Biochimica et Biophysica Acta, 419 (1976) 320–330

BBA 77171

ROLE OF CALCIUM IN EXOCRINE PANCREATIC SECRETION

II COMPARISON OF THE EFFECTS OF CARBACHOL AND THE IONOPHORE A-23187 ON ENZYME SECRETION AND CALCIUM MOVEMENTS IN RABBIT PANCREAS

V V A M SCHREURS, H G P SWARTS, J J H H M DE PONT and S L BONTING

Department of Biochemistry, University of Nymegen, Nymegen (The Netherlands)

(Received July 4th, 1975)

SUMMARY

1 The secretory effects of carbachol and the ionophore A-23187 on the isolated rabbit pancreas and rabbit pancreas fragments are compared in order to obtain more insight in the involvement of calcium in the stimulus-secretion coupling of pancreatic enzyme secretion

2 The divalent cation ionophore A-23187 mimicks the effect of carbachol on pancreatic enzyme secretion in both preparations

3 The action of the ionophore is dependent on the presence of extracellular calcium The carbachol effect is much less dependent on calcium, as it occurs even in a Ca^{2+}-free medium containing 10^{-4} M ethyleneglycol-*bis*-(β-aminoethylether)-*N,N'*-tetraacetic acid

4 Carbachol causes a marked increase in the $^{45}Ca^{2+}$ efflux from pre-loaded pancreas fragments in both a normal Krebs-Ringer bicarbonate medium and this Ca^{2+}-free medium

5 Although the tissue still contains about 50 % of its original $^{45}Ca^{2+}$ content, at the time of stimulation, the ionophore has little or no effect on the $^{45}Ca^{2+}$ efflux This indicates that the cytoplasmic $^{45}Ca^{2+}$ concentration is very low, and hence that most of the $^{45}Ca^{2+}$ must be sequestered in one or more intracellular stores

6 It is concluded that both substances stimulate pancreatic enzyme secretion by increasing the cytoplasmic calcium concentration, through an increase in the calcium permeability of the plasma membrane in the case of the ionophore, and through a release of Ca^{2+} from intracellular stores in the case of carbachol

INTRODUCTION

Pancreatic enzyme secretion, stimulated by pancreozymin or cholinergic agents, is always accompanied by the secretion of divalent cations, of which Ca^{2+} has

Presented in preliminary form at the conference on stimulus-secretion coupling in the gastrointestinal tract, Titisee, May 27–29, 1975

Abbreviation EGTA, ethyleneglycol-*bis*-(β-aminoethylether)-*N,N'*-tetraacetic acid

been most extensively investigated When pancreas tissue preloaded with $^{45}Ca^{2+}$ is stimulated, there is also a transiently increased $^{45}Ca^{2+}$ efflux [1–4] By comparing Ca^{2+} movements in the isolated rabbit pancreas with those in rabbit pancreas fragments, we have shown that this $^{45}Ca^{2+}$ efflux occurs over the serosal membrane and is not associated with the enzymes secreted over the luminal membrane [4] The increased $^{45}Ca^{2+}$ flux over the serosal membrane can be explained by either of two mechanisms an increased permeability of the serosal membrane or a release of Ca^{2+} from intracellular stores Both mechanisms would result in an increased cytoplasmic Ca^{2+} concentration

Previous studies with the divalent cation ionophore A-23187 show that this compound also stimulates pancreatic enzyme secretion, probably mediated by an increase of the cytoplasmic Ca^{2+} concentration [5–7] It is not known whether the ionophore also mimicks the cholinergic effect on the $^{45}Ca^{2+}$ efflux

In order to obtain more insight into the involvement of Ca^{2+} in the stimulus-secretion coupling of pancreatic enzyme secretion, we have compared the simultaneous effects of carbachol and the ionophore on the efflux of $^{45}Ca^{2+}$ and amylase from rabbit pancreas fragments preloaded with $^{45}Ca^{2+}$ Both stimuli appear to lead to an increase in the cytoplasmic Ca^{2+} concentration The ionophore increases the Ca^{2+} permeability of the plasma membrane, while carbachol seems to act by a release of Ca^{2+} from intracellular stores

MATERIALS AND METHODS

Chemicals Carbachol, the carbamyl analogue of acetylcholine was purchased from Brocades-ACF, Holland $^{45}CaCl_2$ (1 μCi/0 037 mg Ca^{2+} per ml) was supplied by the Radiochemical Centre, Amersham Insta-gel and Aquasol were obtained from Packard Instrument Company, Inc and New England Nuclear, respectively The ionophore A-23187, a generous gift of Eli Lilly and Co , USA, was dissolved by first adding approx 50 μl acetone/mg and then adding 1 ml ethanol Verapamil (Isoptin) and D-600 were obtained from Knoll AG, Ludwigshafen, Germany

