CALCIUM AND PANCREATIC SECRETION

I. Subcellular Distribution of Calcium and Magnesium in the Exocrine Pancreas of the Guinea Pig

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ABSTRACT

The distribution of calcium and magnesium has been studied in the acinar cells of the pancreas of the guinea pig. Most of the magnesium was found to be associated with the rough microsomes (probably bound to the ribosomes) and with the postmicrosomal supernate. In contrast, calcium was distributed among all the particulate fractions, primarily the mitochondria, microsomes (especially smooth surfaced), zymogen granules, and the plasmalemma, and was low in the postmicrosomal supernate. Most of the calcium recovered in the particulate fractions was found to be membrane bound. The highest concentrations were found in the membranes of the zymogen granules and in the plasmalemma.

By means of control experiments using ^{45}Ca as the tracer, it was established that a considerable redistribution of calcium occurs during homogenization and cell fractionation. At least some of the resulting artifacts were estimated quantitatively and the data were corrected accordingly. The biochemical results were confirmed with the cytochemical antimonate technique carried out on the tissue as well as on isolated fractions.

The role of calcium associated with the zymogen granules and with their limiting membranes is discussed in relation to the architecture of the granule and to the functionality of the pancreatic juice.

It has been clearly established in a number of cell systems that calcium plays an essential role in several steps of the secretory process (1–4). Thus, calcium appears to be involved (*a*) in the intracellular packaging of secretion products (5, 6), (*b*) in the regulation of the permeability of the plasmalemma, including the permeability changes occurring after stimulation of secretion (1–4, 7, 8), and (*c*) in the complex series of events which transduce the stimulation of the secretory cell into its specific secretory response (stimulus-secretion coupling) (1–4, 7–12).

Since the pioneer work of Douglas and his associates on the adrenal medulla, the calcium acting in the stimulus-secretion coupling was assumed to be of extracellular origin (9–11). This

origin was envisaged in the pancreas as well because in this tissue as in the medulla secretion depends on the availability of extracellular calcium (13–16). However, recent studies on the kinetics of secretion inhibition in Ca^{2+}-free media and on the transcellular movements of the ion (17, 18) suggest that in the pancreas the role of the extracellular calcium would be limited to the preservation of the plasmalemma function, whereas the stimulus-secretion coupling would be effected primarily through the intracellular store(s) of the metal: the secretory response would be triggered by a sudden calcium redistribution within the acinar cells, resulting in a temporary rise of a readily exchangeable pool (17, 18). Thus, the transduction mechanisms operating in the pancreas would be somewhat analogous to those previously identified in skeletal muscle cells, whose physiological response is known to be mediated through the release of Ca^{2+} from intracellular storage organelles (excitation-contraction coupling) (19, 20).

This interpretation requires the exsistence in pancreatic acinar cells of cytoplasmic organelles capable of accumulating and releasing calcium according to the functional state of the gland. This problem has been considered in our study: in the present report we will describe the intracellular distribution of calcium in the pancreas of the guinea pig, as revealed by an investigation carried out by the combined cytochemical and cell fractionation techniques.

MATERIALS AND METHODS

Male albino guinea pigs weighing 450–550 g (gift of Sigurtà Drug Co., Milan, Italy) were starved for 18–20 h, with water given ad libitum, and then sacrificed by a blow over the head followed by cardiosection. The pancreases were rapidly removed and immersed in ice-cold 0.3 M sucrose.

Cell Fractionation

The following subcellular fractions were isolated according to the techniques described previously (21, 22): zymogen granules (ZG),[1] crude mitochondria (cMt), total, rough-surfaced and smooth-surfaced microsomes (TM, RM, and SM, respectively), plasma membranes (PlM), and postmicrosomal (PM) supernate. In order to obtain a partially purified mitochondrial fraction (pMt), the cMt were washed twice (resuspension in 0.3 M sucrose followed by reisolation by differential centrifugation [12,000 rpm/10 min, Spinco 40 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) 13,500 g_{max}/10 min]).

ZG, RM, and SM were subfractionated in order to yield the corresponding membrane and soluble fractions according to the scheme described previously (21), except that a 10 mM Tris-HCl buffer, pH 8, containing 200 mM NaCl was used for lysing the organelles instead of the usual 200 mM $NaHCO_3$ solution, pH 8.

During this work the morphology and biochemistry of the isolated fractions were repeatedly checked. The results did not differ significantly from those we reported in previous publications (21–23).

Calcium and Magnesium Assay

The calcium and magnesium content of homogenates and cell fractions was assayed with a Hilger and Watts atomic absorption spectrometer, model Atomspek (Hilger & Watts Ltd., London, England) after deproteinization with cold 10% TCA and washing of the precipitate with 5% TCA. In order to overcome interferences, all the samples contained 1% La^{3+} and were adjusted at pH 1.5 before the assay; furthermore, samples containing concentrated sucrose were diluted to give final concentrations <0.6 M.

All the glassware and the plasticware used in this work was carefully washed with either 2 N HCl or 10 mM EDTA, pH 7.5, and then rinsed repeatedly with deionized distilled water. The ion contents of all the solutions used was carefully determined. They turned out to be 46 and 1.3 nmol of calcium and magnesium, respectively, per mol of sucrose; 8.75 nmol of calcium/liter in the Tris-HCl + NaCl buffer. The experimental results were corrected accordingly.

Other Analytical Procedures

Protein was determined by the modified Folin reaction of Lowry et al. (24), using bovine serum albumin as standard. ^{45}Ca radioactivity was determined by dissolving the samples in 10 ml of Packard Instagel (Packard Instrument Co., Inc., Downers Grove, Ill.) and counting in an SL 30 Intertechnique liquid scintillation spectrometer. Correction for quenching was made by external standardization.

Enzyme Assays

Cytochrome *c* oxidase was assayed as described by Cooperstein and Lazarow (25), and trypsin and chymotrypsin as described by Hummel (26). For activating trypsinogen and chymotrypsinogen, homogenates and

[1] *Abbreviations used in this paper:* cMt, crude mitochondrial fraction; EGTA, ethylene glycol bis(β-aminoethyl ether *N,N,N',N'*)tetraacetic acid; GC, Golgi complex; PlM, plasma membrane; PM supernate, postmicrosomal supernate; pMt, purified mitochondrial fraction; RER, rough-surfaced endoplasmic reticulum; RM, rough-surfaced microsomes; SM, smooth-surfaced microsomes; TM, total microsomes; ZG, zymogen granules.

cell fractions were incubated at +2°C with porcine enterokinase in 0.1 M Tris-HCl buffer, pH 7.8, containing 0.08 M NaCl and 0.1 M $CaCl_2$. Details on the kinetics of trypsin activation will be given elsewhere.

Electron Microscopy

Tissue fragments and isolated cell fractions were fixed and processed for conventional electron microscopy as described previously (21). Furthermore, a number of experiments were carried out in order to investigate the intracellular localization of cations by the cytochemical technique of Komnick and Komnick (27), as modified by Spicer et al. (28). To this end the specimens were fixed either with 1% OsO_4 containing 0.05 M potassium pyroantimonate pH 7.5 for 2 h or with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 2 h, then washed four times in 0.3 M sucrose and postfixed in 1% OsO_4 containing 0.05 M potassium pyroantimonate, pH 7.5, for 2 h. The specimens were dehydrated in ethanol and embedded in Epon 812 (Fisher Scientific Co., Fair Lawn, N. J.). Stained and unstained thin sections were examined for electron-opaque precipitates. The lack of specificity of the antimonate procedure made necessary an analysis on the nature of the precipitates. This was carried out by two different approaches: unstained grids were treated at 50°C for 10–60 min with the Ca^{2+} chelator ethylene glycol bis(β-aminoethyl ether *N,N,N',N'*)-tetraacetic acid (EGTA), 5 mM, pH 7.4, then stained with uranyl acetate and lead citrate and examined in the electron microscope. Furthermore, unstained thick (~0.5 μm) sections of ZG membrane pellets containing antimonate deposits were analyzed for atomic elements (29) with a CAMECA model MS 46 X-ray microanalyzer (CAMECA, Courbevoie, France), operated at an acceleration voltage of 20 kV and at a probe current of 10 μA, according to the technique described by Libanati and Tandler (30). The radiations used were the Lα1 for antimony and the Kα1 for the lighter metals. The analysis was kindly performed by Mr. J. Orrit at the Laboratoires Elf, Service Analyses Physiques, Toulouse, France.

