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Studies on Intracellular Transport of Secretory Proteins in the Rat Exocrine Pancreas

III. Effect of Cobalt, Lanthanum and Antimycin A

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Summary. The effects of cobalt and lanthanum on the secretory process of the rat exocrine pancreas was studied *in vitro* using isolated pancreatic lobules. Cobalt in concentrations between 10^{-3} to 10^{-5} M has no effect on the rate of protein synthesis, intracellular transport, or discharge of zymogen granules, if the total population of stored granules is considered. It has, however, a marked effect on the release of newly packed zymogen granules which are formed during incubation in 10^{-3} M $CoCl_2$. Determination of specific radioactivity in amylase released under the stimulation of 5×10^{-6} M carbamylcholine and of total proteins retained in the zymogen granule fraction during stimulation indicate that granules formed during incubation in $CoCl_2$ are excluded from discharge.

Lanthanum, on the other hand, has a differential effect on protein synthesis, intracellular transport, and discharge. Incorporation of tritiated leucine into TCA-precipitable proteins is inhibited by 50% at 10^{-3} M $LaCl_3$. Intracellular transport as studied by cell fractionation is not changed during the first 35 min post pulse but is delayed from then on. This late effect is more pronounced if pancreatic lobules are preincubated for 60 min in 10^{-3} M $LaCl_3$. Discharge of amylase and newly synthesized proteins is inhibited dose-dependently up to 80% by 10^{-3} M $LaCl_3$. The effects of both cobalt and lanthanum are not due to an inhibition of cellular respiration. Comparison of these results with the inhibitory action of antimycin A between 10^{-4} to 10^{-8} M concentrations reveals a dose-dependent diminution of the rate of protein synthesis and intracellular transport, while discharge of granules is less energy dependent. The fine structural appearance of pancreatic lobules after 3 hrs incubation in 10^{-3} M $CoCl_2$ is not altered, while in 5×10^{-3} and 10^{-3} M lanthanum acinar lumina are enlarged and the apical cytoplasm contains large vacuoles. At the highest concentration of lanthanum a flocculent electron dense material is observed apposed to the external lamina of the plasma membrane. The distribution of this material on the membrane is described.

Antimycin A leads to cellular changes corresponding to the irreversible inhibition of cellular respiration. It is concluded from the results that cobalt acts on the process of granule formation inside the cell, while lanthanum by its binding to the plasma membrane may alter molecules involved in secretagogue binding and transport systems into the cell.

Key words: Exocrine pancreas — Secretory process — Cobalt — Lanthanum — Intracellular transport — Secretion granules.

Introduction

Calcium ions play an important role in the process of stimulus-secretion coupling in several secretory organs (for review see Rubin, 1970). In two intensively investigated systems (release of acetylcholine from neuromuscular junction and release of catecholamine from adrenal medulla) the process of secretion

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clearly depends on the extracellular concentration of calcium and can be inhibited by magnesium or substituted by barium and strontium. The role of calcium ions in the secretory process of the exocrine pancreas is still controversial: total omission of extracellular calcium leads to an inhibition of enzyme secretion, but this effect develops slowly (Hokin, 1966). Moreover, in rat exocrine pancreas enzyme secretion is the same at 0.1 mM concentration of extracellular calcium as at 2.5 mM (for review see Case, 1973). The physiological situation is further complicated by the fact that the exocrine pancreatic cell contains large intracellular stores of calcium, mainly in zymogen granules and mitochondria (Clemente *et al.*, 1975). It is therefore assumed, that secretagogues exert their stimulus in the exocrine pancreatic cell by releasing calcium from intracellular stores rather than by influx of extracellular calcium (Case, 1973; Williams and Chandler, 1975). Cobalt and lanthanum ions have been used previously as antagonists to calcium influx in muscle cells and neurons (Weiss, 1974; Baker *et al.*, 1973). In these systems cobalt blocks slow calcium channels, while lanthanum replaces calcium by its higher binding affinity to superficially located calcium sites. Both cobalt and lanthanum also act on secretory systems: *In vitro* incubation of isolated islets of Langerhans in the presence of cobalt ions results in an inhibition of insulin release and calcium uptake (Henquin and Lambert, 1975). *In vivo* injection of cobalt chloride into guinea pigs leads to a progressive degranulation and accumulation of secretory material in the cisternal space of the rough endoplasmic reticulum in exocrine pancreatic cells (Kern and Kern, 1969). Similarly *in vitro* incubation of exocrine pancreatic preparation in the presence of increasing concentrations of lanthanum ions blocks calcium influx and enzyme secretion (Heisler and Grondin, 1973; Chandler and Williams, 1974).

In the course of our own studies on intracellular transport of secretory proteins in the rat exocrine pancreas the effect of cobalt and lanthanum was tested on the different phases of the secretory process (protein synthesis, intracellular transport, discharge of enzymes). The results are compared with the effect of the ions on cellular respiration and with the inhibitory action of antimycin A, a known blocker of intracellular transport kinetics (Jamieson, 1972).

