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CALCIUM AND PANCREATIC SECRETION-DYNAMICS OF SUBCELLULAR CALCIUM POOLS IN RESTING AND STIMULATED ACINAR CELLS

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1 Pulse-chase experiments were carried out on pancreatic tissue lobules incubated *in vitro*, with ^{45}Ca as the tracer, in order to shed some light on the functional significance of the calcium pools associated with the various cell organelles of the acinar cell, especially in relation to stimulus-secretion coupling.

2 The kinetics of tracer uptake and release which were observed in the intact lobules suggest the existence of a number of intracellular pools, whose rate of exchange is slower than that across the plasmalemma.

3 The various subcellular fractions accumulate the tracer in different amounts: some (rough microsomes and postmicrosomal supernatant) showed little radioactivity and some (smooth microsomes and zymogen granule membranes) were heavily labelled; mitochondria and zymogen granules showed intermediate values.

4 The fractions are heterogeneous also in relation to the time course of uptake and release of the tracer: in rough and smooth microsomes and, especially, in the postmicrosomal supernatant both rates were fast; zymogen granules and zymogen granule membranes showed slow rates of uptake and little release during chase; intermediate rates were found in mitochondria.

5 In agreement with previous findings we observed that in ^{45}Ca preloaded lobules, stimulation of secretion (brought about by the secretagogue polypeptide caerulein) results in an increase of the tracer release which seems to be due primarily to the rise of the intracellular concentration of free Ca^{2+} and to the consequent increase of the transmembrane Ca^{2+} efflux. Among the cell fractions isolated from stimulated lobules only the mitochondria exhibited a significantly lower ^{45}Ca level relative to the unstimulated controls.

6 It is concluded that, of the organelle-bound calcium pools, that associated with the mitochondria might be involved in the regulation of the calcium-dependent functions, including stimulus-secretion coupling; the calcium associated with the zymogen granule content probably has a role in the architecture of the organelle and in the functionality of the pancreatic juice, while the calcium bound to the membrane of the granules might be concerned with the regulation of its permeability properties.

Introduction

Calcium is known to play a fundamental role in a number of functions of secretory cells, such as the intracellular packaging of secretion products (Berneis, Pletscher & Da Prada, 1969; Wallach & Schramm, 1971), the regulation of the membrane permeability (Rubin, 1970; Rasmussen, 1970; Matthews, 1970; Dean & Matthews, 1970; Case, 1973; Matthews, 1974) and the transduction of the stimulation into the specific secretory response (stimulus-secretion coupling) (Douglas & Rubin, 1961; Douglas & Poisner, 1963; Douglas, 1968; Rubin, 1970; Rasmussen, 1970; Case, 1973; Matthews, 1974; Thorn, 1974). In relation to this latter function, over the last few years it has become increasingly evident that the coupling is not necessarily dependent on a change of calcium permeability of the plasmalemma. Rather, alterations in the intracellular distribution of Ca^{2+} might be the relevant event in the release process (Milligan & Kraicer, 1971; Nielsen & Petersen,

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1972; Case & Clauson, 1973; Malaisse, 1973; Matthews, Petersen & Williams, 1973; Heisler, 1974; Matthews, 1974; Milligan & Kraicer, 1974). This interpretation is based on the assumption that calcium is compartmentalized within the secretory cells in a number of pools, whose size can be rapidly adjusted in relation to the functional activity. However, up to now this assumption has not been proven because only very few secretory systems have been studied in detail in order to elucidate the functional distribution of calcium within the cells (Borowitz, 1969; Rahwan, Borowitz & Miwa, 1973; Herman, Sato & Hales, 1973; Simpson & Spicer, 1974).

This present investigation has been carried out in order to shed some light on these processes in the acinar cells of the pancreas. In a previous paper (Clemente & Meldolesi, 1975) we showed that in these cells the calcium distribution is very heterogeneous since only a small proportion of the total store is localized in the soluble cytoplasm while most of it is associated with various cytoplasmic organelles, especially the zymogen granules, the smooth microsomes and the mitochondria, and with the plasmalemma. The investigation has now been extended by the use of pancreatic tissue lobules incubated *in vitro* in the presence of ^{45}Ca. In particular we have studied: (a) the extent and the rate of accumulation of the tracer in the different cell organelles and (b) the metabolic stability of the various calcium pools in the resting cell as well as after *in vitro* pharmacological stimulation of secretion.

Methods

The animals used were 450-550 g male albino guinea-pigs (gift of Sigurtà drug Co., Milan, Italy) fasted for 24 h with water given *ad libitum*. The pancreases were rapidly removed from the animals and immersed in ice-cold Krebs-Ringer bicarbonate solution (calcium concentration 2.54 mM), equilibrated at pH 7.4 with 95% O_2 and 5% CO_2 and containing a complete set of amino acids, glucose and 2 mg/ml of bovine serum albumin (incubation medium). Groups of tissue lobules, as intact as possible, were dissected out as described by Castle, Jamieson & Palade (1972), transferred to 100 ml conical flasks containing 10 ml of incubation medium and incubated at 37° C in a shaking bath under an atmosphere of 95% O_2 and 5% CO_2. The tracers used were $^{45}CaCl_2$, L-[4,5-^{3}H]-leucine and D-[1-^{3}H]-sorbitol. At the end of the labelling incubations the lobules were recovered by filtration, rinsed with large volumes of ice-cold non-radioactive incubation medium and resuspended for 1 min in 50 ml of the latter medium. In most experiments they were then incubated at 37° C in non-radioactive incubation medium (chase incubation). Detailed conditions of labelling and of chase incubation of the lobules are described in the figure legends.