RESULTS

Subcellular Distribution of Calcium and Magnesium

The levels of calcium and magnesium in the guinea pig pancreas and in the fractions isolated therefrom are reported in Table I, which also includes data on the distribution of enzymes (the secretory proteolytic zymogens, trypsinogen and chymotrypsinogen, and the mitochondrial marker enzyme cytochrome *c* oxidase). Both elements were found to be highly concentrated in the tissue; however, their subcellular distribution was quite different. Magnesium was mostly recovered in the microsomes and was very low in ZG. Calcium, on the other hand, was nearly equally distributed

TABLE I

Distribution of Chymotrypsinogen, Trypsinogen, Cytochrome c Oxidase, Calcium, and Magnesium in Pancreatic Homogenate and in the Cell Fractions Isolated Therefrom

	Chymotrypsinogen*	Trypsinogen*	Cytochrome *c* oxidase*	Ca	Mg
	nmol/mg protein		*nmol/mg protein*	*nmol/mg protein*	
Homogenate	3.12 ± 0.40 (4)	19.67 ± 1.50 (4)	10.20 ± 1.73 (7)	27.46 ± 1.51 (10)	81.31 ± 7.9 (9)
ZG	7.49 ± 0.68 (6)	46.03 ± 4.68 (4)	5.29 ± 1.97 (4)	36.57 ± 3.05 (7)	9.16 ± 1.33 (7)
cMt	2.12 ± 0.5 (2)	—	47.71 ± 6.59 (3)	32.90 ± 5.00 (5)	72.1 ± 4.00 (5)
pMt	0.70 ± 0.00 (3)	—	101.33 ± 7.02 (3)	27.40 ± 1.85 (4)	31.72 ± 2.04 (4)
TM	1.39 ± 0.12 (4)	—	10.51 ± 1.18 (3)	37.41 ± 3.72 (7)	152.28 ± 9.92 (7)
PlM	0 (2)	—	0 (2)	327.00 ± 98.00 (2)	91.00 ± 49.74 (2)
PM supernate	0.82 ± 0.14 (6)	—	0 (6)	13.56 ± 1.41 (8)	51.18 ± 6.61 (8)

Values given are averages ± SE. Number of experiments is shown in parentheses.

* The figures represent chymotrypsin and trypsin: nanomoles of *N*-benzoyl-L-tyrosine ethyl ester and (*p*-tosyl-L-arginine) methyl ester split per minute at 25°C per milligram protein of the preparations activated as described under Materials and Methods; cytochrome *c* oxidase, nanomoles of cytochrome *c* oxidized per minute at 25°C per milligram protein.

among the three major cytoplasmic particulate fractions, ZG, cMt and TM and was low in the PM supernate. The PIM fraction was rich in magnesium and very rich in calcium.

TM were separated by gradient centrifugation to yield the corresponding RM and SM fractions. The first fraction contains nearly exclusively vesicles derived from the fragmentation of the rough-surfaced endoplasmic reticulum (RER), while the second is primarily accounted for by elements of the Golgi complex (GC) and also included small fragments of PIM, pinocytotic vesicles, etc. As shown in Table II, the bulk of the magnesium turns out to be associated with the RM, whereas calcium is equally distributed between the two fractions. In an attempt to identify the stuctural components (membranes, ribosomes, and soluble segregated material) responsible for ion binding, the two microsomal fractions, as well as the ZG, were subfractionated as described under Materials and Methods. As can be seen in Table II, we found that in RM and SM the bulk of calcium remains associated with the membranes, whereas a large amount of the magnesium is released roughly proportionally to the amount of RNA distributed (not shown).

In ZG (Table III), only a minor proportion of either element remains associated with the soluble subfraction, which accounts for the bulk of the protein and for nearly the total of the ZG-segregated secretory enzymes (21). In contrast, the concentrations found in the ZG membranes separated from the ZG content by differential centrifugation and then purified by sucrose density gradient are higher than in any other fractions and comparable only to those in the PIM: 395.2 nmol of Ca and 200.1 nmol of Mg/mg of protein.

Control Experiments

These experiments were conducted in order to establish whether, and to what extent, the distribution of calcium found in the different cell fractions isolated from the guinea pig pancreas was in-

TABLE II

Distribution of Calcium, Magnesium, Phospholipid, and RNA in RM and SM and in the Membrane Fractions Isolated Therefrom

	Ca	Mg	Phospholipid*	RNA*
	nmol/mg protein		*μg/mg protein*	
RM (4)	45.15 ± 9.08	127.67 ± 19.64	98.6	360.0
RM membranes (3)	75.22 ± 14.36	46.08 ± 14.01	190.4	124.0
SM (3)	39.70 ± 3.31	38.17 ± 13.52	248.0	43.0
SM membranes (3)	95.66 ± 26.78	17.11 ± 8.28	438.0	12.5

Values given are averages ± SE. Number of experiments is shown in parentheses.
* Data from reference 21.

TABLE III

Distribution of Calcium, Magnesium, and Secretory Proteolytic Enzymes in ZG and in the subfractions Isolated Therefrom

	Chymotrypsinogen	Trypsinogen	Ca	Mg
	nmol/mg protein		*nmol/mg protein*	
ZG	7.49 ± 0.68 (6)	46.03 ± 4.68 (4)	36.57 ± 3.05 (7)	9.16 ± 1.33 (7)
ZG soluble	8.14 ± 0.90 (6)	51.74 ± 7.41 (4)	18.97 ± 3.10 (7)	9.34 ± 0.71 (7)
ZG membranes	0 (4)	0 (4)	395.30 ± 60.09 (6)	200.10 ± 40.23 (7)

Values given are averages ± SE. Number of experiments is shown in parentheses.
Data as in Table I.

fluenced by an artifactual redistribution of the element taking place during and after tissue homogenization. In principle, chances of artifacts are possible at all the steps of the various isolation procedures. First of all, it is likely that during and after tissue homogenization the calcium, which in the living cell is associated with the organelles by low affinity binding, might be released. Furthermore, the organelles also might lose calcium as a consequence of either mechanical damage (for instance, of ZG) or a cut-off of an energy-dependent binding (for instance, to the mitochondrial inner membrane) (31, 32). This amount of calcium, which in the living cell is particle bound, will therefore become soluble in the homogenate. On the other hand, it is also likely that upon homogenization a certain proportion of the total soluble calcium (including that originally present in the cell sap, that artifactually released from the organelles, as well as that present in the solutions used) might become adsorbed onto cell structures. Finally, in the case of fractions whose isolation requires numerous centrifugation steps, the possibility that the structures may become loaded with ions adsorbed from the different solutions in which they are successively suspended should be considered.