Materials and Methods

The studies were performed using isolated rat pancreatic lobules incubated in Krebs-Ringer Hepes buffer. The system has been described in detail in two previous communications (Bieger and Kern, 1975; Seybold *et al.*, 1975). In experiments with lanthanum phosphate and sulphate (KH_2PO_4, $MgSO_4$) were omitted from the incubation medium and replaced by the appropriate amounts of $MgCl_2$. The incubation flasks in these experiments were regularly gassed with pure oxygen every 30 min, while in all other experiments they were gassed with carbogen (95% O_2/5% CO_2). The rate of protein synthesis was determined by the incorporation of L-leucine-4,5-^{3}H into trichloroacetic acid (TCA) precipitable proteins after 30, 60 and 90 min incubation. Intracellular transport operations were analyzed using a radioassay according to Jamieson and Palade (1971), in which secretory proteins are pulse labelled with tritiated leucine for 4 min and then their discharge is determined over a period of 2 hrs in the presence of 5×10^{-6} M carbamylcholine as secretagogue. From the same incubations samples are taken at regular intervals and release of amylase is determined.

Furthermore, the intracellular transport of newly synthesized proteins from rough endoplasmic reticulum to zymogen granules (with an intermediate stage in the Golgi complex) was analyzed by cell fractionation in a separate set of experiments. After a standard pulse labelling with tritiated leucine (50 μCi/ml) for 5 min pancreatic lobules were chase incubated

for further 10, 35 and 55 minutes as controls or in the presence of 10^{-3} M $LaCl_3$ or 10^{-3} M $CoCl_2$. At the end of the pulse and at each time point of chase incubation the pancreatic lobules were homogenized in 0.3 M sucrose and subjected to the following steps of centrifugation:

1. 10 min at $600 \times g$ for removal of cellular debris and nuclei.
2. 10 min at $1000 \times g$ to reveal a zymogen granule pellet and supernatant which is spun,
3. 15 min at $8700 \times g$ for removal of mitochondria.
4. The supernatant (about 4.5 ml) is put on top of 1.15 ml 2 M sucrose, which is used as cushion on the bottom of 13 ml polycarbonate tubes. They are filled up with 0.3 M sucrose and spun for 20 min at $160000 \times g$. A band of microsomes (instead of a pellet in other procedures) appears at the interphase between 2 M and 0.3 M sucrose.
5. Material from this band is put on a discontinuous gradient which consists from bottom to top of the following sucrose concentrations: 1 ml of 2 M, 4 ml of 1.3 M, 2 ml of the microsomal band taken from previous step, 4 ml of 1.0 M and filled up to 13 ml with 0.3 M. The tubes are spun at $160000 \times g$ for 2.5 hrs.

Two bands will develop: one at the interphase between 1.0 M and 0.3 M consisting mainly of smooth microsomes (elements of Golgi complex and plasma membrane). The second band appears at the interphase between 1.3 M and 2 M sucrose and comprises mainly of rough microsomes (elements of the endoplasmic reticulum).

Assay Procedures

a) Radioactivity Assays. Samples from incubation and secretion experiments and from particulate fractions were precipitated at 4° C with TCA (10% final concentration) over night and washed twice with 5% TCA. The final pellet was dissolved in 1 ml 1 N NaOH, 0.1 ml of this was used for protein determination and the rest was counted in a Nuclear Chicago scintillation counter (Isocap 300) using unisolve (Koch-Light Lab., Colnbrook, England) as scintillation fluid.

b) Chemical Assays. Proteins were determined by the method of Lowry *et al.* (1951) using crystalline bovine serum albumine as standard. DNA was measured according to Burton (1956) in the modification of Richards (1974). Amylase activity (determined according to Schramm and Dannon, 1961) was measured by the procedure of Bernfeld (1955).

Electron Microscopy

Pancreatic lobules after isolation and after incubation for up to 3 hrs in the presence of increasing concentration of cobalt chloride (10^{-5} to 10^{-3} M), lanthanum chloride (10^{-4} to 5×10^{-3} M) and antimycin A (10^{-6} to 10^{-4} M) were fixed in 2.5% formaldehyde-glutaraldehyde in 0.067 M cacodylate buffer pH 7.2. Standard procedures for dehydration and embedding of tissue and for sectioning and staining were used. The micrographs were taken at a Zeiss EM 9 S electron microscope.

Materials

All chemicals were reagent grade (E. Merck, Darmstadt, Germany). L-amino acids-BSA and antimycin A were obtained from Serva Biochemica, Heidelberg, Germany. L-leucine. 4,5-^{3}H (30–60 mCi/mole) was purchased from Amersham Buchler, Braunschweig, Germany,

Results

a) Biochemical Findings

Table 1 summarizes the effect of increasing concentrations of cobalt, lanthanum and antimycin A on the rate of amino acid incorporation into TCA-precipitable proteins and on $^{14}CO_2$-production from ^{14}C-palmitate. It is well established from previous investigations (Jamieson, 1972; Bieger and Kern, 1975) that both parameters proceed at a linear rate at least for 3 hrs, if pancreatic slices or lobules

Table 1. L-leucine-4,5-^{3}H incorporation in TCA-precipitable proteins and $^{14}CO_2$-production from 1-^{14}C-palmitic acid under control conditions and in the presence of cobalt, lanthanum and antimycin A