Preparation of the rabbit pancreas Male and female New Zealand white rabbits, weighing 2–3 kg, are fasted 24 h before the start of each experiment The animals are killed by a blow on the neck, immediately followed by carotic exsanguination For experiments with the isolated pancreas, the organ is prepared and mounted essentially according to Rothman [8] For experiments with pancreas fragments, the tissue is streched between spleen and rectum, and cut into 250 mg (wet weight) fragments

Incubation medium Both pancreas preparations are incubated in a balanced Krebs-Ringer bicarbonate medium, containing (in mmol/l) Na^+, 143 5, K^+, 4 9, Ca^{2+}, 2 5, Mg^{2+}, 1 2; HCO^-_3, 25 0, $H_2PO^-_4$, 1 2, Cl^-, 130 7, glucose, 5 5 Before incubation the pH of the medium is adjusted to 7 2 by addition of HCl During incubation the medium is continuously gassed with O_2/CO_2 (95 5, v/v) and maintained at 37 °C

Incubation and fraction collection for isolated pancreas The isolated rabbit pancreas is incubated for 1 h after mounting in a bath containing 300 ml incubation medium, in order to achieve steady-state condition. After this period the medium is replaced by fresh medium The secreted fluid is thereupon collected in 5-min fractions

in pre-weighed plastic counting vials From each fraction 10 μl samples are taken for protein and total calcium determination The remaining volume is determined by weighing

Incubation and sample collection for pancreas fragments The pancreas fragments are preloaded for 2 h in 10 ml Krebs-Ringer bicarbonate medium containing 100 μl $^{45}CaCl_2$ (1 μCi/ml) solution and subsequently washed for 15 min in 300 ml Krebs-Ringer bicarbonate medium to remove adhering radioactivity The washed fragments are transferred after fixed periods from one plastic counting vial, containing 5 ml efflux 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 periods have been used From each vial 300-μl samples are removed for the amylase assay The radioactivity left in each vial is measured after mixing the efflux medium with 10 ml Insta-gel The composition of the efflux medium is indicated in the figures The radioactivity left in the tissue is determined by means of the internal standard method after destruction in Hyamine hydroxide 10-X (Packard Instrument Company, Inc) The efflux rate of each fraction is calculated as

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

Assay methods 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 [9] on a micro scale, using bovine serum albumin (Behringwerke) as a standard

Total Ca^{2+} concentrations are determined on a micro scale with a calcium rapid stat kit (Pierce Chemical Company, USA), by which the blue color of the Ca^{2+} complex of methylthymol blue is measured

Amylase activity is determined according to Bernfeld [10] One unit of amylase activity is defined as 1 mg maltose liberated in 3 min at 30 °C

The radioactivity present in the collected fractions is measured in a liquid scintillation analyzer (Philips)

RESULTS

Studies on the isolated rabbit pancreas

The secreted fluid (200-800 μl/h), collected from the cannulated duct of the isolated rabbit pancreas during incubation in Krebs-Ringer bicarbonate medium, has a protein content of 1–3 mg/h and a Ca^{2+} concentration of 30–40 % of that in the bathing medium Addition of 10^{-5} M carbachol to the bathing medium slightly decreases the volume flow, but increases the secretion of protein and Ca^{2+} Previously we have established that the Ca^{2+} content in the secreted fluid is provided by an extracellular Ca^{2+} flux and a protein-associated Ca^{2+} flux, which are both increased by 10^{-5} M carbachol [4] The divalent cation ionophore A-23187 (10^{-5} M) mimicks the secretory effects of carbachol, but the effects are smaller and tend to last longer

Calcium requirement of stimulation

Fig 1A shows the effects of changing the medium composition on the secretory action of the ionophore The protein and Ca^{2+} contents of the secreted fluid are

