Br. J. Pharmac. (1974), 52, 387-392

CALCIUM EFFLUX AND SECRETION OF α-AMYLASE FROM RAT PANCREAS

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1 The efflux of ^{45}Ca from rat pancreas was investigated in relation to the secretion of α-amylase.

2 The ^{45}Ca space in the pancreas was increased 1.6 times when pancreatic tissue was incubated in media in which the extracellular calcium concentration was reduced from 1.0 to 0.05 mM.

3 Carbachol increased the rate of ^{45}Ca efflux from the tissue and this effect was associated with an increase in the release of α-amylase. Dibutyryl cyclic adenosine monophosphate (AMP) also evoked a secretory response, but did not alter the rate of ^{45}Ca efflux. In combination with carbachol, the dibutyryl analogue of cyclic AMP reduced the carbachol-stimulated increase in ^{45}Ca efflux while enhancing the release of secretory protein.

4 The stimulatory effect of carbachol on ^{45}Ca efflux was observed when pancreatic tissues were incubated in media containing a high concentration of ethyleneglycolbis (β-aminoethyl)-*N,N'*-tetraacetic acid (EGTA).

5 Atropine blocked the effects of carbachol on both ^{45}Ca efflux and secretion of α-amylase.

6 It was concluded that intracellular calcium can and may sustain stimulus-secretion coupling in the rat exocrine pancreas.

Introduction

The events known as 'stimulus-secretion coupling' (Douglas & Rubin, 1961) with calcium described as the linking intermediate between the two processes, are well documented for the release of transmitter substances from neuromuscular junctions and synapses, of hormones from endocrine glands, and of water and protein from exocrine glands (see Rubin, 1970). In so far as the exocrine pancreas is concerned, extracellular calcium is a limiting factor between cell excitation and the release of digestive protein in response to cholinergic agents (Robberecht & Christophe, 1971; Heisler, Fast & Tenenhouse, 1972).

The role of intracellular calcium in 'excitation-contraction coupling' (Sandow, 1965) in skeletal muscle is well established (Hasselbach, 1964) and Rasmussen (1970) has postulated that the translocation of this ion from cellular organelles such as mitochondria or microsomes, may be an important factor in the physiological response of other cell types. Indeed, Heisler *et al.* (1972) have suggested that under certain conditions intracellular calcium can support secretion of protein from the pancreas stimulated with carbachol. Heisler & Grondin (1973) have recently reported that carbachol increases the rate of efflux of ^{45}Ca from pancreatic fragments when influx of the isotope is presumably blocked by La^{+++}.

The aim of the present study was to investigate further, the effect of carbachol on these rapid changes in calcium metabolism, especially in respect to the secretion of α-amylase from the pancreas.

Methods

Animals

Female Sprague-Dawley rats, 180-225 g were decapitated and the pancreas excised, and trimmed free of adherent fat and mesentery in chilled incubation medium. Each pancreas was cut into fragments weighing 10-20 mg.

Measurement of ^{45}Ca space

Pancreatic fragments (~100 mg wet weight) were incubated for 30 min at 37°C in 2 ml of Krebs-Ringer bicarbonate (pH 7.4) containing either

0.05, 0.1 or 1.0 mM $CaCl_2$. Radioactive ^{45}Ca (0.5 μCi/ml) was also present in the medium. Incubation was terminated by filtration through cheese-cloth and the tissues washed twice with the appropriate incubation medium.

The tissues were homogenized in 3 ml of 3 mM $CaCl_2$ and the protein concentration was determined in an aliquot of 200 μl by the Biuret method (Kabat & Mayer, 1967). The homogenate was acidified with an equal volume of 10% trichloroacetic acid and extracted for 60 min at room temperature prior to centrifugation. Radioactivity (^{45}Ca taken up) was subsequently determined in a 200 μl aliquot of the supernatant, counts being corrected for background and efficiency. The values for ^{45}Ca space were calculated according to Goodman, Weiss, Weinberg & Pomarantz (1972) and were obtained by dividing the amount of ^{45}Ca taken up per g protein of tissue, by the concentration of ^{45}Ca per ml of incubation medium.

^{45}Ca uptake

The uptake of ^{45}Ca was determined by incubation of pancreatic tissue at 37°C for various periods of time in 2 ml Krebs-Ringer bicarbonate buffer containing 0.05 mM $CaCl_2$ (0.5 μCi ^{45}Ca/ml). Termination of incubation and measurement of radioactivity were performed as described above. Uptake is expressed as d/min of ^{45}Ca per mg tissue protein.