At the end of the incubations the lobules were homogenized in 0.3 M sucrose and cell fractions were prepared according to the procedures described elsewhere (Meldolesi, Jamieson & Palade, 1971), modified as indicated by Clemente & Meldolesi (1975). The incubation media were dissolved in 10 ml of Packard Instagel and transferred to disposable plastic vials. When a separate assay of the protein-incorporated radioactivity was needed, the samples were precipitated at 4° C with 10% tricloroacetic acid (TCA) and washed as described previously (Meldolesi, 1974a). Aliquots of the TCA-soluble and insoluble fractions were dissolved in 10 ml of Packard Instagel. All samples were counted in a SL30 Intertechnique liquid scintillation spectrometer (Intertechnique, Plaisir, France). In single label counting the efficiency of counting ^3H and ^{45}Ca was over 40% and 80%, respectively; in double label counting, 25% and 60%. Corrections for quenching and spill were made by external standardization. Protein was determined according to Lowry, Rosebrough, Farr & Randall (1951) on TCA-precipitates dissolved in 1 N NaOH, with crystalline bovine serum albumin as standard.

Materials

The sources of most of the materials used are given by Meldolesi & Clemente (1975). L[4,5-^{3}H] leucine (sp. act. 32 mCi/μmol) and D[1-^{3}H] sorbitol (sp. act. 6.8 mCi/μmol) were purchased from New England Nuclear, Langen, Germany. Caerulein was the kind gift of the Farmitalia Laboratories for Basic Research, Milan, Italy.

Results

Kinetics of calcium uptake and release

The exchange of calcium in pancreatic acinar cells was estimated by determining the uptake and the release of ^{45}Ca in tissue lobules incubated *in vitro*. As can be seen in Figure 1, the uptake of the tracer proceeds at a relatively slow rate, so that after 15 min of incubation the tissue radioactivity exceeds by only a factor of approx 2.5 the contribution of the extracellular space. Thereafter the accumulation of the tracer continues, and almost reaches a plateau after approximately 180 minutes. If the results are recalculated on the basis on the calcium concentration of the guinea-pig

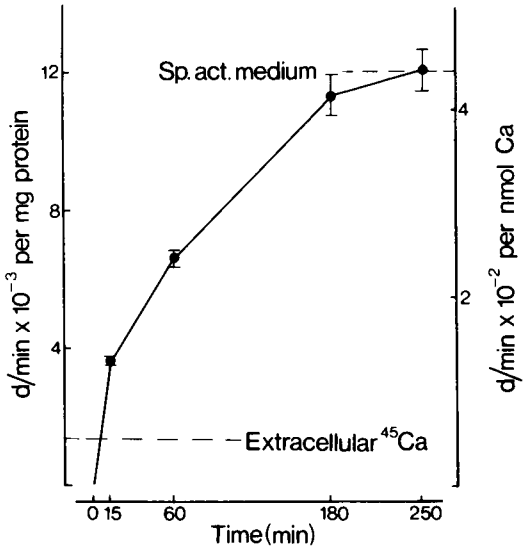

Figure 1 Time course of ^{45}Ca uptake in guinea-pig pancreas tissue lobules incubated for the time interval indicated on the abscissa scale in standard incubation medium containing 0.5 μCi of the tracer/ml. After incubation the lobules were thoroughly washed as described in the text but *not* incubated in chase medium. The ^{45}Ca content of the tissue is expressed as d/min per mg protein (left ordinate scale) as well as specific radioactivity (right ordinate scale) calculated on the basis of the calcium concentration of the pancreatic tissue reported previously (27.46 nmol/mg protein (Clemente & Meldolesi, 1975)). The extracellular calcium was calculated assuming a uniform distribution of the tracer in the extracellular compartment and knowing the size of the sorbitol space of the lobules, which amounts to 0.193 ml/g of tissue = 1.2 μl/mg protein. The latter values were determined in separate experiments in which comparable pancreatic tissue lobules were incubated for various intervals in incubation medium containing D-[1-^{3}H]-sorbitol (2 μCi/ml) (not shown in figures). The values are the mean of 4 experiments; vertical lines show s.e. mean.