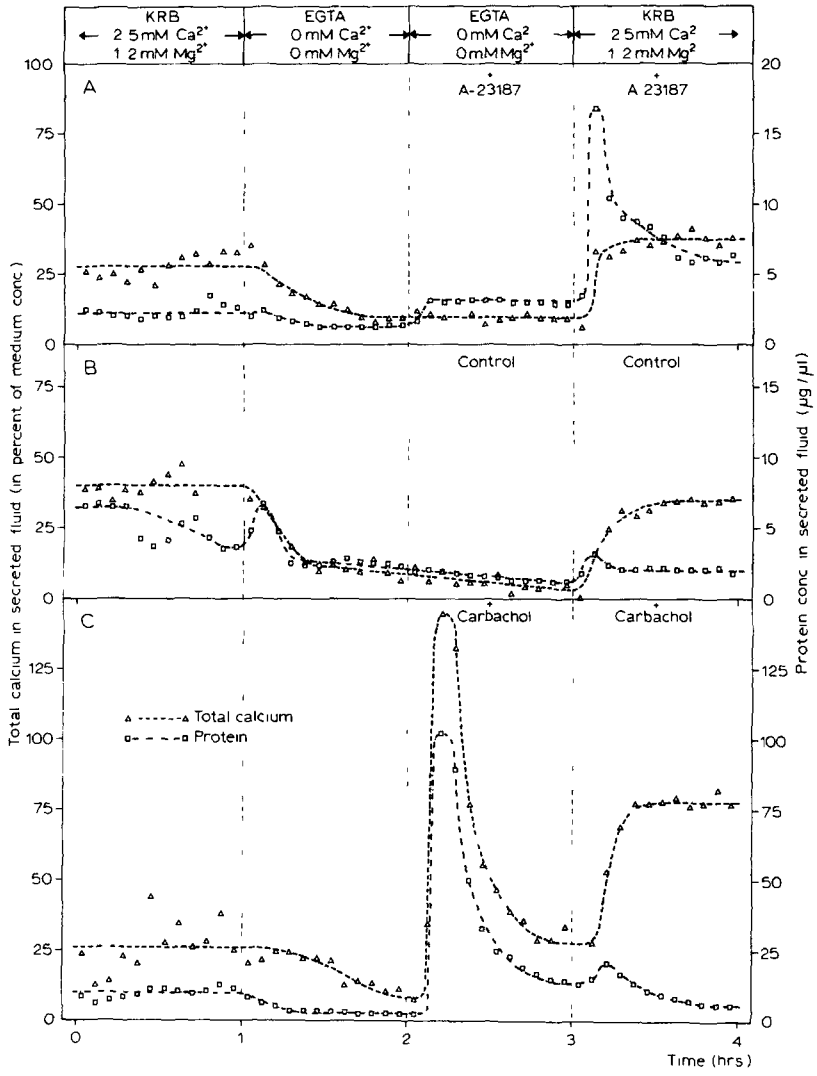

Fig 1 Protein and Ca^{2+} contents of the secreted fluid collected from the cannulated duct of the isolated rabbit pancreas The isolated organ is incubated in normal Krebs-Ringer bicarbonate (KRB) medium during the first and the fourth hour and in EGTA medium during the second and the third hour of the experiment During the third and the fourth hour 10^{-6} M ionophore A-23187 (A) and 10^{-5} M carbachol (C) are added ($n = 4$), while no addition have been made in the control experiment (B)

analyzed in 5-min fractions The basal enzyme secretion is hardly affected upon replacing the Krebs-Ringer bicarbonate medium with a medium (EGTA medium), consisting of Krebs-Ringer bicarbonate minus Ca^{2+} and Mg^{2+} to which 10^{-4} M ethyleneglycol-*bis*-(β-aminoethylether)-*N,N'*-tetraacetic acid (EGTA) is added The Ca^{2+} secretion is, however, strongly diminished This confirms our previous finding [4] that the basal Ca^{2+} concentration of the secreted fluid is mainly dependent on

the Ca^{2+} concentration of the bathing medium Addition of 10^{-6} M ionophore to the EGTA medium has only minor effects on the protein and Ca^{2+} contents of the secreted fluid Subsequent incubation in an ionophore-containing Krebs-Ringer bicarbonate medium yields increases in the enzyme secretion as well as in the total Ca^{2+} concentration of the secreted fluid The latter increase seems to be faster than in the control experiment (Fig 1B) This can be explained by the fact that in this case Ca^{2+} originates from two sources, one directly from the bath via an extracellular route and one in which Ca^{2+} is secreted along with the enzymes In the control experiment the extracellular route contributes most of the secreted Ca^{2+}

When towards the end of the experiment the Krebs-Ringer bicarbonate medium is replaced by one lacking Mg^{2+} the stimulation of protein and Ca^{2+} secretion remains unchanged When Ca^{2+} instead of Mg^{2+} is omitted, no increase in protein and Ca^{2+} secretion is observed (not shown) Changing of the media in the absence of a stimulus also results in changes in protein and Ca^{2+} secretion but those in the protein secretion are relatively small (Fig 1B)

When carbachol is added to the EGTA medium, an immediate increase in both protein and Ca^{2+} output is observed This suggests that the need for Ca^{2+} in the medium is much less pronounced for carbachol than for the ionophore Subsequent incubation in a carbachol-containing Krebs-Ringer bicarbonate medium does not give an additional increase in enzyme secretion but the Ca^{2+} concentration rises again, however, to a higher level, which is normal after stimulation [4] Control experiments show that the small amounts of acetone and ethanol added with the ionophore solution do not affect the protein and Ca^{2+} secretion

