release of $^{45}Ca^{2+}$ from an intracellular store. Both mechanisms would normally cause an increase in the cytoplasmic calcium concentration, which may trigger pancreatic enzyme secretion. The first mechanism would utilize extracellular calcium, while the second would use of calcium from an intracellular store. Much conflicting evidence exists on the relative importance of both calcium sources for the increase of

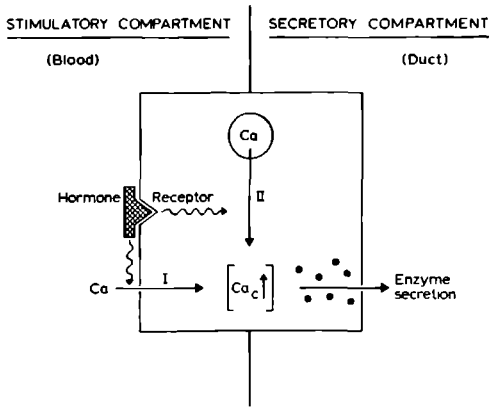

Fig. 8.1. Mechanisms by which the hormone-receptor interaction may increase the cytoplasmic calcium concentration, which in turn may trigger pancreatic enzyme secretion: I. an increase of the permeability of the serosal membrane, causing an influx of extracellular calcium; II. a release of calcium from an intracellular store.

the cytoplasmic free calcium concentration. Omission of calcium from the bathing medium or the perfusion fluid abolishes the stimulated enzyme secretion in slices of pigeon pancreas (Hokin, 1966), rat pancreas (Robberecht and Christophe, 1971; Heisler et al, 1972; Case and Clausen, 1973) guinea pig pancreas (Benz et al, 1972) and the perfused cat pancreas (Argent et al, 1973). The effects of calcium omission are slow to develop and only partly reversible. Moreover it remains unclear whether the abolition of the secretory effects must be attributed to the depletion of extracellular calcium or to the presence of the complexing agent EGTA. A stimulated $^{45}Ca^{2+}$ uptake is absent in rat (Case and Clausen, 1973), mouse (Williams and Chandler, 1974) and pig pancreas fragments (Schreurs et al, unpublished results). Only Heisler and Grondin (1973) have

reported a carbachol stimulated $^{45}Ca^{2+}$ uptake in rat pancreas fragments using the "lanthanum-method". This observation is, however, not confirmed by Chandler and Williams (1974) who have used the same method.

When extracellular calcium is completely removed from the bathing medium with 0.5 mM EGTA present, the amylase release of rat pancreas fragments is virtually abolished, but the accelerated $^{45}Ca^{2+}$ efflux persists (Case and Clausen, 1973). This important observation indicates that the $^{45}Ca^{2+}$, which is released, does not consist of protein-associated calcium. The efflux of $^{45}Ca^{2+}$ cannot be due to an exchange reaction with intracellular calcium in the absence of extracellular calcium. The inhibitory effect of calcium-free media does not influence the initial effects of the stimulant. Normal responses are obtained in media containing as little as 0.1 mM Ca^{2+}, and in our experiments even in the presence of 10^{-4} M EGTA (section 7.3.3.). The combined evidence suggests that stimulation of enzyme secretion may be influenced by extracellular calcium, but does not strictly depend on its presence.

The absence of a stimulatory magnesium flux (section 7.3.1.) strongly suggests that the increase of the cytoplasmic calcium concentration is a specific event in the stimulus-secretion coupling of pancreatic enzyme secretion. Comparison of the effects of carbachol and the ionophore A-23187 on the enzyme secretion and the $^{45}Ca^{2+}$ efflux indicates that carbachol stimulates pancreatic enzyme secretion by increasing the cytoplasmic calcium concentration through a specific release of Ca^{2+} from an intracellular store (chapter 7).