At the moment, artifacts of the first type (release of calcium from the particles) cannot be quantitatively evaluated. Therefore, their correction is unfeasible. In contrast, adsorption of soluble calcium onto particles can be quantitated by determining the subcellular distribution of ^{45}Ca added either to the homogenization fluid or to the other solutions used during cell fractionation. Table IV summarizes the data obtained in the experiments in which ^{45}Ca was added to the homogenization fluid and in which the fractions were isolated according to the usual procedures. The redistribution of the tracer turned out to be very extensive: only a minor proportion (~18%) of the dose added was recovered in the final PM supernate. Most of it was found associated with the particulate fractions, especially with the RM. Lower levels were found associated with the SM and cMt, while in ZG the amount of binding appears relatively minor (radioactivity per milligram protein = ⅓ of the homogenate).

These data on the recovery of the ^{45}Ca added to the homogenate can be used to calculate the total amount of calcium artifactually adsorbed by each fraction, provided (*a*) the specific radioactivity of the adsorbing calcium pool is known and (*b*) the

TABLE IV

Recovery of ^{45}Ca Added to the Homogenization Fluid in Subcellular Fractions Isolated from the Pancreas of the Guinea Pig

	cpm/mg protein
Homogenate	115,000
ZG (4)	22,490 ± 3,900
cMt (2)	110,500 ± 14,300
pMt (3)	33,830 ± 4,050
TM (3)	183,850 ± 21,000
RM (2)	287,000 ± 40,000
SM (3)	77,660 ± 15,000
PlM (1)	5,000
PM supernate (4)	83,320 ± 2,110

Fresh pancreas tissue (~1.2 g) was homogenized in 10 vol of 0.3 M sucrose containing 1 μCi/ml of ^{45}Ca. The fractions were isolated from the homogenate according to the usual procedures.

Values given are averages ± SE. Number of experiments is shown in parentheses.

^{45}Ca adsorbed at the moment of the homogenization is not exchanged during cell fractionation with unlabeled calcium present in the solutions used. In order to determine the specific radioactivity of the adsorbing calcium pool, the PM supernate obtained from a homogenate made in 0.3 M sucrose containing ^{45}Ca was submitted to column chromatography on Sephadex G-25 (Pharmacia Fine Chemicals Inc., Uppsala, Sweden), under pH and ionic conditions analogous with those present in the homogenate: ~27% of the calcium present in the fraction was eluted together with the proteins in the void volume of the column and was therefore considered nonexchangeable (Table V). The fraction included in the column was considered exchangeable and its specific radioactivity was used for the correction of the adsorption artifacts.

The second assumption is clearly valid for those fractions, such as the cMt and the TM, which are isolated directly from the homogenate by differential centrifugation, and therefore do not come into contact with other solutions. Thus, for these fractions the amount of adsorbed calcium was calculated as indicated in Table VI. The corre-

TABLE V

Sephadex Chromatography of the PM Supernate

	A_{280}	Ca	Mg	^{45}Ca
	%	%	%	%
Void volume	52.64	27.20	1.86	8
Retained fraction	34.00	75.00	87.00	100
Recovery	86.64	102.20	88.86	108

Pancreas tissue was homogenized in 10 vol of 0.3 M sucrose containing 1 μCi of ^{45}Ca/ml (see Table IV) and the PM supernate isolated by high-speed centrifugation. A sample (1.5 ml) was loaded onto a column (35 cm in length, ½ cm in diameter) filled with Sephadex G-25 coarse. Elution buffer: 2(*N*-morphine) ethane sulfonic acid 10 mM, pH 6.5, containing 16 mM KCl. Flow rate: 20 ml/h, at 2°C. The samples corresponding to the void volume and to the fractions retained in the column were pooled and analyzed for absorbancy at 280 nm (protein), calcium, magnesium, and ^{45}Ca.

TABLE VI

Calcium Distribution in Pancreatic Cell Fractions: Estimation and Correction of Adsorption Artifacts

	Calcium adsorption*	Corrected calcium concentration‡
	nmol/mg protein	
cMt	10.85	22.05
TM	17.58	19.83

* The amount of calcium adsorbed onto the pancreatic organelles during cell fractionation was calculated on the basis of the results reported in Tables IV and V, according to the criteria illustrated in the text.

‡ The corrected calcium concentration data were obtained by subtracting the adsorbed calcium from the total calcium of the fractions (Table I).

sponding corrected data on the calcium distribution are also included in that table.

However, in the case of the fractions whose isolation requires repeated washings and reisolations, such as the membranes of the ZG, RM and SM, and the PlM, most of the ^{45}Ca originally adsorbed at the moment of the homogenization was released from the particles in the course of their isolation. There are two possible explanations of this finding: either the adsorbed ^{45}Ca was simply removed from the membranes during their purification or it was exchanged with unlabeled calcium present in the solutions used. Were the first explanation true, correction of the distribution results should be made only in relation to the small amount of ^{45}Ca recovered in the fractions; however, if ^{45}Ca was exchanged, a larger correction factor, which cannot be deduced according to the simple criteria used so far, should be used.

This problem was investigated in detail only in the case of the ZG membrane, because we were particularly interested in establishing whether the large calcium concentration associated with that structure is an intrinsic property or is largely due to the ion redistribution. The experimental protocol that we developed was as follows:

Since the procedure used for the isolation of the ZG membranes includes five successive changes of the solutions in which the particles are suspended, the pancreatic tissue obtained from four guinea pigs was divided into five equal portions which were processed in parallel for the isolation of ZG membranes. At each of the five steps of the isolation procedure which involve a change of the solutions, one of the five aliquots was exposed to ^{45}Ca. We found (Table VII) that all of the five isolated ZG membrane samples retained some radioactivity, showing that detectable amounts of calcium are adsorbed onto the membranes at each step and carried through the whole isolation procedure. The amount of calcium adsorbed at each step was calculated as mentioned above, knowing the specific radioactivity of free calcium of the different suspending solutions used (in the case of sucrose and salt solutions the free calcium was assumed to correspond to the total calcium, whereas in the case of the ZG lysate the free calcium fraction was estimated by column chromatography as already described for the PM supernate [Table VIII]).

The calculated amounts of calcium bound to the ZG membranes at each of the various steps of the homogenization procedure are reported in Table VII. Their sum (44.36 nmol/mg protein) represents the maximum possible artifactual loading of the fraction and is only ~11.5% of its total calcium content. Therefore these results strongly suggest that the high calcium concentration of the ZG membrane fraction is not an artifact but represents a genuine feature of that membrane type.

Cytochemical Localization of Calcium

The cytochemical antimonate technique proposed by Komnick and Komnick (27) was used to investigate the intracellular localization of calcium in pancreatic acinar cells. This procedure is based

TABLE VII

Adsorption of Calcium onto ZG Membranes during Cell Fractionation

	Calcium adsorption	
	cpm/mg protein	*nmol/mg protein*
^{45}Ca added in		
0.3 M sucrose (homogenization)	$1.53\ 10^4$	1.52
0.3 M sucrose (resuspension of 1,000-*g* pellet)	$4.4\ 10^4$	6.25
Tris buffer (lysis of ZG)	$1.29\ 10^5$	0.80
1 M sucrose (gradient flotation)	$2.33\ 10^5$	20.57
Distilled water (dilution of floating ZG membrane band)	$1.99\ 10^7$	15.22
Cumulative adsorption		44.36

Samples containing membranes were exposed separately to ^{45}Ca at each of the five steps of the isolation procedure involving a change in the suspension fluid. Isolation of ZG membranes was carried out separately on each sample. The amount of calcium adsorbed was calculated from the recovered counts per minute as described in the text. The sum of the amounts of calcium adsorbed at each step of the isolation procedure represents the maximum possible calcium adsorption of the fraction.