Experiments with rabbit pancreas fragments

The ionophore experiments with the isolated rabbit pancreas suggest that pancreatic enzyme secretion can be triggered by an increase of the cytoplasmic Ca^{2+} concentration, as seems to be the case in several other secretory processes [11] To determine the origin of this Ca^{2+} on stimulation with cholinergic agents, we have investigated whether the increase is due mainly to an increase in the permeability of the plasma membrane or to a release of Ca^{2+} from intracellular stores Therefore we have carried out parallel experiments with carbachol and A-23187 on rabbit pancreas fragments These are used instead of the isolated organ, since in the latter preparation Ca^{2+} movements over the serosal membrane cannot be studied

The pancreas fragments are incubated for 2 h in the presence of $^{45}Ca^{2+}$ and are then transferred to a series of plastic counting vials, each containing 5 ml efflux medium The composition of the efflux medium is indicated in the figures The efflux of both $^{45}Ca^{2+}$ and amylase has been determined The results are described in the next two sections

Secretory effects in Krebs-Ringer bicarbonate efflux medium

The experiment of Fig 2A shows a monotone decrease of the $^{45}Ca^{2+}$ efflux in normal Krebs-Ringer bicarbonate medium, while the amylase efflux remains relatively constant under these circumstances When 10^{-5} M carbachol is added to the efflux medium, there is an immediate, large increase in both effluxes The normal $^{45}Ca^{2+}$ efflux rate is restored in about 30 min after stimulation The amylase efflux remains relatively high during the rest of the experiment The increased $^{45}Ca^{2+}$

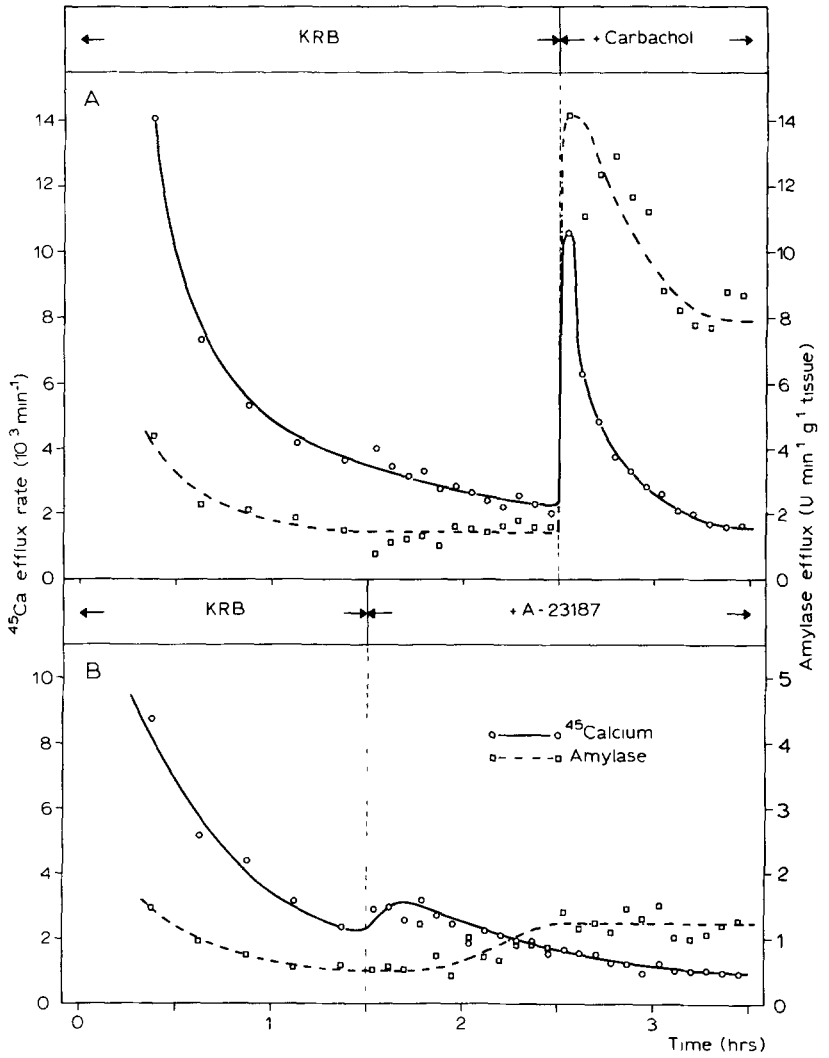

Fig 2 Effects of 10^{-5} M carbachol (A) and 10^{-6} M ionophore A-23187 (B) on the $^{45}Ca^{2+}$ and amylase efflux from pre-loaded rabbit pancreas fragments in Krebs-Ringer bicarbonate (KRB) efflux medium Mean values of 4 consistent experiments

efflux clearly shows that pancreatic Ca^{2+} metabolism is affected upon stimulation by cholinergic drugs Previously [4] we have shown that the $^{45}Ca^{2+}$ flux increase represents a Ca^{2+} movement across the serosal membrane.