		PROTEINSYNTHESIS			CELLULAR RESPIRATION		
		Leucine-^{3}H incorporation in % of controls			^{14}CO production from ^{14}C-palmitic acid in % of controls		
		30	60	90 min	30	60	90 min
control		2140 100 %	3630 100 %	5810 $\frac{CPM}{\mu g\ DNA}$ 100 %	1150 100 %	3160 100 %	5100 $\frac{CPM}{mg\ protein}$ 100 %
Cobalt	10^{-3}M	97 ± 6,8	96 ± 9,1	96,3 ± 10,8	81,5 ± 11,8	72,1 ± 14,5	80,7 ± 8,1
	10^{-4}M	98 ± 8,1	94 ± 4,9	103 ± 4,8	79,2 ± 10,7	78,9 ± 16,8	85,7 ± 12,4
	10^{-5}M	101,5 ± 4,9	98 ± 6,7	101 ± 11,1	104,8 ± 8,6	100,9 ± 9,1	96,1 ± 14,8
Lanthanum	10^{-3}M	72,5 ± 7,1	59,4 ± 5,2	50,7 ± 8,2	91 ± 11,4	84 ± 3,9	88 ± 10,7
	10^{-4}M	94 ± 6,4	92,2 ± 5,8	90,7 ± 7,4	92,8 ± 10,6	104,1 ± 8,9	98,1 ± 6,8
Antimycin A	10^{-4}M	6,2 ± 0,8	3,4 ± 1,1	13,1 ± 0,7	5[1]		
	10^{-6}M	24,2 ± 2,4	17,5 ± 1,7	15,1 ± 1,3	15[1]		
	10^{-8}M	101,2 ± 8,9	95,1 ± 7,4	98,4 ± 6,8	88[1]		

[a] The data of $^{14}CO_2$-production with antimycin A are given for comparative reasons and are taken from the study of Jamieson (1972), incubation time was 37 min.

are incubated *in vitro*. In Table 1 the respective values for controls at 30, 60 and 90 minutes incubation are expressed as 100% each, the absolute values for each time point in cpm/μg DNA or cpm/mg protein are listed also. It can be seen that the addition of cobalt chloride in concentrations between 10^{-3} to 10^{-5} M in the incubation medium does not lead to a significant interference with L-leucine incorporation into protein. This is despite the finding that 10^{-3} and 10^{-4} M cobalt inhibit $^{14}CO_2$-production from ^{14}C-palmitate uniformly by 20%.

The addition of lanthanum to the incubation medium, however, leads to a pronounced effect on protein synthesis which at 10^{-3} M concentrations is reduced by 40% during the first 60 min and by 50% after 90 min. 10^{-4} M lanthanum inhibits amino-acid incorporation by 5–10% at all time points. In contrast to this marked effect of lanthanum on protein synthesis cellular respiration is only blocked by the highest concentration (5×10^{-3} M) but remains unaffected at 10^{-3} and 10^{-4} M.

In comparison to this differential effect of cobalt and lanthanum on protein synthesis and cellular respiration antimycin A affects both processes to the same extent in a dose-response relationship. This is well established from the studies of Jamieson (1972) and is shown here only for comparative reasons (Table 1). Similar differences between cobalt and lanthanum can be observed, if their effect on intracellular transport and enzyme release are tested *in vitro*. Cobalt in concentrations between 10^{-3} to 10^{-5} M has no effect on the discharge of amylase stimulated by 5×10^{-6} M carbamylcholine over a period of 2 hrs (Fig. 1). If the release of newly synthesized proteins is studied after a standard pulse labelling of 4 min their discharge under the same conditions is markedly inhibited at 10^{-4} M cobalt by 30% and at 10^{-3} M by 80% from control levels.

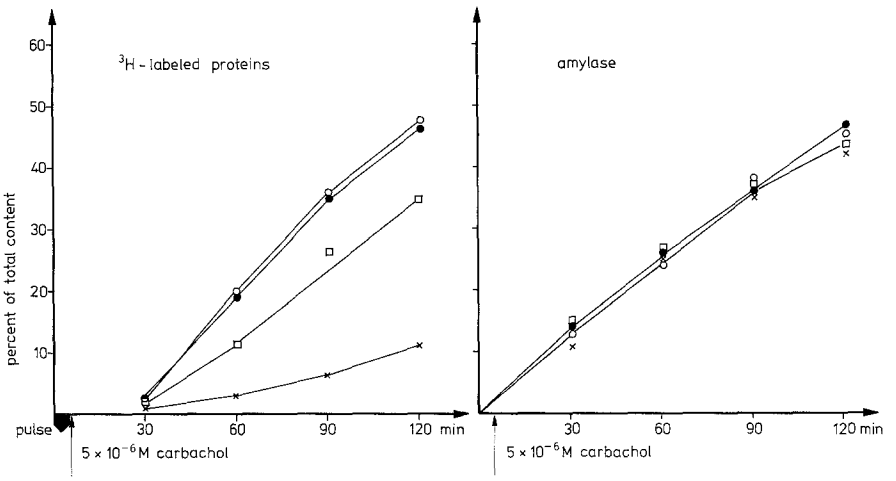

Fig. 1. Carbamylcholine (5×10^{-6} M) stimulated discharge of newly synthesized proteins (4 min pulse) compared with discharge of amylase in the presence of increasing concentration of $CoCl_2$. Results are expressed as percent of the sum of amylase or protein radioactivity in medium and tissue, incubation time is 2 hrs. •—• control, ×—× 10^{-3} M $CoCl_2$, ▫—▫ 10^{-4} M $CoCl_2$, ○—○ 10^{-5} M $CoCl_2$