This raises the question of the identity of the intracellular calcium store. Although the secretory fluxes of calcium and magnesium to the pancreatic fluid behave very similarly, there are marked differences in the intracellular distribution of these cations. Clemente and Meldolesi (1975a) find most of the magnesium in the pancreas associated with the ribosomes and the post-microsomal fraction. In contrast, they find the calcium distributed among all particulate fractions, primarily mitochondria, microsomes, zymogen granules and the plasmalemma, while the calcium content of the post-microsomal supernatant is low. These observations indicate that calcium in the pancreatic acinar cell is, like in other cells, associated with subcellular structures and is virtually not present in the cytoplasm as free diffusible ionic calcium. In pulse-chase experiments, Clemente and Meldolesi (1975b) have further studied the functional significance of the various subcellular calcium pools, especially in relation to the stimulus-secretion coupling of pancreatic enzyme secretion. They have shown that $^{45}Ca^{2+}$ taken up by pancreatic lobules during 3-h incubation is mainly distributed among three cytoplasmic organelles: mitochondria (17 %), golgi complex as the major component of the smooth microsomes (20 %) and zymogen granules (30 %). The relatively fast $^{45}Ca^{2+}$ uptake and release by mitochondria and golgi complex suggest that these subcellular structures may act as an intracellular ion buffer system in the calcium homeostasis of the acinar cell. The slow $^{45}Ca^{2+}$ exchange of the zymogen granules indicates that these organelles are not in direct exchange equilibrium with other intracellular calcium stores. This agrees with

our observation that the protein-associated divalent cations do not exchange with radioactive tracers present in the medium (chapter 5 and 6). Upon stimulation of pancreatic enzyme secretion only the mitochondrial fraction exhibits a significant decrease of its $^{45}Ca^{2+}$ content, suggesting that a release of calcium from mitochondria is mainly responsible for the increase of the cytoplasmic calcium concentration upon stimulation. This conclusion is supported by Argent et al (1975) who have reported that in the pancreas only mitochondria actively accumulate Ca^{2+}.

Little is known so far as to how the initial action of the secretagogue upon the cell surface may cause a release of calcium from mitochondria. Case and Clausen (1973) have reported that the stimulatory effects of pancreozymin and acetylcholine are suppressed when Na^+ is substituted by Li^+. Moreover, acetylcholine increases the Na^+ permeability of the plasmamembrane and depolarizes the acinar cell (Nishiyama and Petersen, 1975). These observations suggest that an increase in the cytoplasmic free calcium concentration and the subsequent induction of the enzyme secretion primarily depend on an influx of Na^+ ions into the cytoplasm. This would agree with the observation of Carafoli et al (1974) that Na^+ ions may promote the specific release of calcium from isolated mitochondria. There is, however, also some evidence that cAMP may have this effect (Borle, 1974; Matlib and O'Brien, 1974). In efflux experiments, the $^{45}Ca^{2+}$ released from the mitochondria will increase the $^{45}Ca^{2+}$ gradient across the plasmamembrane leading to an increase of the $^{45}Ca^{2+}$ efflux rate.

The mechanism by which an increased cytoplasmic calcium con-

centration can lead to exocytosis is still unknown. It has been suggested that the increased cytoplasmic calcium concentration may directly promote the binding and the fusion of the zymogen granule and the apical plasma membrane by neutralizing the repulsive surface charges of the membranes (Schulz et al, 1975). It is, however, unlikely that the role of calcium in the stimulus-secretion coupling can be completely explained by this effect, since it does not explain the specificity for calcium ions. Magnesium ions, which are present in considerable concentration in the cytoplasm, would also have this effect.

There is also a possibility that cGMP may be involved. The guanylate cyclase activity in a number of tissues is mainly located in the cytoplasm (Hardman et al, 1973), while calcium ions seem to be closely related to the control of the cGMP levels in these tissues (Schultz et al, 1973). In pancreas tissue cGMP levels are reported to increase in response to cholinergic agents and pancreozymin (Robberecht et al, 1974; Haymovitz and Scheele, 1976; Albano et al, 1976; Christophe et al, 1976). Exogenous dibutyryl cyclic GMP and cyclic 8-bromo-GMP cause a weak but definite stimulation of pancreatic enzyme secretion (Robberecht, 1974; Albano et al, 1976). The importance of calcium ions in the regulation of the cGMP levels in the exocrine pancreas has recently been studied by Christophe et al (1976). Pancreozymin, carbachol and the ionophore A-23187 produce a rapid and transient increase in the cGMP levels in isolated guinea pig pancreas acinar cells. In calcium depleted cells only the ionophore fails to stimulate the cGMP levels. These results indicate that the cGMP levels are

increased due to activation of the guanylate cyclase by calcium.