Since 10^{-6} and 10^{-5} M A-23187 appear to have about the same effect on pancreas fragments (not shown), we have in further experiments used the lower concentration Addition of 10^{-6} M A-23187 to normal Krebs-Ringer bicarbonate efflux medium has only a slight effect on the $^{45}Ca^{2+}$ efflux, and gives a small and slow increase in the amylase efflux (Fig 2B)

Secretory effects in EGTA efflux medium followed by divalent cation-containing media

Since the effect of the ionophore on the enzyme secretion is more pronounced when A-23187 is added in EGTA medium and Ca^{2+} is added thereafter [5] we have applied the same procedure to study the effects of carbachol and A-23187 in rabbit

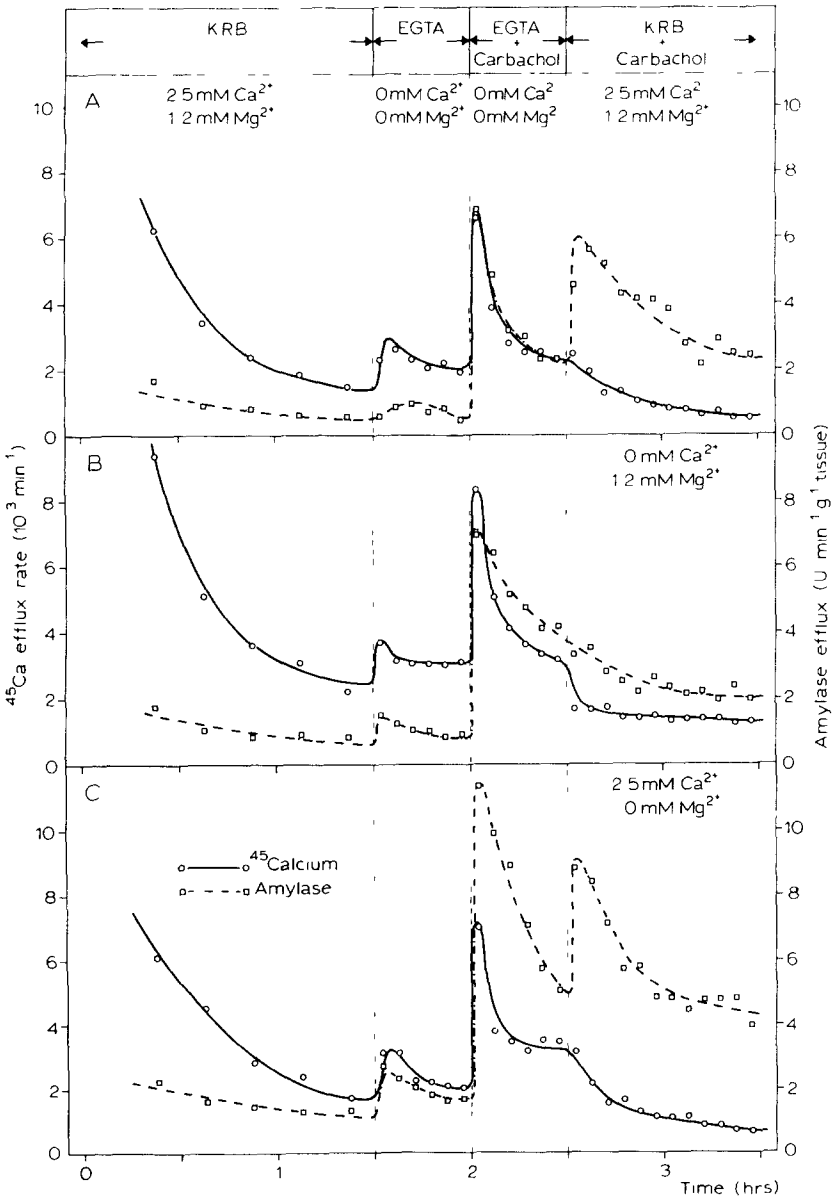

Fig 3 Effects of 10^{-5} M carbachol on the $^{45}Ca^{2+}$ and amylase efflux from pre-loaded rabbit pancreas fragments in EGTA medium followed by divalent cation-containing media 2.5 mM Ca^{2+} + 1.2 mM Mg^{2+} (A), 0 mM Ca^{2+} + 1.2 mM Mg^{2+} (B) and 2.5 mM Ca^{2+} + 0 mM Mg^{2+} (C) Mean values of 4 consistent experiments

pancreas fragments These experiments also supply information about the influence of extracellular Ca^{2+} and Mg^{2+} on the $^{45}Ca^{2+}$ and amylase efflux rates