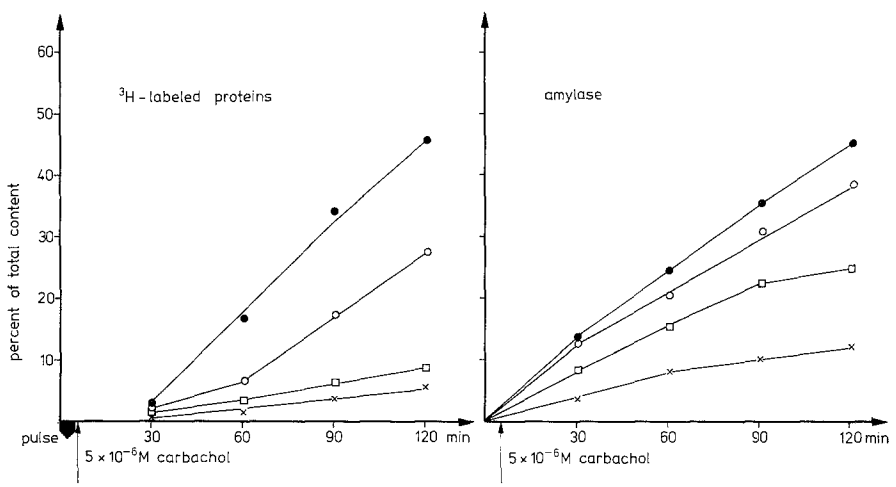

Fig. 2. Effect of increasing concentrations of $LaCl_3$ on discharge of newly synthesized proteins and amylase stimulated by 5×10^{-6} M carbamylcholine. Data are expressed as in Fig. 1. •—• control, ×—× 5×10^{-3} M $LaCl_3$, ▫—▫ 10^{-3} M $LaCl_3$, ○—○ 10^{-4} M $LaCl_3$

Lanthanum in concentrations between 5×10^{-3} M to 10^{-4} M has similar inhibitory effect on release of amylase and newly synthesized proteins, which both decrease dose-dependantly, although release of amylase (representing the total pool of stored zymogen granules) is significantly less affected (Fig. 2). In comparison to these results with cobalt and lanthanum, antimycin A, a known inhibitor of cellular respiration, blocks release of newly synthesized proteins dose-

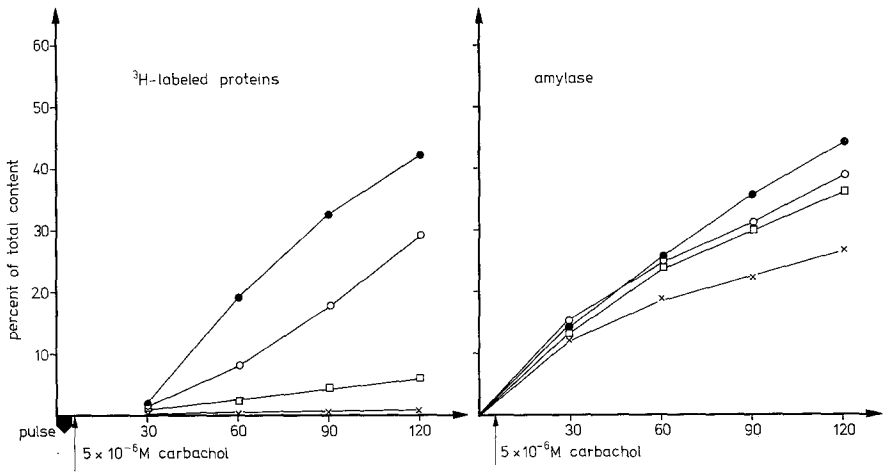

Fig. 3. Effect of increasing concentrations of antimycin A on discharge of labelled proteins and amylase. Same conditions as in Figs. 1 and 2. •—• control, ×—× 10^{-6} M antimycin A, □—□ 10^{-7} M antimycin A, ○—○ 10^{-8} M antimycin A

dependantly up to 95% at 10^{-6} M (Fig. 3). It has only slight effects on the release of amylase at 10^{-8} and 10^{-7} M and more pronounced (50% inhibition) at 10^{-6} M. These results demonstrate the differential energy dependancy of the different intracellular steps of the secretory process: protein synthesis and intracellular transport being more sensitive to supply of energy than is the final discharge of granules. Comparing the secretion experiments in the presence of cobalt and lanthanum (Figs. 1, 2) it could be concluded that cobalt acts predominantly on the intracellular transport and has little or no effect on discharge while lanthanum inhibits mainly the release mechanism of zymogen granules by exocytosis.

This assumption was further tested in cell fractionation experiments. Pancreatic lobules were pulse labeled for 5 min in tritiated leucine and then further chase incubated as controls or in the presence of 10^{-3} M $LaCl_3$ and 10^{-3} M $CoCl_2$. Fractions of zymogen granules, rough microsomes and smooth microsomes were prepared and the specific radioactivity was determined (Fig. 4). Under control conditions there is a progressive decrease from rough microsomes and a linear increase in zymogen granules, with a crossing-over of both curves between 35 and 55 min of chase incubation. The smooth microsomal fraction shows a peak of radioactivity at 10 min post pulse which corresponds to an accumulation of newly synthesized proteins in elements of the Golgi-complex around that time point. Addition of lanthanum during chase incubation has no significant effect on the translocation of labelled proteins from rough microsomes to zymogen granules during the first 35 min post pulse. Thereafter lanthanum inhibits only the appearance of radioactive proteins in the zymogen granule fraction, not the translocation from rough microsomes into smooth microsomes (Fig. 4, middle panel). To analyze this late effect of lanthanum further pancreatic lobules are preincubated for 60 min in the presence of 10^{-3} M $LaCl_3$, then pulse labelled