^{45}Ca efflux and assay of α-amylase

Pooled pancreatic fragments were preincubated for 30 min at 37°C in 10 ml Krebs-Ringer bicarbonate buffer containing 0.05 mM $CaCl_2$ and 0.5 μCi/ml ^{45}Ca. The tissue was removed by filtration, washed twice with 10 ml of ^{45}Ca-free medium and subdivided in 25 ml flasks containing 2 ml of isotope-free buffer and the various test agents. These flasks were incubated for various time periods up to 30 min; unincubated samples of tissue ('zero time') were taken before and after distribution of the tissue.

Following incubation, the medium was removed by aspiration and the tissue homogenized in 3 ml 3 mM $CaCl_2$. Protein concentration and radioactivity were determined as described above. Efflux of ^{45}Ca is expressed as a percentage of the specific activity (d/min of ^{45}Ca per mg protein) with respect to the specific activity in the 'zero time' samples (= 100%).

An aliquot of the aspirated medium (10-50 μl) was diluted to 0.5 ml with 4 mM $CaCl_2$ and used in the α-amylase assay. A 100 μl aliquot of the homogenate (prior to being acidified) was diluted to 5.0 ml with 3 mM $CaCl_2$ and 0.5 ml of this solution was used in the α-amylase assay. The method of Bernfeld (1951) was used to measure α-amylase. Release of the enzyme is expressed as the percent of the total (medium + tissue) enzyme found in the medium.

Drugs

Carbamylcholine chloride (carbachol), dibutyryl cyclic AMP and atropine sulphate were purchased from Sigma Chemical Co. (St Louis, Mo.). ^{45}Ca (15.6 μCi/μg) was purchased from Amersham/Searle Corp. (Oakville Ont.).

Results

^{45}Ca space studies

A previous report by Heisler *et al.* (1972) has indicated that the spontaneous release of α-amylase is calcium-dependent. In fact, the quantity of enzyme released by unstimulated tissues approaches the amounts measurable following carbachol stimulation. At a reduced extracellular Ca^{++} concentration, however, the pancreas still responds adequately to secretagogues and at the same time differences in secretory outputs between stimulated and unstimulated tissues can be observed. Since almost all experiments described here were performed with 0.05 mM Ca^{++} in the incubation media it was first necessary to observe the resultant changes produced in ^{45}Ca space, and the time required to load the tissue adequately with radiocalcium for subsequent efflux studies. These data are illustrated in Table 1 and Fig. 1, respectively.

The ^{45}Ca space in the pancreas is increased as the concentration of Ca^{++} in the bathing medium is reduced (Table 1); the greatest increase in ^{45}Ca space is observed at the lowest concentration of Ca^{++} used.

The time required to preload the pancreas maximally with ^{45}Ca, is 20 min (Figure 1). The

Table 1 Effect of calcium concentration on ^{45}Ca space in fragments of rat pancreas

Concentration of calcium (mM)	*^{45}Ca space (ml/g)*
0.05	10.30 ± 0.41
0.10	9.24 ± 0.36
1.0	6.38 ± 0.70

Values are means with s.e. of 3 observations.

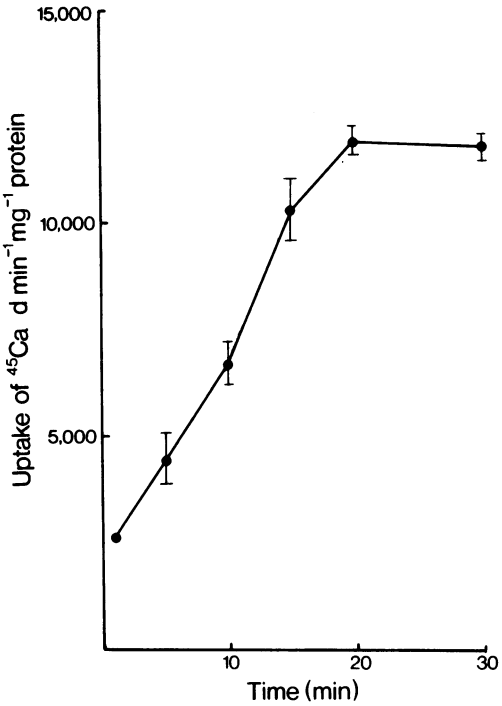

Fig. 1 Uptake of ^{45}Ca by rat pancreatic fragments as a function of time. Values are means with s.e. of 3 observations.

initial rate of uptake is linear for 20 min, a plateau being observed thereafter.