TABLE VIII

Gel Chromatography of ZG Soluble (Content) Fraction

	A_{280}	Ca	Mg	^{45}Ca
	%	%	%	%
Void volume	82.5	21.60	6.5	4.4
Retained fraction	0	69.90	107.35	84.5
Recovery	82.5	91.50	113.85	88.9

Pancreas tissue was homogenized in 10 vol of 0.3 M sucrose containing 1 μCi of ^{45}Ca/ml (see Table IV) and ZG isolated. A sample (1.5 ml) of the material solubilized by treating isolated ZG with 10 mM Tris-HCl buffer, pH 8, containing 200 mM NaCl, was loaded onto a column (35 cm in length, 1.2 cm in diameter) filled with Sephadex G-25 coarse. Elution buffer was Tris-HCl 10 mM, NaCl 200 mM, pH 8.0. Flow rate was 20 ml/h at 2°C. The samples corresponding to the void volume and to the fractions retained in the column were pooled and analyzed for absorbancy at 280 nm (protein), calcium, magnesium, and ^{45}Ca.

on the ability of a number of cations to form insoluble precipitates when reacted with potassium pyroantimonate. The identification of calcium as the major cation present in the deposits associated with some of the labeled structures was established by studying the effect of the Ca^{2+} chelator EGTA as well as by X-ray microprobe analysis of the precipitates.

Exposure of pancreatic tissue fragments (either unfixed or fixed with glutaraldehyde) to Komnick's fluid (1% OsO_4 containing 0.05 M potassium pyroantimonate, prepared according to Spicer et al. [28]) resulted in the deposition of granular precipitates. The reaction was quantitatively different from cell to cell; however, patterns of precipitation in relation to specific organelles were recognizable. Thus, large, patchy deposits were usually associated with the PIM, particularly with its outer aspect in its basal and lateral portions (Fig. 1), as well as with the matrix of many mitochondria (Fig. 2). Finer precipitates were found in the nucleus, particularly in the interchromation regions. The cytoplasmic regions occupied by the RER and ribosomes showed little reaction or were completely negative (Fig. 2). The GC exhibited deposits associated with its membranes as well as with its fibrillar content (Figs. 2 and 3). The condensing vacuoles were always labeled. A considerable precipitation occurred along the inner surface of their limiting membrane; also, the content of the organelles consistently exhibited granular deposits (Figs. 2–4). ZG were usually negative (Fig. 3). However, in some cases, a line of heavy precipitate was found running along the inner surface of the ZG membrane. (Figs. 1 and 4).

Isolated ZG, RM, and SM membrane fractions were also reacted with potassium pyroantimonate. Most of the isolated ZG membranes appeared labeled. Deposits were often prevailingly located on one side of the membrane; however, in some cases, they were distributed equally on both sides (Fig. 5). Only a few SM membranes exhibited heavy precipitates, while the others were slightly labeled or completely negative. Even less reaction product was found associated with the RM membranes.

The deposits found in the tissue and in the isolated fractions were solubilized by exposure to

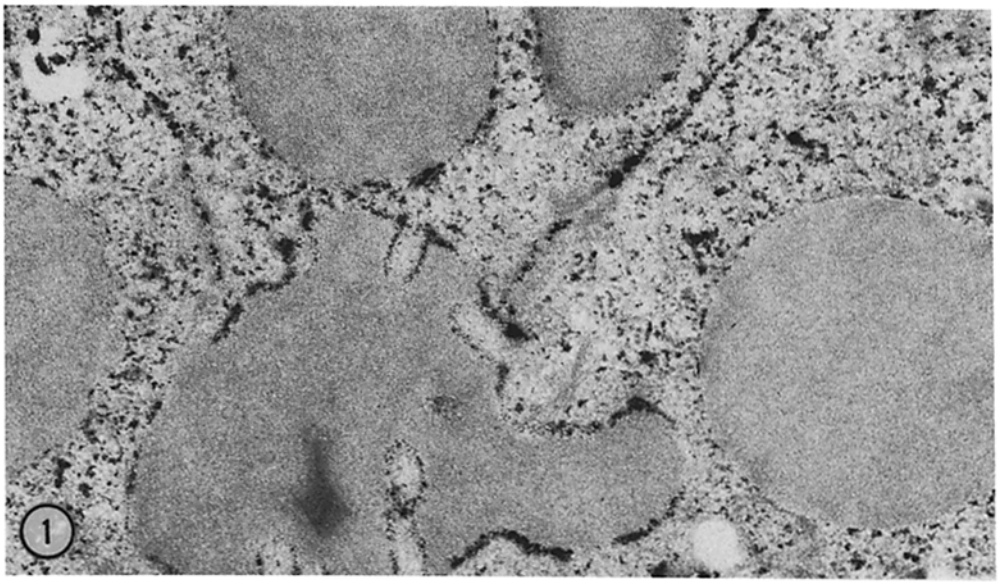

FIGURE 1 Guinea pig pancreas fixed with 1% OsO_4 containing 0.05 M potassium pyroantimonate. Heavy granular deposits of antimonate are aligned at the outer surface of the plasmalemma of four adjacent acinar cells, both in the lateral and in the luminal regions. Four ZG exhibit a discontinuous line of precipitation associated with their limiting membranes. × 35,000.

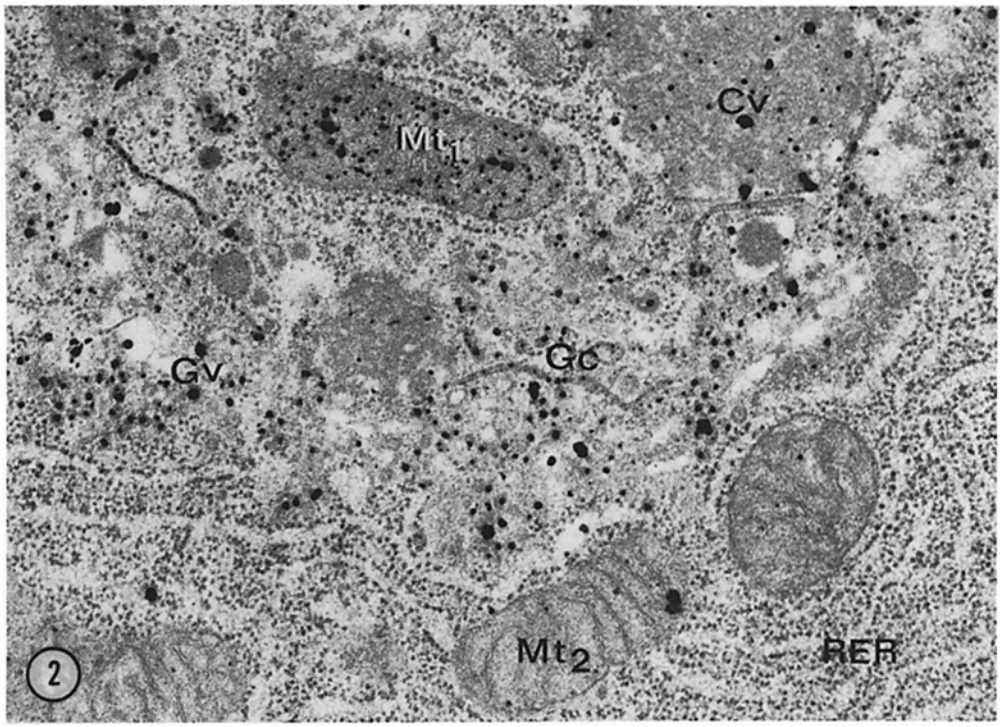

FIGURE 2 The field illustrates the distribution of antimonate deposits in the Golgi region of a pancreatic acinar cell fixed with glutaraldehyde and then with 1% OsO_4 containing 0.05 M potassium pyroantimonate. Many vesicles (*Gv*) and cisternae (*Gc*) of the Golgi complex as well as one condensing vacuole (*CV*) appear labeled. The rough-surfaced endoplasmic reticulum (*RER*) is completely negative. Four mitochondria are evident: one (Mt_1) exhibits a heavy precipitate; the others are almost unlabeled. × 30,000.