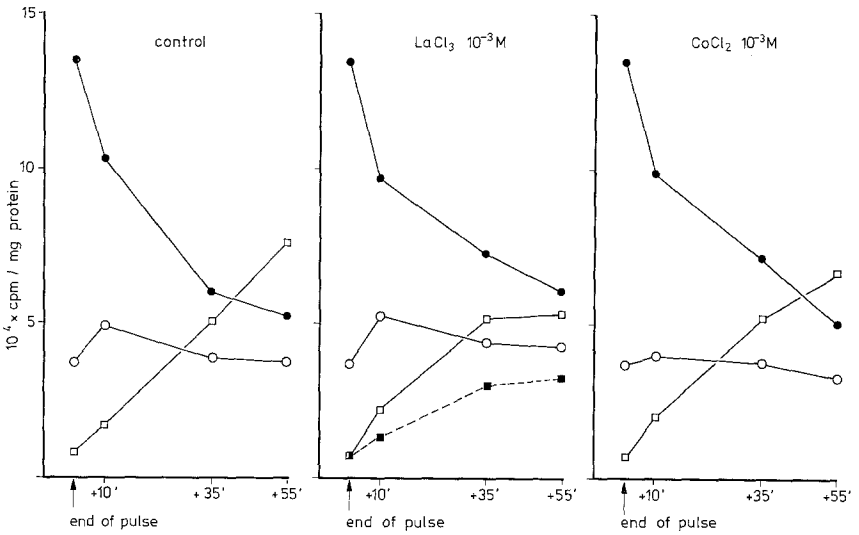

Fig. 4. Graphic presentation of cell fractionation studies. Sets of pancreatic lobules were pulse labelled for 5 min with leucine-^{3}H and chase incubated for an additional 10, 35 and 55 min under control conditions or in the presence of 10^{-3} M $LaCl_3$ or 10^{-3} M $CoCl_2$. The specific protein radioactivity is shown in the respective fractions of rough microsomes, smooth microsomes and zymogen granules. In the middle panel results are shown from lobules preincubated before the pulse for 60 min in 10^{-3} M $LaCl_3$. Only the specific activity in the zymogen granules is drawn. ▪---▪ zymogen granules (60 min preincubation with 1 mM $LaCl_3$), ▫—▫ zymogen granules; •—• rough microsomes; ○—○ smooth microsomes

and further chase incubated with the same concentration of $LaCl_3$ for an additional time of 10, 35 and 55 min. The same time-table is performed with control lobules in the absence of $LaCl_3$. It can be seen that after preincubation in lanthanum the specific activity at the end of the pulse in the total homogenate is about half of that in controls due to an inhibition of protein synthesis described earlier. The rate of disappearance from rough microsomes is not changed compared to controls, but the appearance of radioactivity in the zymogen granule fraction is inhibited by 50% (Fig. 4, middle panel).

Incubation of pancreatic lobules in 10^{-3} M $CoCl_2$ after pulse labelling has no effect on intracellular transport if studied by cell fractionation (Fig. 4, right panel). From these data it is concluded that intracellular transport operations for newly synthesized proteins are performed regularly in the presence of $CoCl_2$. The secretion experiments, however, indicate that cobalt interferes with the discharge of newly packed zymogen granules but not with "old" ones. This possibility can be elucidated by the following experiment: pancreatic lobules are pulse labelled for 5 min and then chase incubated in the presence of 5×10^{-6} M carbamylcholine for an additional 60, 120 and 180 min. It can be expected that under control conditions newly synthesized proteins are transported to the Golgi complex and packed to labelled zymogen granules which are discharged in increasing concentrations from 30 min on. The specific radioactivity in a given enzyme fraction (e.g. amylase) should therefore increase in the medium during

Table 2. Discharge of amylase and pulse-labelled amylase stimulated by 5×10^{-6} M carbamylcholine in the medium under control conditions and in the presence of 10^{-3} M $CoCl_2$

conditions		Discharge of amylase during stimulated incubation (5×10^{-6} M carbachol) amylase U/ml	labeled amylase CPM / ml	amylase specific radioactivity; CPM/U % (of 3hrs)
control	1 h	88 ± 3	2.600 ± 140	20,1
	2 h	148 ± 7,4	11.700 ± 910	54,1
	3 h	214 ± 20,7	31.400 ± 2.100	100
$CoCl_2$ 1 mM	1 h	86 ± 5,4	510 ± 85	4,1
	2 h	137 ± 23,5	5.250 ± 640	13,5
	3 h	191 ± 16,8	9.078 ± 1740	32,4

prolonged incubation. If cobalt inhibits preferentially the release of newly synthesized proteins discharge of e.g. amylase should remain the same but the specific radioactivity in the enzyme fraction should be lower. Concomitant to this secretion pattern in the medium the specific radioactivity of newly synthesized protein in the intracellular pool (namely in zymogen granules) should first increase under control conditions (during the first hour of incubation) and then decrease due to the random release of "old" and newly formed granules. In the presence of cobalt, however, the specific radioactivity in the zymogen granule fraction should increase consistently due to the preferential release of "old" zymogen granules and the retention of new ones.