Response to carbachol and dibutyryl cyclic AMP

The efflux of ^{45}Ca, and the release of α-amylase in response to carbachol (10^{-5} M) alone, or together with dibutyryl cyclic AMP (10^{-3} M) are illustrated in Figure 2. Carbachol greatly increased the rate of efflux of ^{45}Ca from the pancreas in comparison to that observed in unstimulated tissue. The dibutyryl analogue of cyclic AMP had no apparent effect on the rate of efflux, when compared to controls. When both agents were used in combination, however, the nucleotide reduced the carbachol effect on the rate of efflux of isotope during the first 15 min, but by the end of the observation period, the amount of ^{45}Ca remaining in the tissue was similar to that found in the presence of carbachol alone. An increase in the release of α-amylase was observed after 5 min of stimulation with carbachol. The cyclic nucleotide also stimulated enzyme secretion, but to a lesser extent than with carbachol; a 15 min lag phase before the onset of this response, was also observed. The response to dibutyryl cyclic AMP and carbachol together was greater than the individual responses, the increase in release of α-amylase, also being observed after only 5 min of incubation.

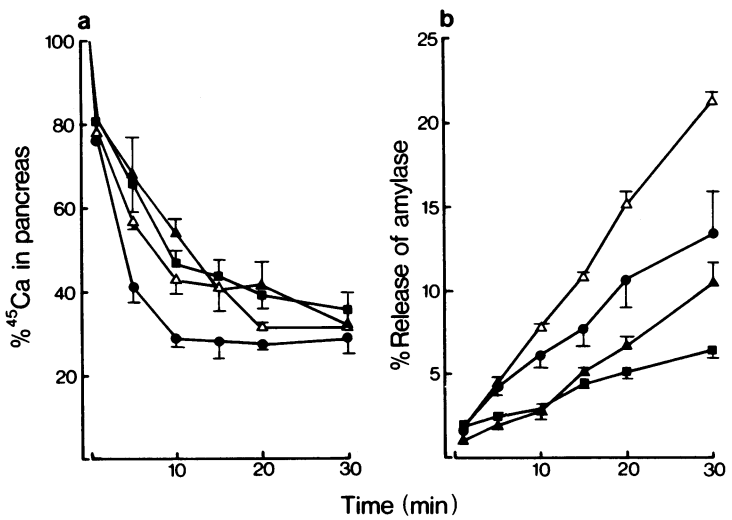

Fig. 2 Efflux of ^{45}Ca (a) and release of α-amylase (b) from rat pancreatic fragments as a function of time. Unstimulated tissue (■); tissues exposed to carbachol 10^{-5} M (●), dibutyryl cyclic AMP 10^{-3} M (▲) and carbachol 10^{-5} M + dibutyryl cyclic AMP 10^{-3} M (△). Values are means with s.e. of 3-5 observations.

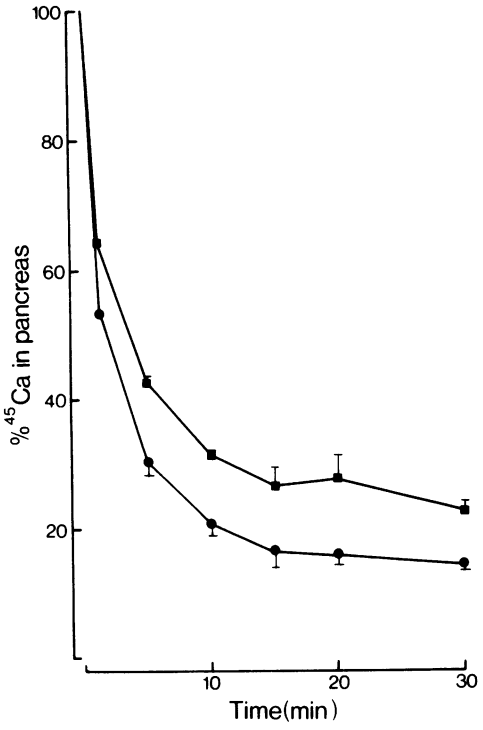

Fig. 3 Efflux of ^{45}Ca from rat pancreatic fragments as a function of time. Tissues exposed to EGTA 10^{-3} M (■) and to EGTA 10^{-3} M + carbachol 10^{-5} M (●). Values are means with s.e. of 2-4 observations.