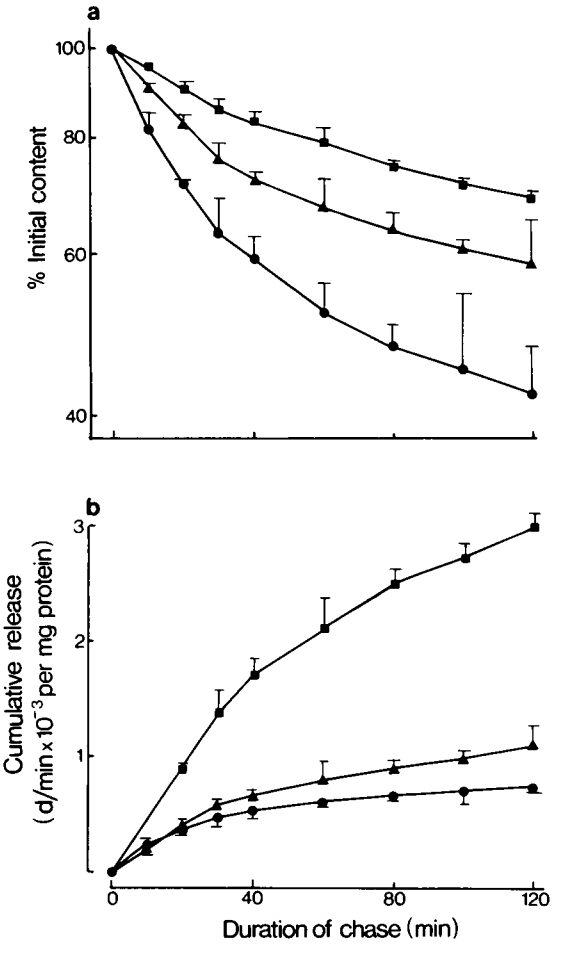

Figure 2 Effect of the length of the time of labelling with ^{45}Ca (0.5 μCi/ml) on the release of the tracer from pancreas tissue lobules during chase incubation. The data are plotted as (a) % calcium content of the tissue and (b) as cumulative release. Lobules obtained from the same animal were labelled for either 15 (●), 40 (▲) or 180 (■) minutes. After washing as described in the text the lobules were sequentially transferred to flasks containing chase medium with changes occurring after 5, 15, 25, 35, 45, 65, 85, 105 and 125 minutes. The radioactivity released during the first 5 min of chase incubation is not included in the results because it was considered to be primarily of extracellular origin. The values shown are the mean of 6 experiments; vertical lines show s.e. mean.

pancreas (27.46 nmol/mg protein (Clemente & Meldolesi, 1975), Figure 1) it becomes clear that this plateau corresponds to an almost complete equilibration of the intra and extracellular calcium pools, as suggested by the similar specific radioactivity of the tissue and incubation medium.

The release experiments are summarized in Figure 2, in which the efflux of ^{45}Ca from the pancreas lobules loaded for different time intervals is plotted against time. Figure 2a shows the time course of the changes of the ^{45}Ca content of the lobules during washout in chase medium, expressed as a percentage of the initial content; Figure 2b shows the cumulative release from the incubated lobules. On the basis of our sorbitol space data (see legend to Figure 1) we calculated that the ^{45}Ca released during the first 5 min of