Addition of 10^{-5} M carbachol 30 min after changing from the Krebs-Ringer bicarbonate medium to the EGTA medium still causes a considerable increase in both

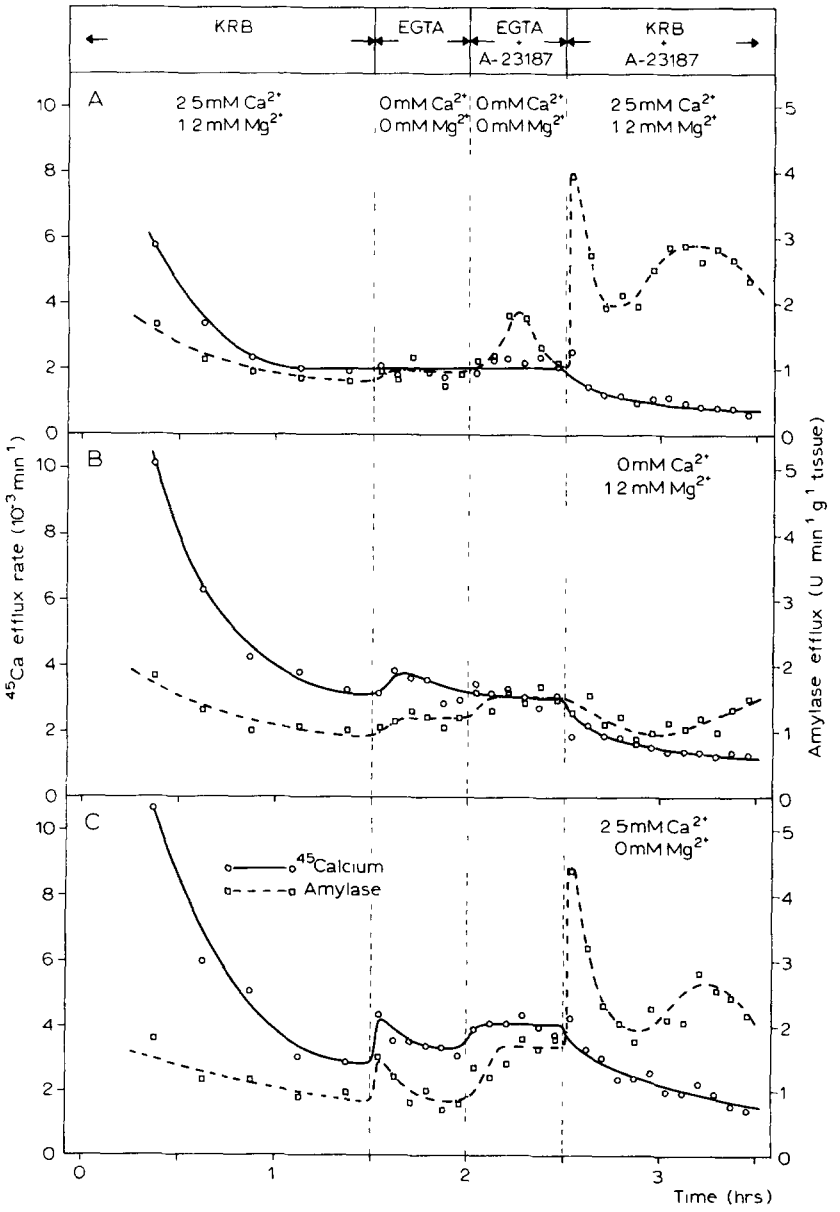

Fig 4 Effects of 10^{-6} M ionophore A-23187 on the $^{45}Ca^{2+}$ and amylase efflux from pre-loaded rabbit pancreas fragments in EGTA medium followed by divalent cation containing media 2 5 mM Ca^{2+} +1 2 mM Mg^{2+} (A), 0 mM Ca^{2+} +1 2 mM Mg^{2+} (B) and 2 5 mM Ca^{2+} +0 mM Mg^{2+} (C) Mean values of 4 consistent experiments

effluxes (Fig 3) When after another 30 min the medium is replaced by a normal Krebs-Ringer bicarbonate medium, an additional increase in amylase secretion is noticed, but the $^{45}Ca^{2+}$ efflux rate is slightly diminished When, 30 min after addition of 10^{-5} M carbachol, a Krebs-Ringer bicarbonate medium without Ca^{2+}, instead of normal Krebs-Ringer bicarbonate medium is added no extra increase in amylase efflux is observed (Fig 3B) Omission of Mg^{2+}, instead of Ca^{2+} from the latter medium gives essentially the same efflux pattern as in normal Krebs-Ringer bicarbonate medium (Fig 3C), indicating that Mg^{2+} in the medium does not play a role in triggering enzyme secretion