Table 2 summarizes results from such experiments, in which after pulse labelling and chase incubation in the presence of carbamylcholine as secretagogue the specific radioactivity of amylase in the medium was determined after glycogen precipitation according to Schramm and Loyter (1966). This specific radioactivity under control conditions increases at a linear rate and is inhibited by 10^{-3} M cobalt by 95% at 60 min, by 85% at 120 min and by 70% at 180 min incubation. These results correspond to the cell fractionation data, in which the specific radioactivity was determined in the zymogen granule fraction after 20, 45, 90, 120 and 180 min chase incubation in the presence of carbachol (Fig. 5). In controls the specific activity in the zymogen granule fraction increases up to 90 min post pulse and then decreases due to the release of both granule populations. In the presence of 10^{-3} M cobalt, however, the specific activity increases at a linear rate obviously due to a consistent release of "old" zymogen granules and an accumulation of the newly formed granules.

b) Morphological Findings

Incubation of isolated pancreatic lobules in the presence of 10^{-3} to 10^{-5} M concentrations of $CoCl_2$ does not lead to any structural alterations of the exocrine cells (Fig. 6). Occasional vacuoles in the Golgi area most likely represent condensing vacuoles which are less well preserved after 3 hrs *in vitro* incubation.

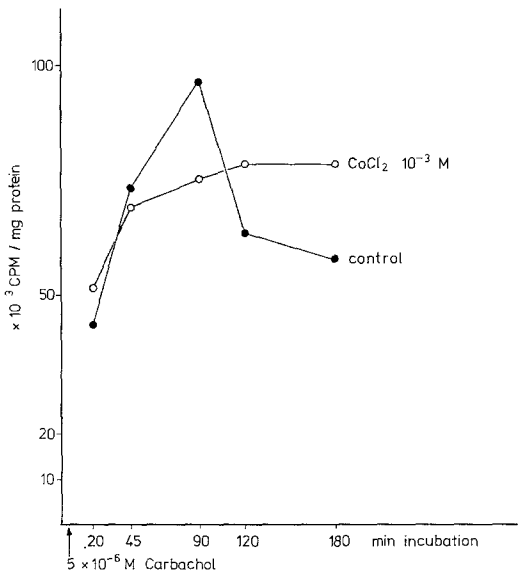

Fig. 5. Specific activity in zymogen granule fractions isolated from lobules which, after pulse-labelling for 5 min are incubated for up to 3 hrs in the presence of 5×10^{-6} M carbamylcholine. For each time point a separate set of lobules was incubated and fractionated according to standard procedures

Addition of carbamylcholine (5×10^{-6} M) to the incubation medium leads to degranulation, the remaining granules are not different in their fine structure.

Incubation in the presence of lanthanum (5×10^{-3} M, 10^{-3} M), however, prevents the degranulation induced by carbamylcholine. The acinar lumina after prolonged incubation appear distended with very few and short microvilli present. The apical cytoplasm contains 2 to 3 large vacuoles (Fig. 7a). Especially after incubation in the highest concentration of $LaCl_3$ (5×10^{-3} M) the exocrine pancreatic cells are outlined by electron dense material, which is deposited in the extracellular space and at the basal part of the plasma membrane. This material is well preserved after incubation in 5×10^{-3} M $LaCl_3$. The electron dense deposits are evident in thin sections without counter-staining with uranylacetate or lead citrate. They are restricted exclusively to the extracellular space and are never found in the cytoplasm or attached to inner membranes or cell organelles. The finestructure and distribution along the different parts of the plasma membrane of these electron dense deposits can best be analyzed in oblique and tangential sections through the plasma membrane (Fig. 7b). They consist of flocculent or globular aggregates which reach a thickness of 0.1 to 0.2 μ at the basal plasmamembrane or between two adjacent acini (Fig. 8a). At the lateral plasma-membrane these aggregates are densely packed and fill the extracellular space up to the tight junctions occluding it from the acinar lumen (Fig. 8b). Higher magnification of cross sections through the lateral plasma-membrane reveal the attachment of the electron dense material to the external lamina of the triple-layered membrane (Fig. 8c). Sections running tangential to the external lamina