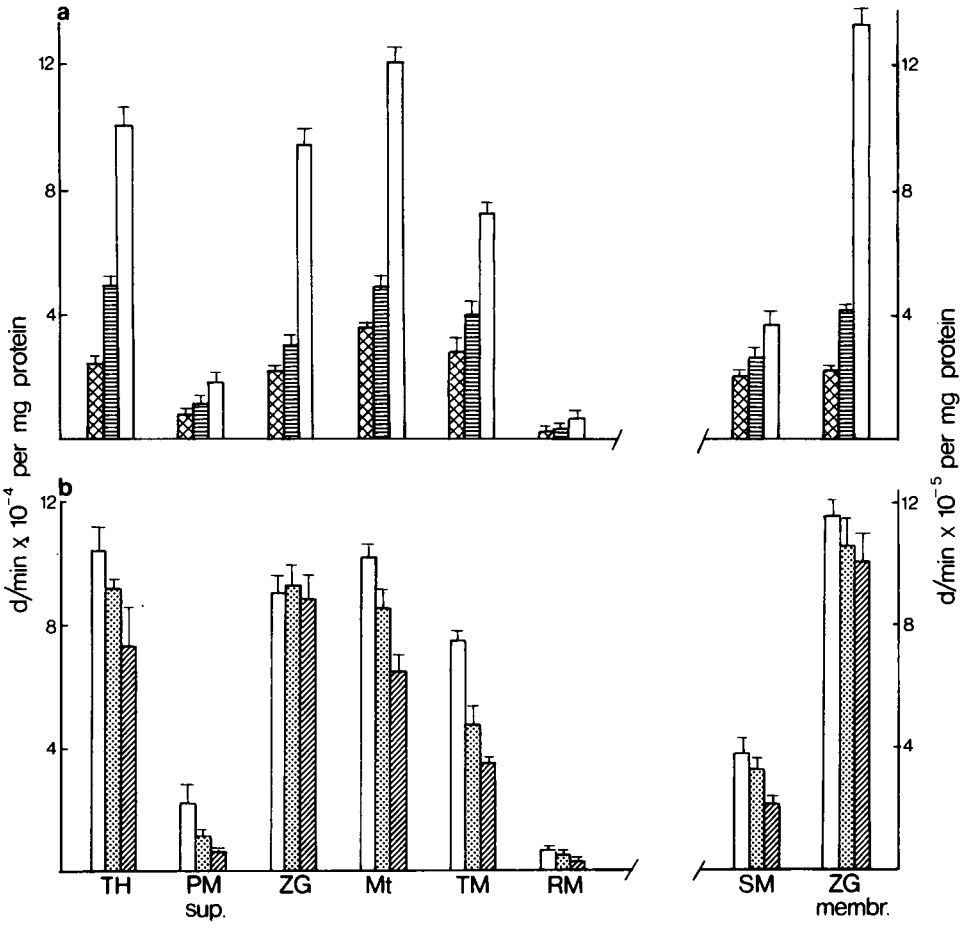

Figure 3 Distribution of ^{45}Ca in subcellular fractions isolated from pancreatic tissue lobules: (a) effects of the length of the labelling time and (b) of the chase time. In the experiments summarized in (a) the lobules obtained from 3 animals were pooled, then divided into 3 sets which were labelled with ^{45}Ca (5 μCi/ml) for either 15 (double diagonally hatched columns) 60 (horizontally hatched columns) or 180 (open columns) min. At the end of the labelling incubation the lobules were thoroughly washed, incubated in chase medium for 5 min, washed again and then homogenized. In (b) all three sets of lobules were labelled with ^{45}Ca (5 μCi/ml) for 3 hours. At the end of the labelling incubation the lobules were thoroughly washed and incubated in chase medium for either 5 (open columns), 45 (stippled columns) or 120 (diagonally hatched columns) min (with changes of medium occurring after the first 5 min and every 10 min thereafter) before washing and homogenization. Cell fractions were isolated from the homogenates by differential and density gradient centrifugation. TH = total homogenate; PM Sup. = postmicrosomal supernate; ZG = zymogen granules; MT = mitochondria; TM, RM and SM = total, rough-surfaced and smooth-surfaced microsomes; ZG membr. = zymogen granule membranes. Results are mean of 3 experiments; vertical lines show s.e. mean.

chase incubation corresponds to the bulk of the extracellular radioactivity. As a consequence, the radioactivity released during this time was not included in the results, i.e. time zero of Figure 2 corresponds to the 5 min chase incubation and the ^{45}Ca radioactivity remaining in the tissue at a given time (Figure 2a) is expressed as a percentage of the total content of the lobules at that time. Moreover, the cumulative release data do not include the contribution of the 0-5 min chase medium. It is probable, therefore, that the results shown in Figure 2 are primarily due to the release of the tracer originating from intracellular sources. As can be seen in the figure, such an efflux proceeds at a relatively slow rate and is greatly dependent on the length of the labelling time:

proportionally, the release is slower in the lobules labelled for 40 min than in those labelled for 15 min, and is even slower in those labelled for 3 hours. In all cases a constant slope is not obtained, even after 120 min of chase incubation.

Taken together, the results of the uptake and release experiments appear consistent with the idea that the intracellular calcium of the pancreas is distributed in a number of pools, whose rate of exchange is considerably slower than that across the plasmalemma. Analogous conclusions were reached previously by others (Case & Clausen, 1973; Matthews *et al.*, 1973; Heisler, 1974).

Experiments involving cell fractionation

The pancreatic homogenates obtained from tissue lobules exposed for various times to ^{45}Ca or incubated for various times in chase medium after prolonged labelling with the tracer were submitted to cell fractionation in order to estimate the kinetic features of the calcium pools associated with the different cell organelles. The characterization of the fractions used is reported in detail in previous publications (Meldolesi *et al.*, 1971; Clemente & Meldolesi, 1975). However, in the present investigation we have observed that the levels of radioactivity found in the crude mitochondrial pellet, isolated from labelled tissue lobules by differential centrifugation, remains practically unchanged after repeated resuspensions and reisolations (partially purified mitochondria). Thus, these two mitochondrial fractions are considered together in this paper.

The various fractions under study were found to accumulate the radioactivity in very different amounts (Figure 3). In particular, the rough-surfaced microsomes and the postmicrosomal supernatant are labelled to only a small extent during *in vitro* incubation of tissue lobules with ^{45}Ca; the zymogen granules and the mitochondria show levels of radioactivity comparable to those of the total homogenate, whereas the smooth-surfaced microsomes and, especially, the zymogen granule membranes show very high levels. Also in the time course of the uptake and release of the tracer a considerable heterogeneity was observed among the cell fractions. Some of them, such as the postmicrosomal supernatant, the rough and smooth-surfaced microsomes, accumulate the tracer during the labelling at a rate faster than that of the homogenate (Figure 3a). It is interesting to note that in these same fractions the rate of release of ^{45}Ca is also fast, as observed in the experiments reported in Figure 3b, in which the lobules were labelled first for 3 h (in order to reach an almost complete equilibration of all the different intracellular calcium pools with the incubation