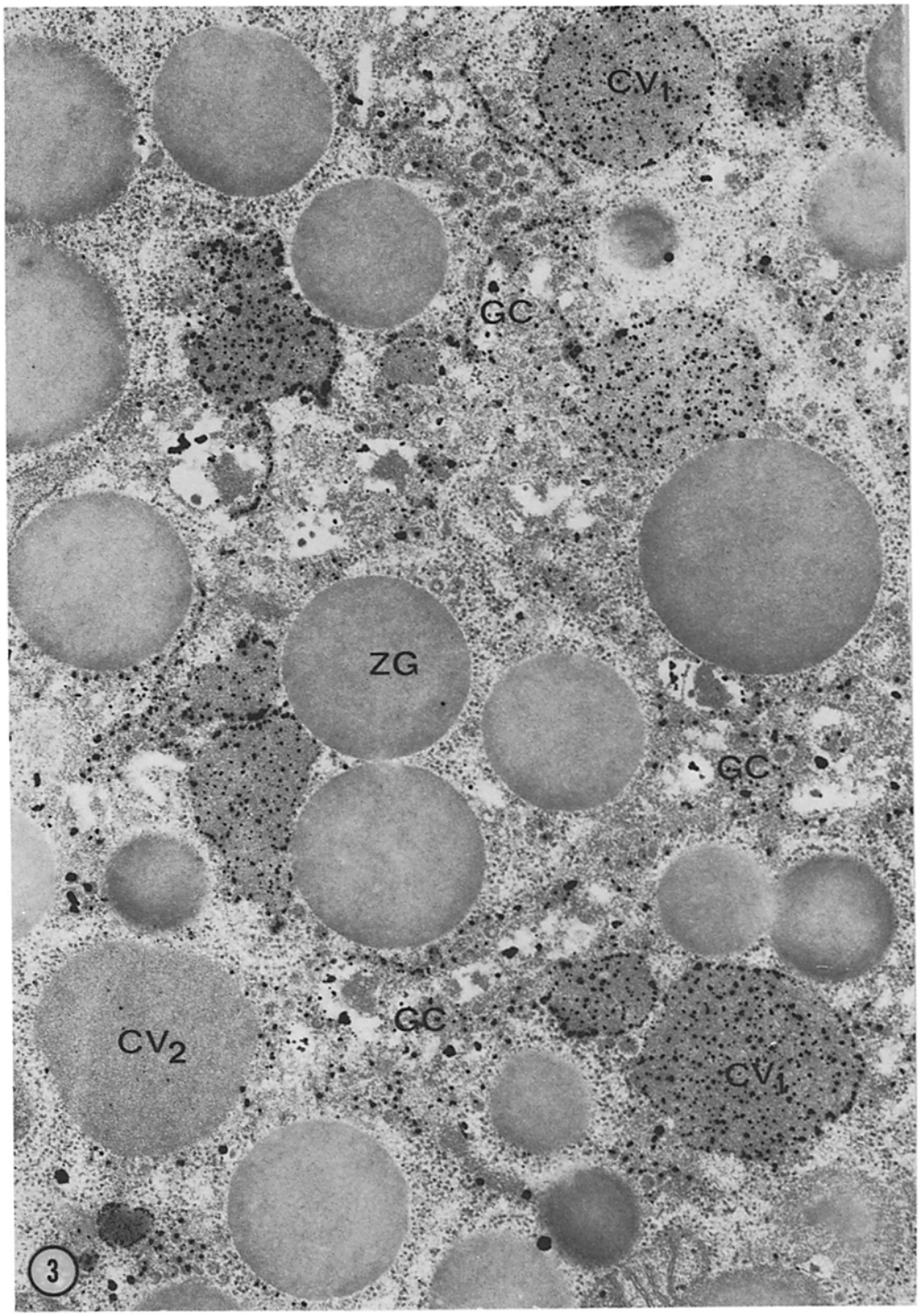

FIGURE 3 Guinea pig pancreatic acinar cell fixed with glutaraldehyde and then with 1% OsO_4 containing 0.05 M potassium pyroantimonate. In a Golgi area, numerous condensing vacuoles (CV_1) exhibit a heavy precipitate, associated especially with their limiting membranes. Analogous deposition occurs in the cisternae and vesicles of the Golgi complex (GC). One other mature condensing vacuole is slightly labeled (CV_2). Numerous mature zymogen granules (ZG) are entirely negative. × 27,000.

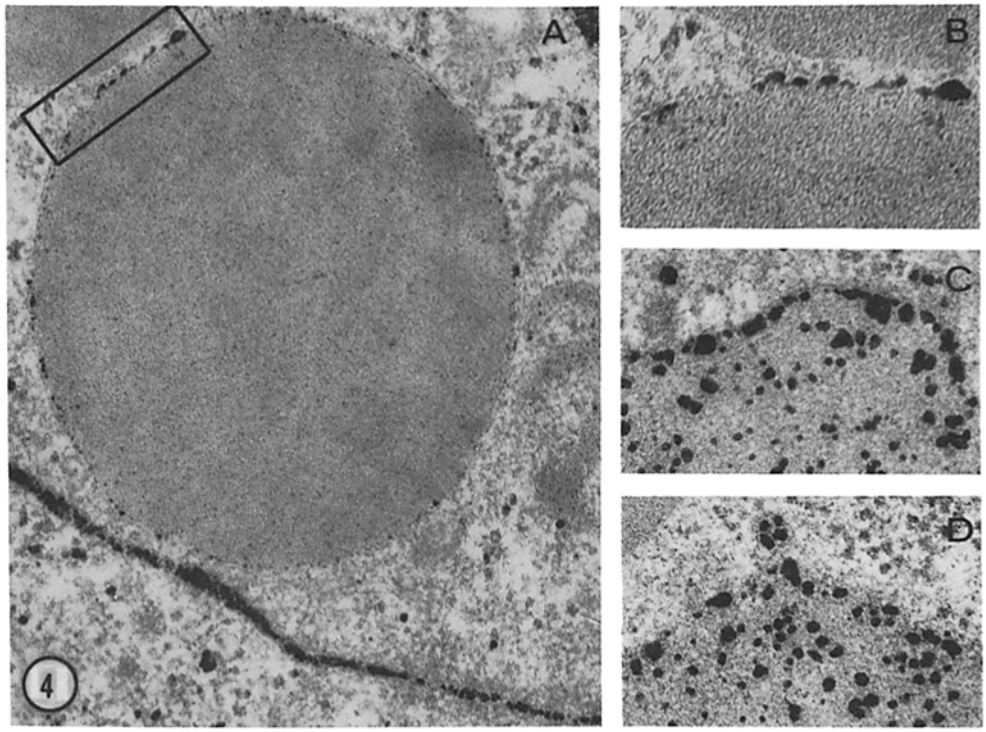

FIGURE 4 This figure shows that the electron-opaque antimonate deposits associated with the membranes of the ZG and condensing vacuoles are localized at the inner surface of the organelles. Fig. 4. A and B refer to one ZG fixed with 1% OsO_4 containing potassium pyroantimonate. Fig. 4 B is an enlargement of the boxed area in Fig. 4 A. Fig. 4 C and D, to two condensing vacuoles fixed with glutaraldehyde and then with OsO_4 + potassium pyroantimonate. A, × 65,000; B, × 120,000; C, × 60,000; and D, × 72,000.