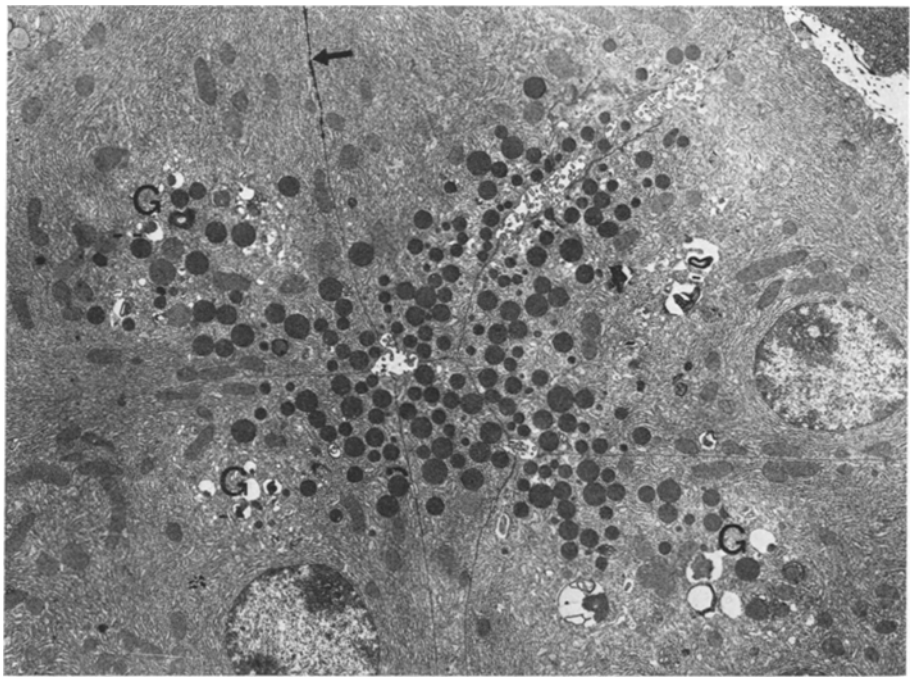

Fig. 6. Low power micrograph of a pancreatic lobule incubated for 3 hrs in 10^{-3} M $CoCl_2$. Note the preservation of fine structure and a few enlarged condensing vacuoles in the Golgi-region (*G*). In this series of experiments 1% tannic acid was added to the fixation medium which produces electron dense deposits in some areas of the extracellular space (arrow). Magnification, ×4500

show that the material is not uniformly distributed over the outside of the membrane but forms clusters and sometimes spares out circular areas of about 400 Å in diameter (Fig. 8d).

The structural alterations induced by antimycin A correspond to the inhibition of cellular respiration. After 3 hrs incubation in 10^{-4} or 10^{-5} M concentrations the exocrine cells are crowded with zymogen granules, the regular arrangement of the rough ER is distorted (Fig. 9a). Cell nuclei are irregularly outlined and show a condensation of chromatin material along the nuclear membrane. The karyoplasm contains dense particles measuring 500–600 Å in diameter (Fig. 9b). The ER has broken down to vesicles and shows finger-print like arrangements, the cisternal space is enlarged (Fig. 9c). Mitochondria show a fragmentation of the cristae and round electron dense deposits (Fig. 9b, c).

Fig. 7a and b. Structural alterations induced by incubation for 3 hrs in 5×10^{-3} M $LaCl_3$. (a) The pancreatic cells are fully granulated, the acinar lumen (*L*) appears widened with few microvilli projecting from the apical plasma membrane. The cytoplasm contains several large vacuoles (*V*) which contain globular and membraneous material. The cell borders are outlined by electron dense material which is most pronounced at the basal plasma membrane (arrows). (b) Intercellular space between 3 exocrine cells. The plasma membrane is cut obliquely (at the right) and tangentially (at the left). Magnification, ×20000