Response to EGTA

Since the previous efflux experiments were performed in the presence of added extracellular Ca^{++}, it was possible that the carbachol effect on efflux may have been an indirect response arising from an initial increase in the rate of influx of Ca^{++}. To test this possibility, pancreatic fragments were pre-loaded with ^{45}Ca as described in the methods section, but efflux was studied in media to which no Ca^{++} was added but in which ethyleneglycolbis (β-aminoethyl)-*N,N'*-tetraacetic acid (EGTA) (10^{-3} M) was present. Since it had previously been demonstrated that EGTA inhibited the secretory response to carbachol (Heisler *et al.*, 1972), only the effect of the chelator on the efflux pattern was studied. The data are summarized in Figure 3. Carbachol stimulated the rate of ^{45}Ca efflux as observed previously. It was also noted that EGTA itself, stimulated loss of ^{45}Ca from the tissues as indicated by the amount of ^{45}Ca remaining in both EGTA-treated and non-treated tissues at the various times (cf. controls, Figure 2).

Effect of atropine

The effects of atropine (10^{-5} M) on the efflux of ^{45}Ca, and secretion of α-amylase in response to carbachol, are illustrated in Figure 4. Atropine inhibited both the carbachol-stimulated rate of efflux of ^{45}Ca, and the secretion of α-amylase,

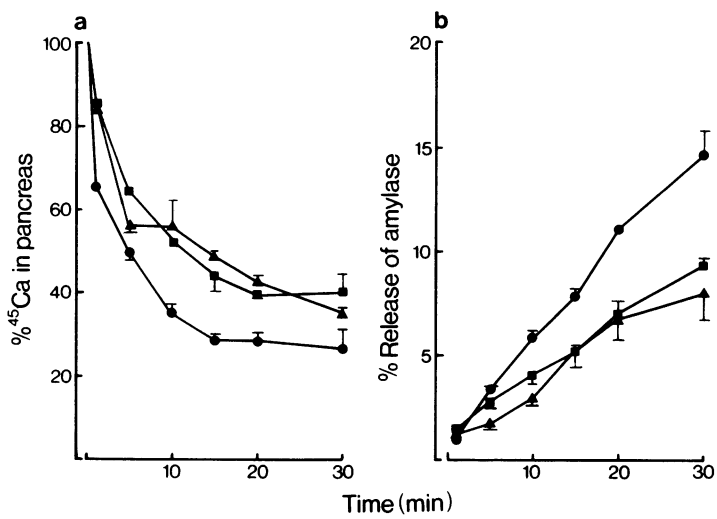

Fig. 4 Efflux of ^{45}Ca (a) and release of α-amylase (b) from rat pancreatic fragments as a function of time. Tissues exposed to atropine 10^{-5} M (▲), carbachol 10^{-5} M (●), and atropine 10^{-5} M + carbachol 10^{-5} M (■). Values are means of 2 observations.

while by itself, it did not influence these events (cf. controls, Figure 2).

Discussion

In the pancreas, a reduction in extracellular Ca^{++} from 1.0 to 0.05 mM increases ^{45}Ca space 1.61 times. To our knowledge, similar ^{45}Ca space studies have not been performed in other secretory organs. In aortic smooth muscle, however, Goodman *et al.* (1972) have demonstrated that a reduction in extracellular Ca^{++} from 1.5 to 0.1 mM results in a fivefold increase in ^{45}Ca space. The relative variations in ^{45}Ca spaces between the pancreas and vascular smooth muscle may reflect differences in the Ca^{++} self-exchangeability in the various cellular Ca^{++} pools in the two tissues, i.e. the pancreas may contain cellular Ca^{++} pools, not found in muscle, which exchange very slowly with ^{45}Ca. A most likely Ca^{++} store, in this respect, may be the zymogen granule. α-Amylase, for example, is a Ca^{++}-metalloenzyme which binds 1 g atom of Ca^{++} per mol (Vallee, Stein, Sumerwell & Fisher, 1959).

Ca^{++} is secreted into pancreatic juice in direct relation to the enzyme following stimulation with pancreozymin (Zimmerman, Dreiling, Rosenberg & Janowitz, 1967; Goebell, Steffen & Bode, 1972). That the ^{45}Ca effluxed from the pancreas in the current experiments is not complexed to the α-amylase secreted, is suggested by a number of observations. In all the experiments, a plateau in the rate of loss of ^{45}Ca from the tissues was observed after 10 to 20 min of incubation; the stimulated rate of release of α-amylase in contrast, was still linear following this time period and continues to be so for up to 60 min (Heisler *et al.*, 1972). The ability of carbachol to stimulate the efflux of ^{45}Ca in the presence of EGTA 1 mM, a concentration which is 13.3 times greater than that required to inhibit the secretory response to carbachol (Heisler *et al.*, 1972), further substantiates the belief that the ^{45}Ca lost from the cell is not derived from a zymogen pool.