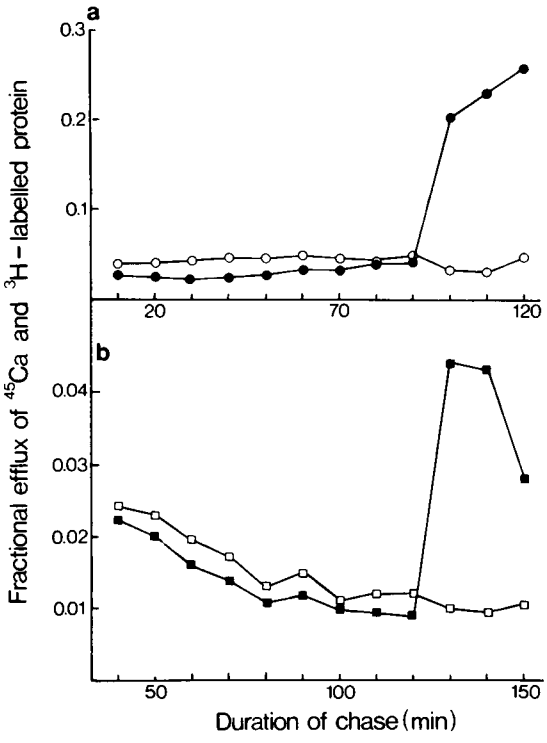

Figure 4 Effect of caerulein on the release of (a) ^{3}H-labelled proteins and (b) ^{45}Ca from pancreatic tissue lobules. The lobules were first labelled with ^{45}Ca (0.5 μCi/ml) for 60 min, then washed and reincubated in chase medium for the total time indicated on the abscissa scale of (b) with changes of medium occurring every 10 minutes. The medium used from the 20th to the 30th min of this incubation contained L-[4, 5-^{3}H]-leucine (5 μCi/ml). Caerulein (10^{-8} M) was added to half of the flasks (● and ■) during the last three 10 min intervals of incubation (120-150 min of chase incubation with respect to the end of ^{45}Ca labelling). The other flasks acted as controls (○ and □). At the end of the incubations the lobules were homogenized. Aliquots of the media and homogenates were analyzed for TCA insoluble ^{3}H and for ^{45}Ca radioactivity. The results are expressed as fractional efflux, i.e., as the ratio of the radioactivity released to the total radioactivity at each time point. The values shown are the average of 2 similar experiments.

medium) and then reincubated up to 2 h in chase medium before cell fractionation. A different behaviour was observed in other fractions: the mitochondria have rates of uptake and release comparable with those of the homogenate; in zymogen granules and in their membranes the rate of accumulation is slow and there is little release during chase.