EGTA, suggesting that they are primarily accounted for by calcium salts (Fig. 5). Furthermore, sections of ZG membrane pellets were also studied by X-ray microanalysis. The results indicate, among the cations, the presence of calcium, magnesium, and potassium. Of these, calcium is the majority component, with a calcium-to-magnesium ratio of ~2.5. This value is not too far from that obtained by biochemical analysis of fresh ZG membrane preparations (Table III).

DISCUSSION

A high intracellular level of both calcium and magnesium seems to be a peculiar characteristic of many secretory tissues, exocrine as well as endocrine. Thus, concentrations comparable to those we observed in the pancreas of the guinea pig have been previously found in the salivary glands (parotid, sublingual, and submandibular) (5, 33, 34), as well as in the adrenal medulla (35), whereas much lower levels have been found in tissues which are not so highly specialized towards secretion, such as the liver (36).

The high level of magnesium found in the pancreas seems to be essentially related to its high protein-synthesizing capacity. Thus, a large proportion of the element was found associated with the RM, the fraction containing the ribosomes, whose affinity for magnesium is well-known. In contrast, in the case of calcium, most of the large intracellular stores appear associated with a number of different cell organelles. However, the data on the subcellular distribution of the element should be considered with caution since a large artifactual redistribution definitely occurs during cell fractionation. As already mentioned, the artifacts are mainly of two types: (*a*) release from the particles of calcium either loosely bound or originated from damaged or de-energized sources, and (*b*) adsorption of exchangeable calcium onto cell structures. At least for some of our fractions, the adsorption artifacts have been evaluated and corrected. In contrast, no adequate correction of the

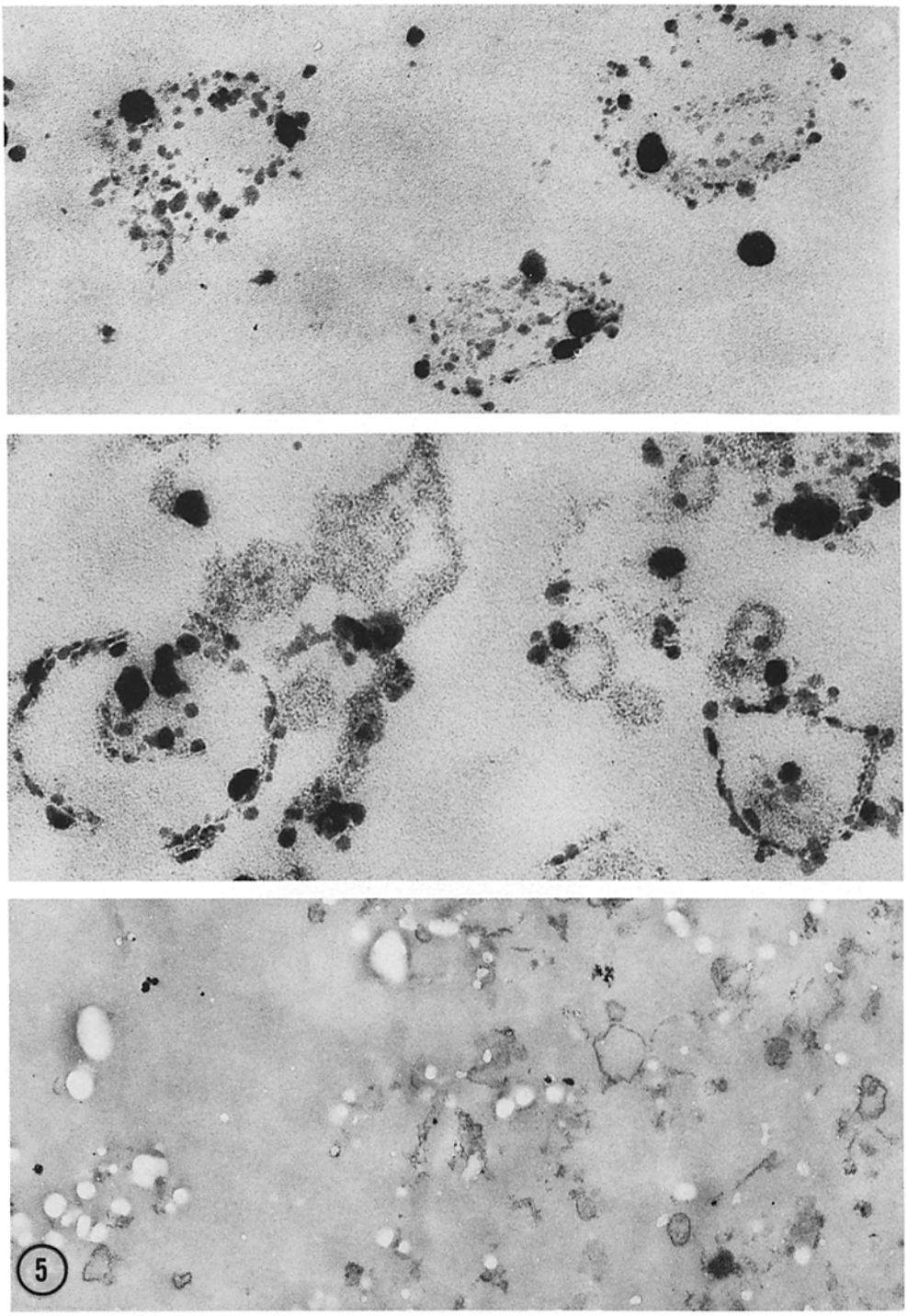

FIGURE 5 Isolated ZG membranes fixed with 1% OsO_4 containing 0.05 M potassium pyroantimonate. The figure illustrates the morphology of the membranes: unstained (upper panel), doubly stained with uranyl acetate and lead citrate (middle panel), and treated with 5 mM EGTA for 30 min at 50°C before staining (lower panel). The dense precipitates visible in the unstained preparations are always associated with the membranes and are solubilized by the Ca-chelating agent. Upper and middle panels, × 145,000, and lower panel, × 46,000.

release artifacts could be envisaged[2]. It appears likely that the latter would modify the calcium level of some of the fractions especially: the PM supernate (where at least some of the calcium released from the particles is expected to be recovered) and the fractions which are able to bind the ion by an energy-dependent mechanism. On the basis of the results obtained in different cell systems, the latter should include the mitochondria (31, 32, 40) as well as the SM (40–42) and the PIM (37, 38). All the other fractions are probably affected to a slight degree.

Furthermore, it should be emphasized that in a number of secretory systems, including the exocrine pancreas, the calcium fraction which appears to be directly involved in the stimulus-secretion coupling is the free, ionic calcium of the soluble cytoplasm (1–4, 7–18), which most likely represents a very small proportion of the total tissue calcium. Therefore, the experimental approach followed in the biochemical as well as in the cytochemical experiments that we have carried out is adequate to give information primarily about the distribution of bound Ca^{2+} (physiologically or artifactually). Hence, the findings that we have reported can refer to stimulus-secretion coupling only indirectly.

Even with these limitations our results provide some interesting data on the intracellular localization of calcium. First of all, it appears that in the pancreas, as well as in other tissues, most of the element is not soluble in the cytoplasm, but is bound to subcellular structures, particularly to membranes. Furthermore, the concentration of calcium in different membranes is quite different: some of them, such as the PIM and the ZG membranes, appear much more rich in calcium than the others.