Disodium edetate (EDTA), and presumably EGTA, is known to act extracellularly (Bianchi, 1965). The exposure of vascular smooth muscle to EDTA produces a sustained increase in the rate of ^{45}Ca efflux, an effect which has been attributed to an inhibition of re-uptake or re-binding of ^{45}Ca at extracellular sites (Bianchi, 1965; Goodman & Weiss, 1971). The observation that carbachol increased the loss of ^{45}Ca from the pancreas in the presence of EGTA suggests that the ion which is lost is derived from a cellular calcium compartment inaccessible to the chelator. In all probability, in the absence of EGTA, carbachol also causes a qualitatively similar outward movement of Ca^{++} from the same cellular site.

In the present study, dibutyryl cyclic AMP slows the carbachol-increased rate of ^{45}Ca efflux while enhancing the secretory response to carbachol. The observations that Ca^{++} is a limiting factor in pancreatic secretion (Robberecht & Christophe, 1971; Heisler *et al.*, 1972) and that with carbachol, ^{45}Ca accumulates in the rat pancreas prior to the onset of the secretory process (Heisler & Grondin, 1973), suggest the following hypothesis. An 'activation' site exists within the pancreas at which Ca^{++} triggers secretory events. A certain turn-over rate of Ca^{++} at this site would account for the basal release of enzyme and a secretagogue-stimulated increase of this turn-over rate would regulate the augmented secretory response. Changes in turn-over rate would depend on the ability of secretagogue to influence the transmembrane flux of Ca^{++} and/or the processes which affect the subcellular distribution of the ion. With carbachol, Ca^{++} is sequestered at the 'activation' site and is rapidly removed by efflux. With dibutyryl cyclic AMP, the lag phase in the onset of the secretory response may be explained by the fact that the nucleotide does not directly promote sequestration of Ca^{++} to the 'activation' site, but rather acts to limit its loss from this site. When carbachol and dibutyryl cyclic AMP are used in conjunction, the nucleotide prevents the removal by efflux, of Ca^{++} sequestered to the activation site by carbachol, and in so doing, enhances the carbachol stimulatory process. The absolute requirement for extracellular Ca^{++} in these events is demonstrated by the inhibitory action of EGTA on secretion in response to carbachol and/or dibutyryl cyclic AMP (Heisler *et al.*, 1972) and by the demonstration that La^{+++}, which blocks the carbachol-stimulated uptake of ^{45}Ca by the pancreas, also inhibits the secretory response to the same agent (Heisler & Grondin, 1973). Finally, Robberecht & Christophe (1971) found that in the absence of extracellular Ca^{++} the basal secretion of amylase is diminished.

Atropine, like dibutyryl cyclic AMP, slows the carbachol-stimulated efflux of ^{45}Ca from the pancreas, but unlike the nucleotide, it inhibits the release of α-amylase by carbachol. The ability of atropine to block the cholinergic-induced secretory response in mouse pancreas, while leaving unaffected the response to pancreozymin (Kulka & Sternlicht, 1968) suggests that this agent moderates the observed carbachol effects in the current studies by competition at carbachol receptor sites rather than by exerting a nucleotide-like effect on events elsewhere in the cell.

Though it is impossible to ascertain from this study whether either intra- or extracellular Ca^{++} is

used preferentially during the secretion process in the rat pancreas, the observation that the output of α-amylase in response to carbachol is the same at a wide range of external Ca^{++} concentrations (Heisler *et al.*, 1972) suggests that at low extracellular ionic concentrations, intracellular Ca^{++} may sustain the release of secretory protein.

In the pancreas identification of either the cell compartment from which carbachol stimulates Ca^{++} efflux or the site at which Ca^{++} activates secretion has not been made. Stimulus-secretion coupling, however, has invariably been compared to the analogous series of events of excitation-contraction coupling. In excited muscle, the sarcoplasmic reticulum releases Ca^{++} which in turn interacts with the contractile proteins to initiate the myogenic response (Mommaerts, 1971). The possibility that the pancreatic endoplasmic reticulum and zymogen vesicle are structural correlates to muscle sarcoplasmic reticulum and contractile proteins appears well worth investigating.

The author wishes to thank Mr G. Grondin for his excellent technical assistance, and Miss C. Picard for her aid in preparing the manuscript. This work was supported by grants from the Medical Research Council of Canada and the Medical Research Council of Quebec.

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(Revised February 22, 1974)