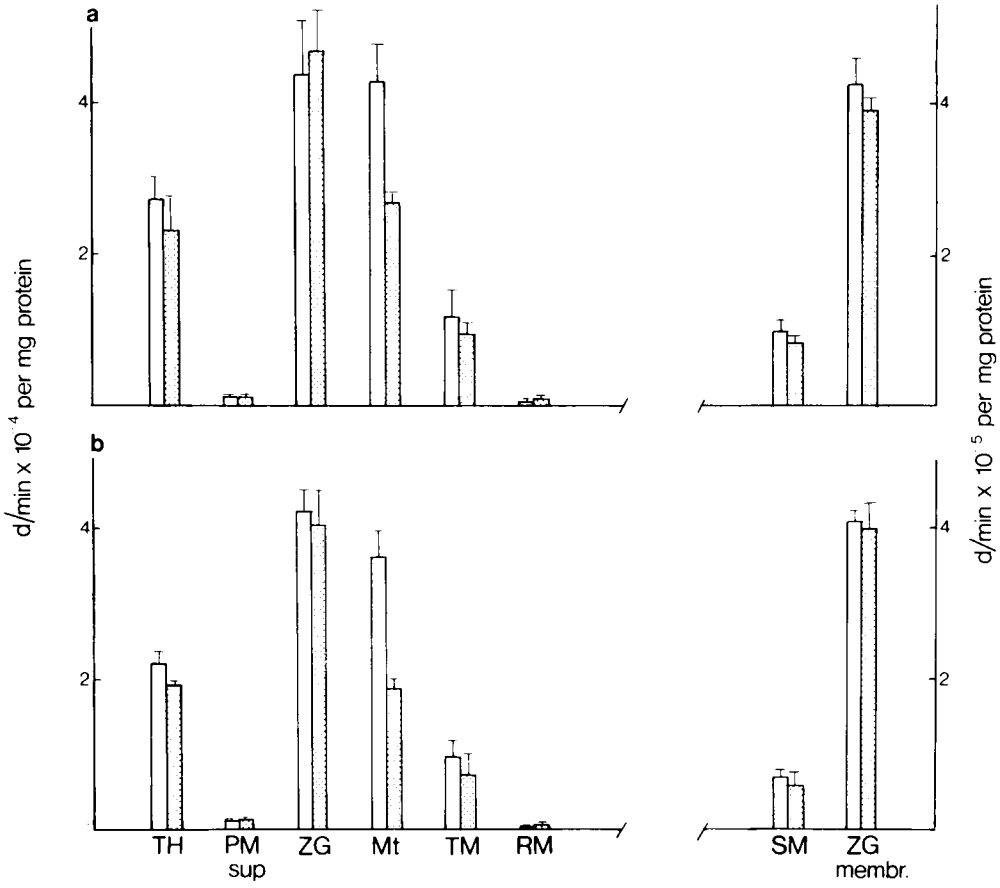

Figure 5 Effect of caerulein on the subcellular distribution of ^{45}Ca in pancreatic tissue lobules. Sets of lobules were labelled *in vitro* with ^{45}Ca (5 μCi/ml) for 60 min and then incubated in chase medium for a total of 120 min, with changes of medium occurring every 10 minutes. Half of them were then incubated in non-radioactive medium with (stippled columns) or without (open columns) caerulein (10^{-8} M) for either (a) 5 min or (b) 30 minutes. Stimulated and control lobules were washed, homogenized and fractionated by centrifugation. TH = total homogenate; PM sup. = postmicrosomal supernate; ZG = zymogen granules; Mt = mitochondria; TM, RM and SM = total, rough-surfaced and smooth-surfaced microsomes; ZG membr. = zymogen granule memebranes. The results shown are the mean of 3 experiments; vertical lines show s.e. mean.

Experiments on pharmacologically stimulated lobules

It has been previously reported that, in preparations of rat pancrease incubated *in vitro*, the stimulation of secretion results not only in the release of the secretion products segregated within the zymogen granules, but also in an efflux of calcium from the acinar cells, most probably occurring through the plasmalemma (Case & Clausen, 1973; Matthews *et al.*, 1973; Heisler, 1974; Chandler & Williams, 1974). The existence of this phenomenon was verified in the pancreas of the guinea-pig by using tissue lobules first doubly labelled with ^{45}Ca and with L-[3,4-^{3}H]-leucine, and then incubated in chase medium long enough (a) to have the ^{3}H-labelled exportable proteins transported to the granules and (b) to minimize the variations of the fractional efflux of the ^{45}Ca. Finally these lobules were stimulated with caerulein, a polypeptide of known pancreozymin-like activity (Bertaccini, De Caro, Endean, Erspamer & Impicciatore, 1969; Meldolesi, 1970). As shown in Figure 4, the stimulation of secretion produces approximately a four-fold increase in the release of both the ^{45}Ca and the ^{3}H-labelled

proteins. The efflux of ^{45}Ca cannot be due entirely to discharge by exocytosis, concomitantly with the proteins, because the ^{45}Ca/TCA-insoluble-^{3}H ratio observed in the medium is considerably (approximately four-fold) higher than that found in the zymogen granules isolated from doubly labelled pancreatic lobules (not shown in figures).

In order to investigate the intracellular events which are the basis of the increased calcium efflux triggered by the stimulation, we compared the distribution of the ^{45}Ca in subcellular fractions isolated from pancreatic lobules incubated for either 5 or 30 min with or without caerulein after *in vitro* loading with the tracer followed by chase incubation. As can be seen in Figure 5, no significant changes in radioactivity were found in the isolated fractions except for the mitochondria. In the latter fraction the exposure to the secretagogue drug produced approximately a 40% decrease which was evident 5 min after the onset of the treatment and persisted until the 30th minute.

Discussion

Calcium is known to play a critical role in all living cells because it is involved in the regulation of a number of fundamental functions, such as membrane permeability, enzyme activities, contractile processes, cell growth and adhesion. In secretory cells the importance of calcium is even greater because a compelling body of evidence, obtained in a number of different systems, indicates that a sudden rise of the concentration of free Ca^{2+} in the soluble cytoplasm is the common trigger of the secretory response (for reviews see Douglas, 1968; Rubin, 1970; Rasmussen, 1970; Matthews, 1970, 1974; Thorn, 1974). Such an increase in the free Ca^{2+} may occur because of an enhanced membrane permeability induced by the action of either the specific secretagogues (Douglas & Rubin, 1961; Douglas & Poisner, 1962; Douglas, 1968; Rubin, 1970) or calcium ionophores (Prince, Rasmussen & Berridge, 1973; Foreman, Mongar & Gomperts, 1973; Eimerl, Savion, Heichal & Selinger, 1974; Cochrane & Douglas, 1974; Kagayama & Douglas, 1974; Williams & Lee, 1974; Russel, Hansen & Thorn, 1974; Garçia, Kirpekar & Prat, 1975) as well as through a redistribution of the intracellular store, from the organelle-bound to the soluble pools. Recent experiments suggest that in a number of secretory systems, both endocrine and exocrine, including the pancreatic acinar cells, the latter mechanisms might account for the physiological regulation of secretion, i.e., that the free Ca^{2+} concentration of the soluble cytoplasm is controlled primarily by intracellular events (Milligan & Kraicer, 1971; Nielsen & Petersen, 1972; Case & Clausen, 1973; Matthews *et al.*, 1973; Malaisse, 1973; Milligan & Kraicer, 1974; Chandler & Williams, 1974). It is clear therefore that a detailed knowledge of the characteristics of the different intracellular calcium pools represents a necessary prerequisite to the understanding of the secretory process.