Interestingly, the calcium concentration of the membranes does not seem to parallel their capacity for adsorbing the exchangeable calcium of the homogenate. This observation is in line with the idea that in pancreatic acinar cells calcium is distributed in a number of pools of different exchange capacity.

The high concentration of calcium found in the PIM and ZG membranes seems not to depend substantially either on artifactual adsorption or on contamination with other membrane types. In this respect it should be mentioned that when other fractions, such as pMt and TM, are processed according to the technique used for the isolation of the limiting membranes from the ZG, only a small proportion of their membranes are recovered and they contain a relatively low concentration of calcium (not shown). On the other hand, we have excluded the possibility that the calcium recovered in the ZG membranes might have been adsorbed, at the moment of the lysis of the ZG, from a pool originally associated with the content of the organelles, since only a small proportion of the free ^{45}Ca added to the Tris buffer used for lysing the ZG was adsorbed onto the membranes. Hence, the high calcium-binding capacity appears to be an intrinsic property of the ZG membranes. This conclusion is apparently in disagreement with our cytochemical results showing that in most intact pancreatic acinar cells the ZG membranes are negative after Komnick's antimonate reaction. However, isolated ZG membranes always exhibit a very heavy reaction. Since this technique assumes the ready entry of antimonate into the cell structures as well as the persistence of the cations during the fixation for antimonate binding, our cytochemical data might be consistent with the idea that the ZG membrane is relatively impermeable to the antimonate and that in the living cell most of the ZG membrane-bound calcium is localized along its inner surface. The occurrence of deposits on both sides of the isolated ZG membranes might be due

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[2] An approach which has been widely used in a number of cell systems to evaluate the calcium capacity of cell structures (such as PIM [37, 38], microsomes [39, 40], mitochondria [31, 32, 40], etc.) is based on the quantitative measurement of the binding of the ion incubated in vitro with the corresponding isolated fraction.

An obvious limitation of this approach is that the results obtained are hard to correlate with the situations existing in the living cells, since the binding is greatly dependent on a number of conditions (pH, ionic strength, etc.) which in the in vitro experiments are, to a certain degree, arbitrarily selected. However, these experiments provide some valuable information, not only on the amount of ion binding, but also on its characteristics (affinity, chemical groups involved, energy dependency, etc.).

In the course of this study, we have carried out a number of in vitro binding experiments, using RM, SM, and ZG membranes, and pMt isolated from the guinea pig pancreas. We found that all the membranes under study bind considerable amounts of calcium, with different affinities, suggesting the existence of at least two classes of binding sites in each membrane. However, the results were not completely satisfactory because (*a*) they were not reproducible and (*b*) the binding capacity of all membranes rapidly decreased on storage. Thus, this experimental approach was not pursued.

to either relocation of membrane components during cell fractionation or the preferential localization of the small amounts of artifactually adsorbed calcium onto the outer surface of the ZG membrane.

As far as we know, this is the first biochemical demonstration of a high amount of calcium associated with the membrane of a secretory granule. Recently, however, Herman et al. (43), using the antimonate technique coupled with X-ray microanalysis, observed that heavy deposits of Ca antimonate form along the inner surface of the granules of the pancreas endocrine β-cells. Thus, the high Ca-binding capacity might be a characteristic not only of pancreas ZG membranes, but also of other granule membranes and possibly of all of them. This conclusion is strengthened by preliminary results from our laboratory showing that a large amount of calcium is associated also with the membranes of the ZG isolated from the parotid gland of the rat. The molecular components of the membranes responsible for this high level of binding (whether the phospholipids or, more likely, some protein(s) or both) are still unknown.

Since the distribution of protein among the various cell organelles present in pancreatic homogenates is known, at least approximately (44), the data reported in Tables I, III, and VI can be recalculated in order to give a rough estimate of the size of the different intracellular calcium pools. These calculations indicate that, of the total calcium present in 1 g of tissue (4.9 μmol), ~34.6% is localized in ZG, ~5.6% in ZG membranes, ~5% in the PlM, ~6.3% in cMt, ~18.8% in TM, and ~13% in the PM supernate. However, for the reasons extensively discussed above, these data (particularly those concerning the PM supernate) should be considered only indicative and not a real estimate of the situation present in the living cell.

The functional significance of the different calcium pools associated with the various subcellular structures of the pancreatic acinar cell will be considered in detail elsewhere.[3] Here, only the calcium segregated within the ZG will be discussed in relation to its role in the architecture of the granule and to the functionality of the pancreatic juice.

The level of calcium associated with the ZG and, in particular, with their easily solubilizable fraction (which contains virtually the totality of the secretory enzymes and zymogens [Table III and reference 21]) turned out to be much lower then that found in secretory granules isolated from different sources, such as the parotid gland and the adrenal medulla. In these tissues, calcium has been implicated in the molecular arrangement of the granules, i.e., in the complex chemical interactions which occur among the different molecules segregated within the granules and which seem to have a major role in determining the physical and physicochemical characteristics of the organelles (4, 5).

In the pancreas, a definite conclusion on the mechanism(s) acting in the packaging of secretory proteins in ZG has not been reached yet. The well-known observation originally made by Hokin (45) that ZG are stable in distilled water as well as in hypertonic solutions at slightly acidic pH but release their content rapidly on incubation at pH >7, suggests the existence of extensive electrostatic interactions among the segregated charged molecules (the proteins and the other electrolytes) resulting in the formation of very high molecular weight complexes (45, 46). In principle, large polymers might be established by divalent cations acting as bridges between adjacent protein molecules. However, the Ca+Mg/protein molar ratio found in pancreatic ZG (assuming for the secretion proteins an average mol wt of ~30,000) is not far from 1. Thus, one should admit that virtually all the divalent cations of the granules would be permanently involved in the formation of the polymers, and this is very unlikely.

At the present state of knowledge it appears therefore reasonable to assume that, besides calcium bridges, other mechanisms might operate in the molecular organization of pancreatic ZG. In this respect it should be mentioned that, of the secretory protein molecules, some, such as chymotrypsinogen, tend to form polymers (47); others, such as proelastase, are slightly soluble (48). Furthermore, in pancreas ZG, as well as in other secretory granules, the existence of acidic sulfate-containing macromolecules, probably mucopolysaccharides, has recently been reported; these might form complexes with the cationic zymogens (49, 50).

On the other hand, it appears possible that at least some of the calcium bound to the ZG membrane might have a role in anchoring the membrane to the granule content. Such a role is suggested (*a*) by the preferential localization of calcium at the inner surface of the ZG membrane, (*b*) by the existence of a small proportion of the

[3] Meldolesi, J., and F. Clemente. Manuscript in preparation.

ZG-segregated proteins which are bound to the membranes so tightly that they are not solubilized either by ZG lysis or by repeated washing of the purified ZG membrane fraction (51).

Apart from the structural role mentioned so far, Ca^{2+} seems to have other functions in the ZG content and in the pancreatic juice. Several secretory proteins have calcium binding sites. Amylase is known to be a calcium enzyme, i.e., it contains one atom of tightly bound calcium per mol of enzyme (52); trypsinogen and chymotrypsinogen have two binding sites, one of moderate, the other of low affinity, and the interaction with calcium is of great importance for the maintenance of their tertiary structure (53). Finally, a high concentration of calcium is required for the activation of trypsinogen (54, 55). Thus, it it tempting to suggest that the relatively low level of calcium in the ZG content might be a mechanism working in synergy with others (existence of Kazal-type trypsin inhibitors, restriction of the molecular mobility) to prevent the activation of proteolytic zymogens within the granules.