The role of cGMP in pancreatic enzyme secretion is still very speculative. It is suggested that it may interact with a cGMP-dependent protein kinase (Kuo et al, 1971), which is able to activate an enzyme system by phosporylation. Such an activated enzyme system could in turn serve several regulatory functions. Since the physiological substrates of the cGMP-dependent protein kinases are unknown, we can only propose some hypothetical functions for the activated enzyme system. The stimulation of pancreatic enzyme secretion by exogenous dibutyryl cyclic GMP suggests that this nucleotide may have a direct effect on exocytosis. The sharp transient increase of the $^{45}Ca^{2+}$ efflux relative to the amylase release which remains rather high after stimulation (fig. 3.5.) suggests that there exists a feed back mechanism for the calcium release from the mitochondria, in order to prevent depletion of this ion buffer system. cGMP may possibly be involved in this regulation.

Although we have earlier discussed evidence that extracellular calcium is not necessary for the initiation of stimulated enzyme secretion, there are some indications that it is required for the continuation. In fig. 7.4c. it is shown that adding calcium after stimulation in a calcium-free medium causes a second release of amylase. From similar observations, Petersen and Ueda (1976) have reached the same conclusion. Until now it is unclear whether this calcium enters the cytoplasm as "trigger calcium" or that it is extracellularly required for the functional integrity of the acinar cell. The first alternative is supported by recent

$^{45}Ca^{2+}$ uptake studies of Kondo and Schulz (1976), who find a stimulated $^{45}Ca^{2+}$ uptake in isolated acinar cells, even 30 min after stimulation. Preliminary experiments in our laboratory seem to confirm these results. These observations indicate that after the onset of stimulation due to a specific release of calcium from an intracellular store the calcium permeability of the plasmamembrane remains increased - possibly under the influence of the generated cGMP - in order to allow the entrance of extracellular calcium. This calcium may maintain an increased cytoplasmic calcium concentration for the duration of stimulation and may be used to supply the partly depleted calcium stores. The final quenching of the calcium signal, i.e. the removal of calcium from the cytoplasm, is most probably carried out by calcium-activated ATPases present in the plasma membrane (Forget and Heisler, 1976) and in the mitochondria (Argent et al, 1975). A decrease in the calcium concentration of the cytoplasm will also reduce its cGMP level and consequently the original calcium permeability of the plasmamembrane will be restored.

It will be clear that more work is required to establish whether either of these hypothetical functions of calcium - neutralization of surface charges or activation of guanylate cyclase - operates in exocrine pancreatic secretion, and if so, which one.

The exocrine pancreas secretes a fluid, which consists of water, electrolytes and digestive enzymes and which plays an important role in the digestion of food in the intestine. The secretion of water and electrolytes on the one hand and that of the digestive enzymes on the other hand are under separate hormonal and neural control.

Polypeptide hormones and neurotransmitters, such as control the exocrine pancreatic secretion, do not enter their target cell, but their interaction with receptors at the cell surface generates an intracellular substance which is in turn directly responsible for the cellular response. The investigation reported in this thesis is meant to determine whether an increase in the cytoplasmic calcium ion concentration acts as an essential and specific intracellular trigger in the stimulus-secretion coupling of enzyme secretion by the pancreatic acinar cell.

In chapter 1 we summarize the present knowledge about the physiology of the exocrine pancreas and the role of calcium in its secretory processes.

Chapter 2 contains a description of the materials and methods which have been used in our study.

In chapter 3, we have studied the calcium movements in the resting and stimulated pancreas. We find three stimulation-dependent calcium movements in the rabbit pancreas (see fig. 3.6.):

1. an extracellular flux, which runs directly from the bathing medium to the ductular fluid through the so-called "tight-junctions" of adjacent cells.
2. a secretory flux, which runs from an endogenous pool in the acinar cells to the ductular fluid and is associated with the secreted enzymes.
3. a stimulatory flux, which is due to a release of calcium from an intracellular store and which is mainly localized over the basal membrane of the acinar cells.

These calcium movements are further studied and are compared with the magnesium movements in order to check their specificity.