The information which is available on this problem has been mostly obtained by two different experimental approaches. On the one hand, the transcellular fluxes of calcium have been thoroughly studied in a number of secretory systems, under a variety of carefully controlled experimental conditions. The results obtained so far appear always consistent with the existence of a considerable degree of intracellular calcium compartmentalization (Milligan & Kraicer, 1971; Nielsen & Petersen, 1972; Case & Clausen, 1973; Matthews *et al.*, 1973; Malaisse, 1973; Milligan & Kraicer, 1974; Chandler & Williams, 1974). However, the direct identification of the various pools and the elucidation of their specific functional role is beyond the potentialities of these experiments.

Important results have also been obtained by investigating the *in vitro* binding ability of isolated subcellular structures (Poisner & Hava, 1970; Selinger, Naim & Lasser, 1970; Alonso, Bazerque, Arrigo & Tumilasci, 1971; Carafoli, 1973; Smith, Argent & Case, 1974). However, an obvious limitation of this type of study is that the data obtained are hard to correlate with the situation existing in the living cells, since the binding is greatly dependent on a number of conditions, such as the pH and the ionic strength of the suspending media, which in the *in vitro* experiments are selected arbitrarily, at least to a certain degree.

In principle, the approach that we have followed in the present investigation on the exocrine pancreas (and which had been followed previously by others working on the adrenal medulla; Borowitz, 1969), i.e. the study of well characterized subcellular fractions isolated from cells labelled with ^{45}Ca under controlled conditions, appears the most adequate to investigate directly some of the features and functions of the various calcium pools present in a secretory tissue. However, this approach too suffers a severe limitation because an extensive redistribution of the intracellular calcium is known to occur during homogenization and fractionation of the tissue. As discussed in detail in a previous paper (Clemente & Meldolesi, 1975), the quantitative evaluation of this type of artefact is laborious and sometimes not feasible. Thus, all our results should be considered with great caution.

However, we believe that our previous results

(concerning the distribution of calcium in cell fractions isolated from fresh pancreas tissue; Clemente & Meldolesi, 1975) were much more affected by this type of artefact than those reported here. This conclusion is based on the fact that in the present cell fractionation experiments the bulk of the extracellular ^{45}Ca was removed from the lobules by a short incubation in chase medium, (see legend of Figure 2). Thus the influence of this source of artefact was greatly decreased. Moreover, the concentration of ^{45}Ca in subcellular structures is probably not greatly affected by the binding of the non-radioactive calcium present in small amounts in the solutions used. The fact that the binding artefacts do not have a major influence on our results is also indicated by the difference of ^{45}Ca distribution in cell fractions isolated from *in vitro* labelled pancreatic lobules (Figure 3, this paper) relative to the comparable fractions isolated from non-radioactive tissue homogenized in 0.3 M sucrose containing the tracer (Table 4, Clemente & Meldolesi, 1975). It should be noted that the fraction which is most heavily labelled in the latter condition, the rough-surfaced microsomal fraction, is the one which shows the lowest level of radioactivity when isolated from *in vitro*-labelled lobules; the converse is true for the mitochondria and zymogen granule membranes. Finally, the tracer associated with the fractions isolated from the loaded lobules is bound much more tightly than that adsorbed at the moment of homogenization. For instance, the partial purification of the crude mitochondria by repeated washes of the organelles did not appreciably change the radioactivity/protein ratio of the fraction isolated from the lobules but produced a considerable decrease of the same ratio in the fraction exposed to the tracer at the moment of homogenization (see Table 4, Clemente & Meldolesi, 1975). Analogous results were obtained with the zymogen granule membranes.

It seems reasonable to conclude, therefore, that, even if the results reported in the present paper are open to a degree of uncertainty, nevertheless they appear useful since they provide some further indication as to the distribution of calcium in the pancreatic acinar cells and permit some insight into the functional role of the different intracellular calcium pools.

In our work we have found that the concentration of calcium is relatively low in the post microsomal supernatant: for instance, after 3 h of *in vitro* incubation with ^{45}Ca the radioactivity recovered in this fraction/mg protein amounts to 1.8×10^4 d/min which is approximately one sixth of that of the total homogenate. If we assume that at this time the calcium of the supernatant is virtually in equilibrium with that of the incubation medium we can calculate the total calcium concentration of the fraction as approximately 150 nmol/g of tissue. (These calculations are based on the corrected subcellular distribution of protein in the guinea-pig pancreas reported by Meldolesi, 1974b.) However, in all tissues so far investigated, the concentration of the free Ca^{2+} of the soluble cytoplasm has been found to be much lower, $10^{-5} - 10^{-8}$ M (see Rasmussen, 1970; and Thorn, 1974; for reviews). Thus, it is most likely that only a small proportion of the calcium recovered in the post microsomal supernatant is accounted for by the free Ca^{2+}, whereas the bulk of it is contributed by calcium originally complexed with other molecules of the soluble cytoplasm or artefactually released from particulate pools. These considerations explain why there is little change in the ^{45}Ca radioactivity of the postmicrosomal supernatant after pharmacological stimulation of secretion, a condition in which the concentration of only the free Ca^{2+} is expected to rise considerably.