We have recently reported evidence indicating that, in the guinea pig, only ~one-half of the calcium recovered in the pancreatic juice secreted in response to the pharmacological stimulation of the acinar cells originates from the ZG. The rest comes from different sources which might be either the interstitial fluid or a different pathway operating in the acinar cells, or both (56). The existence of multiple sources of calcium in the pancreatic secretion could be one of the numerous mechanisms which contribute to switch the conditions for the activation of the proteolytic zymogens from unfavorable, as in ZG, to favorable, as in the intestinal lumen.

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REFERENCES

1. RUBIN, R. P. 1970. *Pharmacol. Rev.* **22**:389.
2. RASMUSSEN, H. 1970. *Science (Wash. D.C.).* **170**:404.
3. MATTEWS, E. K. 1970. *In* Calcium and Cellular Function. A. W. Cuthbert, editor. MacMillan & Co., Ltd., London. 167.
4. CASE, R. M. 1973. *Digestion.* **8**:269.
5. WALLACH, D., and M. SCHRAMM. 1971. *Eur. J. Biochem.* **21**:433.
6. BERNEIS, K. A., A. PLETSCHER, and M. DA PRADA. 1969. *Nature (Lond.).* **224**:281.
7. DEAN, P. M., and E. K. MATTEWS. 1970. *J. Physiol. (Lond.).* **210**:265.
8. MATTEWS, E. K. 1974. *In* Secretory Mechanisms of Exocrine Glands. N. Thorn and O. H. Petersen, editors. Munksgaard, Copenhagen. 185.
9. DOUGLAS, W. W., and R. P. RUBIN. 1961. *J. Physiol. (Lond).* **159**:40.
10. DOUGLAS, W. W., and A. M. POISNER. 1962. *J. Physiol. (Lond.).* **162**:385.
11. DOUGLAS, W. W. 1968. *Br. J. Pharmacol.* **34**:451.
12. GESCHWIND, I. I. 1969. *In* Neuroendocrinology 1969. W. Ganong and L. Martini, editors. Oxford University Press, Inc., New York. 345.
13. HOKIN, L. E. 1966. *Biochim. Biophys. Acta.* **15**:219.
14. ROBBERECHT, P., and J. CHRISTOPHE. 1971. *Am. J. Physiol.* **220**:911.
15. BENZ, L., B. ECKSTEIN, E. K. MATTEWS, and J. A. WILLIAMS. 1972. *Br. J. Pharmacol.* **46**:66.
16. HEISLER, S., D. FAST, and A. TENENHOUSE. 1972. *Biochim. Biophys. Acta.* **279**:561.
17. CASE, R. M., and T. CLAUSEN. 1973. *J. Physiol. (Lond.).* **235**:75.
18. MATTEWS, E. K., O. H. PETERSEN, and J. A. WILLIAMS. 1973. *J. Physiol. (Lond).* **234**:689.
19. SANDOW, A. 1965. *Pharmacol. Rev.* **17**:265.
20. BIANCHI, C. P. 1970. *In* Protein Metabolism and Biological Function. C. P. Bianchi and R. Hilf, editors. Rutgers University Press, New Brunswick, N. J. 111.
21. MELDOLESI, J., J. D. JAMIESON, and G. E. PALADE. 1971. *J. Cell Biol.* **51**:109.
22. MELDOLESI, J., and D. COVA. 1972. *J. Cell Biol.* **55**:1.
23. MELDOLESI, J., J. D. JAMIESON, and G. E. PALADE. 1971. *J. Cell Biol.* **51**:150.
24. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. *J. Biol. Chem.* **193**:265.
25. COOPERSTEIN, S. J., and A. LAZAROW. 1951. *J. Biol. Chem.* **189**:665.
26. HUMMEL, B. C. W. 1959. *Can. J. Biochem. Physiol.* **37**:1397.
27. KOMNICK, H., and U. KOMNICK. 1963. *Z. Zellforsch. Mikrosk. Anat.* **60**:163.
28. SPICER, S. S., J. H. HARDIN, and W. B. GREENE. 1968. *J. Cell Biol.* **39**:216.
29. MARSHALL, D. J., and T. A. HALL. 1966. *In* Fourth International Congress on X-Ray Optics in Microanalysis. R. Castaing, P. Deschamp, and J. Philibert, editors. Hermann, Paris. 374.
30. LIBANATI, C. M., and C. J. TANDLER. 1969. *J. Cell Biol.* **42**:754.
31. DRAHOTA, Z., E. CARAFOLI, C. S. ROSSI, R. L. GAMBLE, and A. L. LEHNINGER. 1965. *J. Biol. Chem.* **240**:2712.

32. CARAFOLI, E. 1973. *Biochem. Soc. Symp.* **39**:89.
33. DREISBACH, R. H. 1964. *Proc. Soc. Exp. Biol. Med.* **116**:953.
34. FEINSTEIN, H., and M. SCHRAMM. 1970. *Eur. J. Biochem.* **13**:158.
35. BOROWITZ, J. L., K. FUWA, and N. WEINER. 1965. *Nature (Lond.).* **205**:42.
36. THIERS, R. F., and B. L. VALLEE. 1957. *J. Biol. Chem.* **226**:911.
37. SHLATZ, L., and G. V. MARINETTI. 1972. *Biochim. Biophys. Acta.* **290**:70.
38. NIJAR, M. S., and E. T. PRITCHARD. 1973. *Biochim. Biophys. Acta.* **323**:391.
39. TOURY, R. 1973. *Biochim. Biophys. Acta* **307**:607.
40. POISNER, A. M., and M. HAVA. 1970. *Mol. Pharmacol.* **6**:407.
41. SELINGER, Z., E. NAIM, and M. LASSER. 1970. *Biochim. Biophys. Acta.* **203**:326.
42. ALONSO, G. L., P. M. BAZERQUE, D. M. ARRIGO, and O. R. TUMILASCI. 1971. *J. Gen. Physiol.* **58**:340.
43. HERMAN, L., T. SATO, and C. N. HALES. 1973. *J. Ultrastruct. Res.* **42**:298.
44. MELDOLESI., J. 1974. *In* Cytopharmacology of Secretion. B. Ceccarelli, F. Clementi, and J. Meldolesi, editors. Raven Press, New York. 71.
45. HOKIN, L. E. 1955. *Biochim. Biophys. Acta.* **18**:379.
46. GREENE, L. J., C. H. W. HIRS, and G. E. PALADE. 1963. *J. Biol. Chem.* **238**:2054.
47. SMILLIE, L. B., A. G. ENENKEL, and C. MCKAY. 1966. *J. Biol. Chem.* **241**:2097.
48. URAM, M., and F. LAMY. 1969. *Biochim. Biophys. Acta.* **194**:102.
49. TARTAKOFF, A. M., L. J. GREENE, J. D. JAMIESON, and G. E. PALADE. 1974. *In* Cytopharmacology of Secretion. B. Ceccarelli, F. Clementi, and J. Meldolesi, editors. Raven Press, New York. 177.
50. BERG, N. B., and R. W. YOUNG. 1971. *J. Cell Biol.* **50**:469.
51. MELDOLESI, J. 1974. *J. Cell Biol.* **61**:1.
52. STEIN, E. A., J. HSIU, and E. H. FISCHER. 1964. *Biochemistry.* **3**:56.
53. DELAAGE, M., and M. LAZDUNSKI. 1967. *Biochem. Biophys. Res. Commun.* **28**:390.
54. MCDONALD, M. R., and M. KUNITZ. 1941. *J. Gen. Physiol.* **25**:53.
55. DESNUELLE, P., and C. GABELOTEAU. 1957. *Arch. Biochem. Biophys.* **69**:475.
56. CECCARELLI, B., F. CLEMENTE, and J. MELDOLESI. 1975. *J. Physiol. (Lond.).* **244.**