In chapter 4 it is concluded that calcium and other small molecules and ions may permeate directly from the bathing medium to the ductular fluid through an extracellular pathway between adjacent cells. The permeability characteristics of this pathway seem to depend on the size, shape and charge of the permeant. The permeability is also influenced by hormonal and neural stimulation, but the effects depend rather strongly on the applied dose of the stimulant.

The secretory calcium flux described in chapter 5 consists of protein-associated calcium and is directly proportional to the secreted amounts of enzyme protein. This flux is independent of the nature and the concentration of the stimulant used. The calcium ions derive from a single endogenous pool, which in our experiments does not noticeably exchange with tracer ions in the medium.

The exchangeability of the secretory calcium pool is further

examined in chapter 6. It is concluded that the protein-associated cations are immobilized during the intracellular transport of the digestive enzymes, most probably during the conversion of the condensing vacuole to the mature zymogen granule.

Although the extracellular and secretory calcium fluxes are stimulation dependent, it is unlikely that they play a specific role in the stimulus-secretion coupling of pancreatic enzyme secretion, since we find in our study comparable fluxes for magnesium.

In chapter 7, we have further studied the stimulatory calcium flux. This flux is calcium specific, since we do not find a stimulation-dependent increase in the $^{28}Mg^{2+}$ efflux. The increase of the $^{45}Ca^{2+}$ efflux over the basal membrane of the acinar cell can be explained either by an increase of the permeability of this membrane or by an increase of the $^{45}Ca^{2+}$ gradient over this membrane, due to a release of $^{45}Ca^{2+}$ from an intracellular store. Comparison of the effects of carbachol and the divalent cation ionophore A-23187 on enzyme secretion and calcium movements in the rabbit pancreas leads us to the following conclusion. Carbachol and the ionophore A-23187 both stimulate pancreatic enzyme secretion by a specific increase in the cytoplasmic free calcium concentration. The ionophore increases the calcium permeability of the plasma membrane, causing an influx of extracellular calcium, while carbachol releases calcium from an intracellular store.

In chapter 8 we have discussed our results in the light of the most recent literature about the role of calcium in exocrine pancreatic secretion. Upon stimulation calcium is released from an

intracellular store, most probably the mitochondria. This initiates pancreatic enzyme secretion, while extracellular calcium seems to be involved in the continuation of the enzyme secretion. Further experimental work will be needed to elucidate the mechanisms by which an increase of the cytoplasmic free calcium concentration induces exocytosis in the pancreatic acinar cell.

De exocriene pancreas secreteert een sap dat naast water, electrolyten en spijsverteringsenzymen bevat. Dit pancreassap speelt een belangrijke rol bij de vertering van het voedsel in de darm. De secretie van water en electrolyten enerzijds en van spijsverteringsenzymen anderzijds wordt afzonderlijk zowel hormonaal als neuraal gereguleerd.

De polypeptide hormonen en neurotransmitters die de secretie van de exocriene pancreas reguleren dringen de cel niet in, maar hebben een interactie met hun receptoren aan het celoppervlak. Deze interactie brengt intracellulair een verandering teweeg die op zijn beurt direct verantwoordelijk is voor de cellulaire response. In dit proefschrift is onderzocht of een verhoging van de cytoplasmatische calcium concentration fungeert als een essentiele en specifieke intracellulaire prikkel in de stimulus-secretie koppeling van de enzymsecretie door de acineuze pancreascel.

Hoofdstuk 1 geeft een overzicht van de fysiologie van de exocriene pancreas en de rol van calcium in de secretieprocessen.

Hoofdstuk 2 geeft een beschrijving van de gebruikte materialen en methoden.

In hoofdstuk 3 zijn de calcium bewegingen onder basale en gestimuleerde enzymsecretie bestudeerd. In de konijnepancreas vinden wij drie stimulatie-afhankelijke calcium bewegingen (fig. 3.6.):

1. Een extracellulaire flux van calcium, dat vanuit de badvloeistof via de "tight-junctions" tussen naast elkaar gelegen cellen in de ductulaire vloeistof komt.
2. Een secretoire flux van calcium, dat geassocieerd met de gesecreteerde enzymen vanuit een endogene pool in de acineuze cel in de ductulaire vloeistof komt.
3. Een stimulatoire flux van calcium, dat als gevolg van het vrijkomen van calcium uit een intracellulaire pool de acineuze cel voornamelijk via het basale membraan verlaat.