Addition of 10^{-6} M A-23187, 30 min after replacing the normal Krebs-Ringer bicarbonate medium by an EGTA medium, leads to a minor increase in amylase efflux, whereas the $^{45}Ca^{2+}$ efflux is not affected (Fig 4A) When 30 min after addition of the ionophore the EGTA medium is replaced by a Krebs-Ringer bicarbonate medium with or without Mg^{2+} (Figs 4A and 4C), a significant increase in the amylase efflux is found, while the $^{45}Ca^{2+}$ efflux rate decreases like in the control experiment When Ca^{2+} is omitted from the Krebs-Ringer bicarbonate medium, no stimulation of the enzyme secretion is found (Fig 4B) This shows that the stimulating effect of the ionophore A-23187 upon returning to normal Krebs-Ringer bicarbonate medium is due to the presence of Ca^{2+}

When the efflux medium is changed from Krebs-Ringer bicarbonate to the EGTA medium, 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 considerable variability between repeated experiments For example, in Fig 3 each curve is the average of four experiments, which show a two-fold variation in the $^{45}Ca^{2+}$/amylase ratio in the period immediately after addition of carbachol Likewise, the small amylase peak observed in Fig 4A after addition of the ionophore is not observed in Figs 4B and 4C, although the conditions were the same

Secretory effects in the presence of Verapamil and D-600

In several systems the Ca^{2+}-antagonistic compounds Verapamil and D-600 block Ca^{2+} channels in the plasma membrane, thereby inhibiting processes in which Ca^{2+} transport through the plasma membrane plays a role [13–15] In the isolated rabbit pancreas and in rabbit pancreatic fragments preincubation with $5 \cdot 10^{-5}$ M of either compound does not result in an inhibition of the carbachol effects on enzyme and $^{45}Ca^{2+}$ secretion, although this concentration is higher than that able to inhibit Ca^{2+}-induced contractions of isolated rat uterus [13]

DISCUSSION

Previously we have shown that the rabbit pancreas in vitro maintains three distinct Ca^{2+} movements, all of which are influenced upon stimulation of pancreatic enzyme secretion by cholinergic agents [4] One of these Ca^{2+} movements is reflected by an increased $^{45}Ca^{2+}$ efflux from pancreas fragments, preloaded with this tracer, and has also been reported for other species [1–3] We have been able to establish that this movement of Ca^{2+} takes place across the serosal membrane and does not

accompany the secretion of enzymes across the luminal membrane [4] The release of $^{45}Ca^{2+}$ can be due either to an increase in the permeability of the serosal membrane or to a release of Ca^{2+} from intracellular stores Independently of the mechanism involved, this flux seems to indicate that the cytoplasmic Ca^{2+} concentration increases upon stimulation

Recently it has been shown [5–7] that the divalent cation ionophore A-23187 can stimulate pancreatic enzyme secretion This ionophore is known to facilitate the transport of divalent cations across biological membranes [12] Its action on pancreas tissue is Ca^{2+}-dependent and it increases its Ca^{2+} content [6] This suggests that an increase in the cytoplasmic Ca^{2+} concentration may be involved in the stimulus-secretion coupling of pancreatic enzyme secretion The ionophoric effects on the $^{45}Ca^{2+}$ efflux have not yet been studied In the present 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 Ca^{2+} in the stimulus-secretion coupling Our experiments with the isolated rabbit pancreas and with rabbit pancreas fragments clearly show that the stimulating effect of cholinergic agents on pancreatic enzyme secretion can be mimicked by the divalent cation ionophore A-23187 (Fig 1A) In addition, we have shown that this ionophoric action requires the presence of extracellular Ca^{2+} (Figs 1A and 4), in accordance with studies on rat pancreas [5] From Figs 1, 3 and 4 it can be concluded that external Ca^{2+} is required only for the action of the ionophore, but not for that of carbachol These observations suggest not only that an increase in the cytoplasmic Ca^{2+} concentration is a common feature of the action of both stimuli, but also that these agents increase the cytoplasmic Ca^{2+} concentration in different ways