The calcium associated with the cytoplasmic organelles appears to be mostly divided among three different major pools: those associated with the mitochondria, with the Golgi complex (the major component of the pancreatic smooth-surfaced microsomes; Meldolesi *et al.*, 1971) and with the zymogen granules. Since the subcellular distribution of protein in the guinea-pig pancreas is known (Meldolesi, 1974b), it can be calculated that of the total ^{45}Ca taken up by the pancreatic lobules during 3 h of *in vitro* labelling approximately 17%, 20% and 30%, respectively, can be recovered in the corresponding fractions. Little calcium is probably associated in the living cell with the rough endoplasmic reticulum: probably the considerable calcium level found in the rough microsomes by direct assay (Clemente & Meldolesi, 1975) is an artefact, as suggested by the high labelling of that fraction in the adsorption experiments (Table 4, Clemente & Meldolesi, 1975) and by its very low labelling in the present experiments with tissue lobules.

The functional significance of the three major pools is probably quite different. Two of them (those associated with the mitochondria and smooth microsomes) appear to have relatively fast uptake and release properties, compatible with a direct involvement in the regulation of the Ca^{2+}-dependent cellular functions. In this respect it should be noticed that in other tissues the organelles recovered in theses two fractions have been found to be capable of energy-dependent calcium binding (Selinger *et al.*, 1970; Poisner & Hava, 1970; Alonso *et al.*, 1971; Carafoli, 1973).

Furthermore, among the various cell fractions investigated, the mitochondrial fraction was the only one which exhibited a significant decrease in ^{45}Ca early after pharmacological stimulation of secretion with caerulein. Even if the results obtained so far are not sufficient to exclude the contribution of the other cellular pools in the stimulus-secretion coupling, nevertheless they seem to represent the first direct indication that the Ca^{2+} involved in such a process might be primarily of mitochondrial origin. This conclusion seems to correlate well with the recent observation by Nishiyama & Petersen (1975) that in pancreatic acinar cells an increase in Na conductance at the level of the plasmalemma represents the primary event after exposure to secretagogue drugs, because a rise in Na^+ concentration of the medium is known to promote specifically the release of calcium from isolated mitochondria (Carafoli, 1973). Furthermore, it has been recently reported that *in vitro* treatment of pancreas preparations with drugs known to interfere with the mitochondrial calcium binding results in a stimulation of secretion (Smith *et al.*, 1974).

In contrast, the slow kinetics of ^{45}Ca accumulation in zymogen granules during tissue labelling and the stability of the granule-associated radioactivity during chase incubation, even in the presence of secretagogue drugs, strongly suggest that the calcium pool associated with this type of organelle is not directly in equilibrium with the rest of the intracellular calcium store. These findings further confirm the idea that in the pancreas, as previously suggested in other secretory systems (Berneis *et al.*, 1969; Wallach & Schramm, 1971), the role of the calcium segregated within the secretion granules might be concerned not with the regulation of the cellular activity but with the architecture of the granules themselves and with the functionality of the released secretion products (Clemente & Meldolesi, 1975; Ceccarelli, Clemente & Meldolesi, 1975).

The association of large amounts of ^{45}Ca with the membranes of the zymogen granules deserves a special comment. Both the slow rate of accumulation and the stability during chase suggest that this pool is not involved in stimulus-secretion coupling. Rather, it might play an essentially structural role. In pancreatic acinar cells the membranes of zymogen granules are the membranes specifically capable of fusing with and being incorportated into the apical portion of the plasmalemma during exocytosis. In a variety of cell types the plasmalemma has been found to bind considerable amounts of calcium (Forstner & Manery, 1971; Perdue, 1971; Shlatz & Marinetti, 1972; Nijjar & Pritchard, 1973). From cytochemical evidence (Goodford & Wolowyck, 1972; Herman *et al.*, 1973) and from the results of kinetic experiments (Borle, 1969a,b), it appears that a large proportion of this calcium is associated with the outer surface of the plasmalemma, where it is assumed to produce significant changes in membrane organization and properties (Manery, 1966; Burger, Fuji & Hanahan, 1968; Romero & Whittam, 1971). This function of calcium is presumably related to the capacity of the cation to form complexes and to bridge adjacent negative charges thereby producing alterations in the physical state of membrane components which result in the stiffening of the structure and in the decrease of the permeability of the membrane itself (Hanahan, 1966; Burger *et al.*, 1968; Reynolds, 1972).

If one bears in mind that the large calcium pool associated with the zymogen granule membrane appears to be mostly localized at its inner face, i.e., at the face which after exocytosis becomes exposed at the outer surface of the cell (Clemente & Meldolesi, 1975), it is difficult to escape the suggestion that the granule membrane is equipped for becoming a temporary portion of the plasmalemma not only in relation to its specific protein and lipid composition (Meldolesi, De Camilli & Peluchetti, 1974) but also to its calcium 'coating'. Whether the high calcium binding capacity of the zymogen granule and plasma membranes are due to analogous molecular mechanisms is now under investigation.

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