Deze calcium bewegingen zijn nader bestudeerd en vergeleken met de magnesium bewegingen om na te gaan in hoeverre zij specifiek zijn.

In hoofdstuk 4 wordt geconcludeerd dat niet alleen calcium maar ook andere kleine moleculen en ionen langs een extracellulaire weg vanuit het bad naar de ductulaire vloeistof kunnen diffunderen. Hun grootte, vorm en lading zijn daarbij bepalend voor de relatieve permeabiliteit. De permeabiliteit wordt ook beinvloed door hormonale en neurale stimulatie, maar deze effecten zijn sterk afhankelijk van de toegediende dosis.

De secretoire calcium flux, beschreven in hoofdstuk 5, bestaat uit eiwit-geassocieerd calcium en is direct evenredig met de hoeveelheid gesecreteerd enzymatisch eiwit. Deze flux is onafhankelijk van de aard en de concentratie van de gebruikte stimulans. De calcium ionen komen uit een endogene pool, die in onze experimenten niet duidelijk uitwisselt met radioactieve calcium ionen in het incubatie medium.

De uitwisselbaarheid van de secretoire calcium pool is nader

bestudeerd in hoofdstuk 6. Er is geconcludeerd dat de eiwit-geassocieerde kationen worden geimmobilizeerd tijdens het intracellulair transport van de spijsverteringsenzymen en waarschijnlijk bij de overgang van de condenserende vacuolen naar de rijpe zymogeen granula.

Ofschoon zowel de extracellulaire als de secretoire calcium flux stimulatie-afhankelijk zijn, is het onwaarschijnlijk dat zij een specifieke rol spelen in de stimulus-secretie koppeling van de enzymsecretie aangezien wij volledig vergelijkbare fluxen hebben gevonden voor magnesium.

In hoofdstuk 7 hebben wij de stimulatoire calcium flux nader bestudeerd. Deze flux lijkt calcium specifiek te zijn, aangezien wij geen vergelijkbare magnesium flux hebben gevonden. De verhoogde $^{45}Ca^{2+}$ efflux over het basale membraan van de acineuze cel bij stimulatie kan worden verklaard zowel door een verhoging van de permeabiliteit van dit membraan als door een vergroting van de $^{45}Ca^{2+}$ gradient over dit membraan ten gevolge van vrijkomen van $^{45}Ca^{2+}$ uit een intracellulaire pool. Een vergelijking van de effecten van carbachol en het divalent cation ionofoor A-23187 op de enzymsecretie en de calcium bewegingen in de konijnepancreas heeft geleid tot de volgende conclusie. Carbachol en het ionofoor A-23187 stimuleren beide de enzymsecretie door een specifieke verhoging van de cytoplasmatische calcium concentratie. Het ionofoor verhoogt de calciumpermeabiliteit van het plasmamembraan en daardoor de influx van extracellulair calcium, terwijl carbachol het calcium vrijmaakt uit een intracellulaire pool.

In hoofdstuk 8 hebben wij onze resultaten besproken in het

licht van de meest recente literatuur omtrent de rol van calcium in enzymsecretie van de exocriene pancreas. Bij stimulatie wordt calcium vrijgemaakt uit een intracellulaire pool, waarschijnlijk de mitochondriën. Dit leidt tot de initiatie van de enzymsecretie, terwijl extracellulair calcium betrokken lijkt te zijn bij het aanhouden van de enzymsecretie. Verder experimenteel werk zal moeten uitwijzen hoe een verhoging van de cytoplasmatische calcium concentratie de exocytose in de acineuze pancreascel reguleert.

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Wright, E.M. and Pietras, R.J. (1974): Routes of nonelectrolyte permeation across epithelial membranes. J. Membr. Biol., 17, 293-312.

Zimmerman, M.J., Dreiling, D.A.. Rosenberg, I.R. and Janowitz, H.D. (1967): Secretion of calcium by the canine pancreas. Gastroenterol., 52, 865-870.

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S T E L L I N G E N

I

Een verhoging van de cytoplasmatische calciumconcentratie fungeert bij de exocriene pancreas als een essentiële en specifieke intracellulaire prikkel in de stimulus-secretie koppeling van de enzymsecretie.

Dit proefschrift, hoofdstuk 7.