The action of the ionophore is Ca^{2+}-dependent, whereas this dependency is less pronounced for carbachol, which stimulates even in the EGTA medium Moreover, the $^{45}Ca^{2+}$ efflux is scarcely influenced by removal of Ca^{2+} from the efflux medium, which suggests that the $^{45}Ca^{2+}$ efflux is not primarily dependent on a Ca^{2+} exchange mechanism The ionophore, which facilitates the transport of Ca^{2+} across biological membranes, has little or no effect on the $^{45}Ca^{2+}$ efflux, indicating that the cytoplasmic $^{45}Ca^{2+}$ concentration is very low Since at the moment of stimulation the tissue still contains about 50 % of its original $^{45}Ca^{2+}$ content, most of the Ca^{2+} must be sequestered in intracellular stores

Carbachol, even in EGTA medium, causes a marked increase in the $^{45}Ca^{2+}$ efflux Since the cytoplasmic Ca^{2+} concentration is very low, this effect of carbachol cannot be explained by an increased Ca^{2+} permeability of the serosal membrane This means that carbachol releases Ca^{2+} from intracellular stores, which are not attacked by the ionophore This conclusion is further supported by the fact that the Ca^{2+} antagonistic compounds Verapamil and D-600 do not inhibit the action of carbachol

These experiments suggest that an increase in the cytoplasmic Ca^{2+} concentration is an essential step in the physiological stimulus-secretion coupling and that the Ca^{2+} originates from intracellular stores Extracellular Ca^{2+} is not directly involved, but may be important for the secretory process itself This can be derived from Fig 3, which shows that re-addition of Ca^{2+} after stimulation with carbachol in EGTA medium increases the amylase efflux level without affecting the $^{45}Ca^{2+}$ efflux Case and Clausen [1] have shown that stimulation of the enzyme secretion is abolished in a

Ca^{2+}-free medium containing 0 5 mM EGTA Normal responses are, however obtained in a medium containing only 0 1 mM Ca^{2+} Since we have observed that EGTA adsorbs onto the tissue, when this is incubated in a medium containing 1 mM EGTA, it is unclear whether the abolishing of the secretory effects must be attributed to depletion of extracellular Ca^{2+} or to the presence of EGTA itself

The involvement of intracellular Ca^{2+} in the stimulus-secretion coupling is consistent with the absence of an increased $^{45}Ca^{2+}$ uptake by rat pancreas [1] and pig pancreas (Schreurs et al , unpublished observations) upon stimulation The requirement of intracellular Ca^{2+} indicates that Ca^{2+} acts as a third messenger in pancreatic stimulus-secretion coupling The way in which the original stimulus causes the release of Ca^{2+} from the intracellular stores still needs further elucidation

ACKNOWLEDGEMENTS

The authors want to thank Eli Lilly and Co , USA and Knoll AG, Ludwigshafen for their gifts of the ionophore A-23187 and the compound D-600, respectively This investigation was supported in part by the Netherlands Organization for the Advancement of Basic Research (ZWO), through the Foundation of Biophysics

REFERENCES

1 Case, R M and Clausen, T (1973) J Physiol 235, 75–102
2 Matthews, E K , Petersen, O H and Williams, J A (1973) J Physiol 234, 689–701
3 Heisler, S (1974) Brit J Pharmacol 52, 387–392
4 Schreurs, V V A M , Swarts, H G P , de Pont, J J H H M and Bonting, S L (1975) Biochim Biophys Acta 404, 257–267
5 Eimerl, S , Savion, N , Heichal, O and Selinger, Z (1974) J Biol Chem 249, 3991–3993
6 Williams, J A and Lee, M (1974) Biochem Biophys Res Commun 60, 542–548
7 Schreurs, V V A M , de Pont, J J H H M and Bonting, S L (1974) J Cell Biol 63, 304a
8 Rothman, S S (1964) Nature 204, 84–85
9 Lowry, O H , Rosebrough, N J , Farr, A L and Randall, R J (1951) J Biol Chem 193, 265–275
10 Bernfeld, P (1955) In Methods in Enzymology (Colowick, S P and Kaplan N O eds) Vol I p 149, Academic Press, New York
11 Rubin R P (1970) Pharmacol Rev 22, 389–428
12 Reed, P W and Lardy, H D (1972) J Biol Chem 247, 6970–6977
13 Fleckenstein, A , Grun, G , Tritthart, H and Byon, K (1971) Klin Wschr 49, 32–41
14 Russell, J T and Thorn, N A (1974) Acta Endocrinol 76, 471–487
15 Eto S , McMillin Wood, J , Hutchins, M and Fleischer, N (1974) Am J Physiol 226, 1315–1320