II

De bij stimulatie van de enzymsecretie van de exocriene pancreas waargenomen stimulatoire calcium flux wordt niet veroorzaakt door een verhoging van de calciumpermeabiliteit van het plasmamembraan, maar is een secundair gevolg van het vrijkomen van calciumionen uit een intracellulaire pool.

Dit proefschrift, hoofdstuk 7.

Kondo, S. en Schulz, I. (1976): Biochim. Biophys. Acta, 419, 78-92.

III

Gezien de waarschijnlijke betrokkenheid van extracellulair calcium bij het aanhouden van de enzymsecretie is het de vraag of de experimenten waarbij stimulatie is uitgevoerd in een "calcium-vrij" medium voldoende nauwkeurig zijn geweest om de conclusie toe te laten dat extracellulair calcium ook betrokken is bij de initiatie van de enzymsecretie.

Dit proefschrift, hoofdstuk 7.

Hokin, L.E. (1966): Biochim. Biophys. Acta, 115, 219-221.

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IV

De relatie tussen de fysiologische toestand van de exocriene pancreas en de permeabiliteit van de extracellulaire weg waarlangs kleine moleculen en ionen vanuit de extracellulaire naar de ductulaire vloeistof kunnen diffunderen dient nader onderzocht te worden.

Dit proefschrift, hoofdstuk 4.

V

In het verleden is bij de kwantificering van de secretoire calcium en magnesium fluxen in het secreet van de exocriene pancreas te weinig rekening gehouden met de stimuleerbaarheid van de extracellulaire kation fluxen.

Dit proefschrift, hoofdstuk 4 en 5.

Sullivan, J.F., Burch, R.E. en Magee, D.F. (1974): Am. J. Physiol., 226, 1420-1423.

Ceccarelli, B., Clemente, F. en Meldolesi, J. (1975): J. Physiol., 245, 617-638.

VI

Kondo en Schulz hebben onvoldoende grond voor hun conclusie dat de door hun gerapporteerde verhoogde influx van calcium na stimulatie van de exocriene pancreas een oorzakelijk verband heeft met de initiatie van de enzymsecretie.

Kondo, S. en Schulz, I. (1976): Biochim. Biophys. Acta, 419, 78-92.

VII

Door Rothman en Isenman wordt niet afdoende bewezen dat de exocriene pancreas haar spijsverteringsenzymen secreteert vanuit twee parallele intracellulaire pools.

Rothman, S.S. en Isenman, L.D. (1974): Am. J. Physiol., 226, 1082-1087.

VIII

Bij het gebruik van N-ethylmaleimide als modificatiereagens voor eiwitten moet er rekening mee worden gehouden dat het niet alleen met sulfhydrylgroepen maar ook met aminogroepen kan reageren.

Bloch, R. (1974): J. Biol. Chem., 249, 1814-1822.

IX

De experimenten van Giotta bewijzen onvoldoende dat het Na-K-ATPase een $\alpha_2\beta_2$ subeenheden structuur heeft.

Giotta, G.J. (1976): J. Biol. Chem., 251, 1247-1252.

X

Bij de bepaling van de stimulatie door HCO_3^- op de Mg-ATPase activiteit in erythrocyten ghosts, dient rekening gehouden te worden met een effect van de anionen uit de gebruikte buffer.

Duncan, C.J. (1975): Life sciences, 16, 955-966.

XI

Het verschil tussen het inhibitiegedrag van ouabaine op het Na-K-ATPase en het K^+ gestimuleerde p-nitrofenylfosfatase moet geweten worden aan een verschil in de bindingskinetiek van ouabaine onder de omstandigheden van de bepaling.

Yoshida, H., Nagai, K., Ohashi, T. en Nakagawa, Y. (1969): Biochim. Biophys. Acta, 171, 178-185.

Walker, J.A. en Wheeler, K.P. (1975): Biochim. J., 151, 439-442.

XII

Het onderzoek naar de regulatie van het eiwitmetabolisme bij herkauwers is van algemeen maatschappelijk belang.

XIII

Het principe "Die dan leeft, die dan zorgt" biedt weinig perspectief aan hen die menen dat de ouderen in onze samenleving onvoldoende levensruimte wordt gelaten.

Nijmegen, 22 october 1976 Victor Schreurs