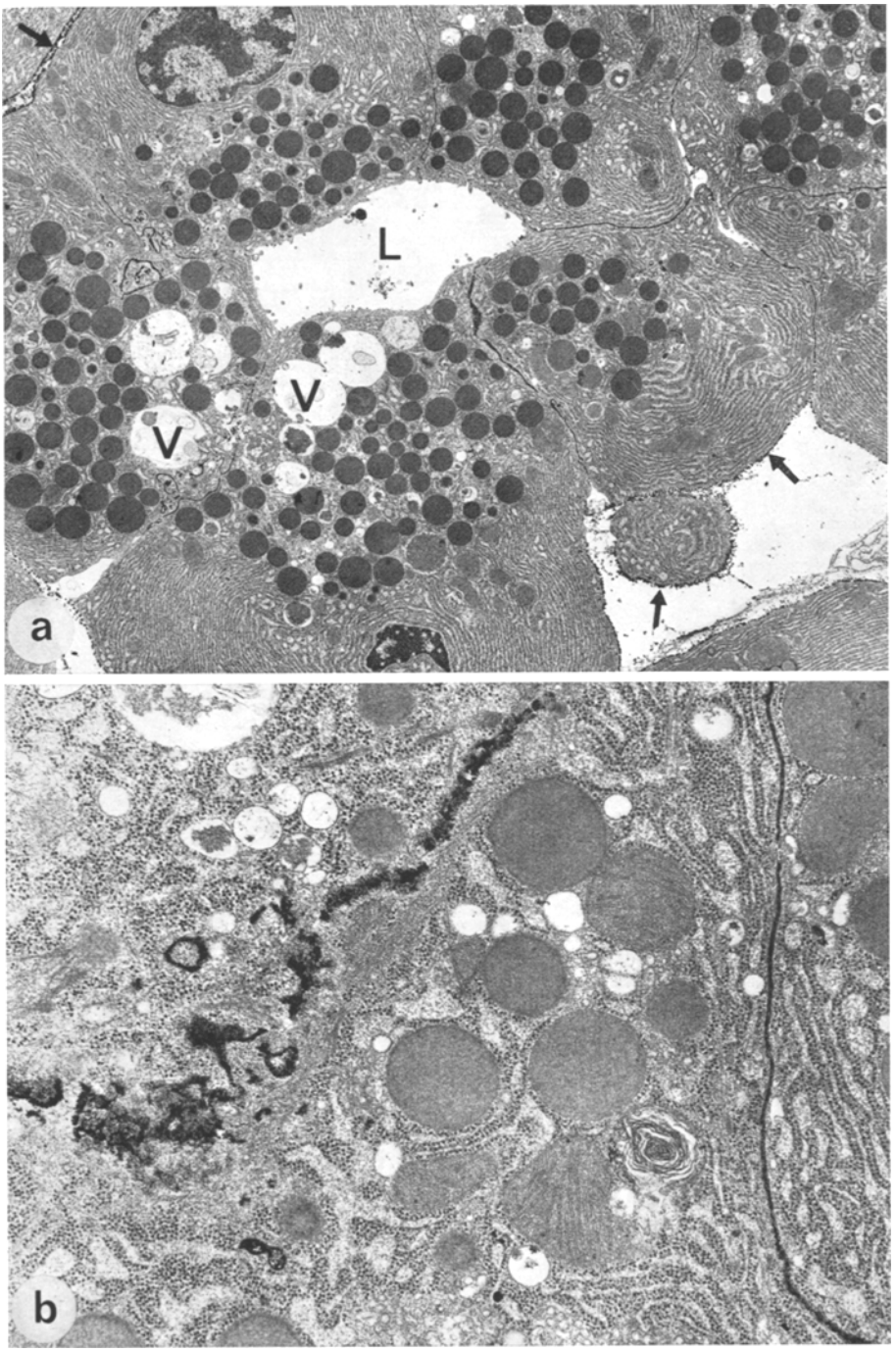

Fig. 7a and b

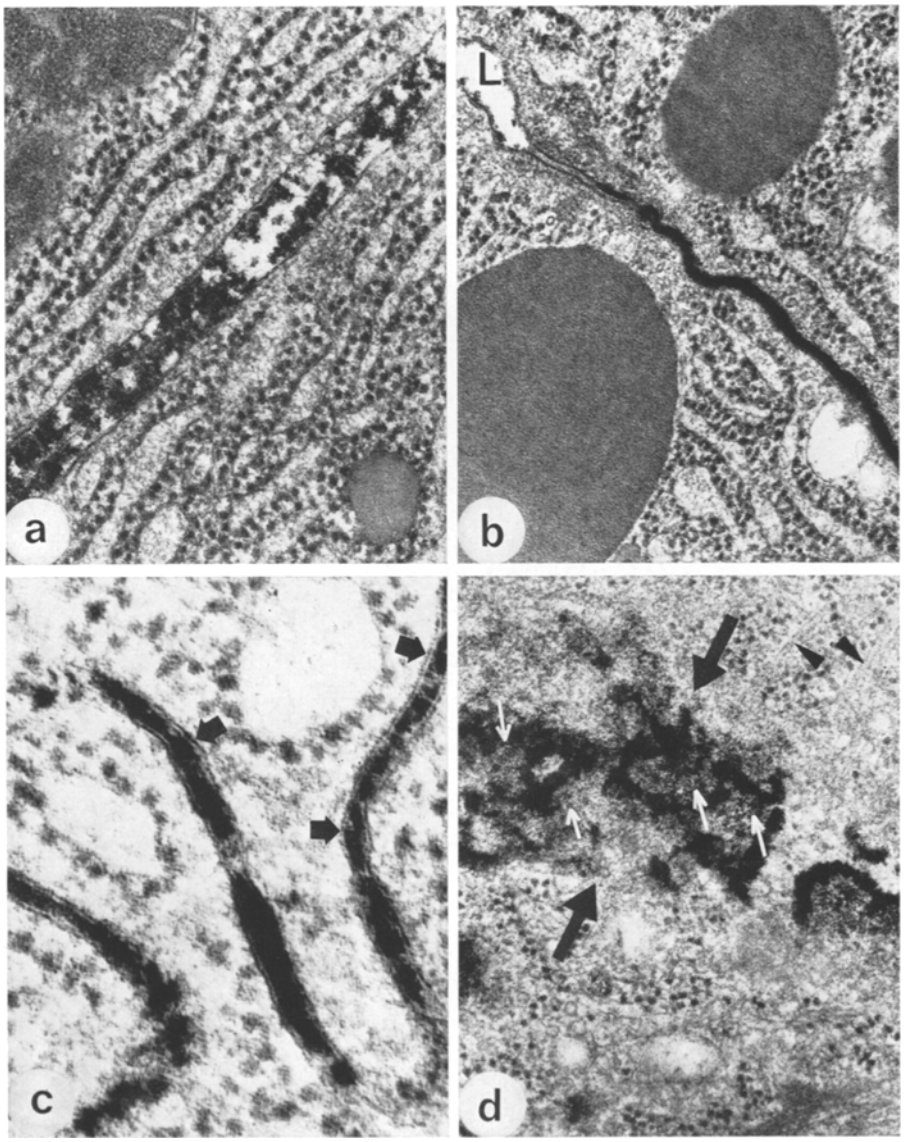

Fig. 8a—d. The lanthanum deposits at the external lamina of the plasma membrane at higher magnification. (a) At the basal plasma membrane between two neighboring acini the lanthanum staining material forms globular aggregates about 0.1 μ in thickness. Magnification, ×55000. (b) Lanthanum deposits fill the extracellular space, but do not penetrate into tight junction, which seal it off from the acinar lumen (*L*). Magnification, ×55000. (c) At high resolution the lanthanum deposits can be observed apposed to the external lamina of the plasma membrane. The triple-layered structure of the membrane is evident in areas where lanthanum staining material is sparse (arrows). Magnification, ×120000. (d) Distribution of lanthanum staining material on the surface of the plasma membrane which is cut tangentially. The outline of the membrane is indicated by large arrows, microtubules can be seen ending in the membrane (arrow heads). Lanthanum deposits spare out circular areas of 300 to 400 Å (white arrows). Magnification, ×55000

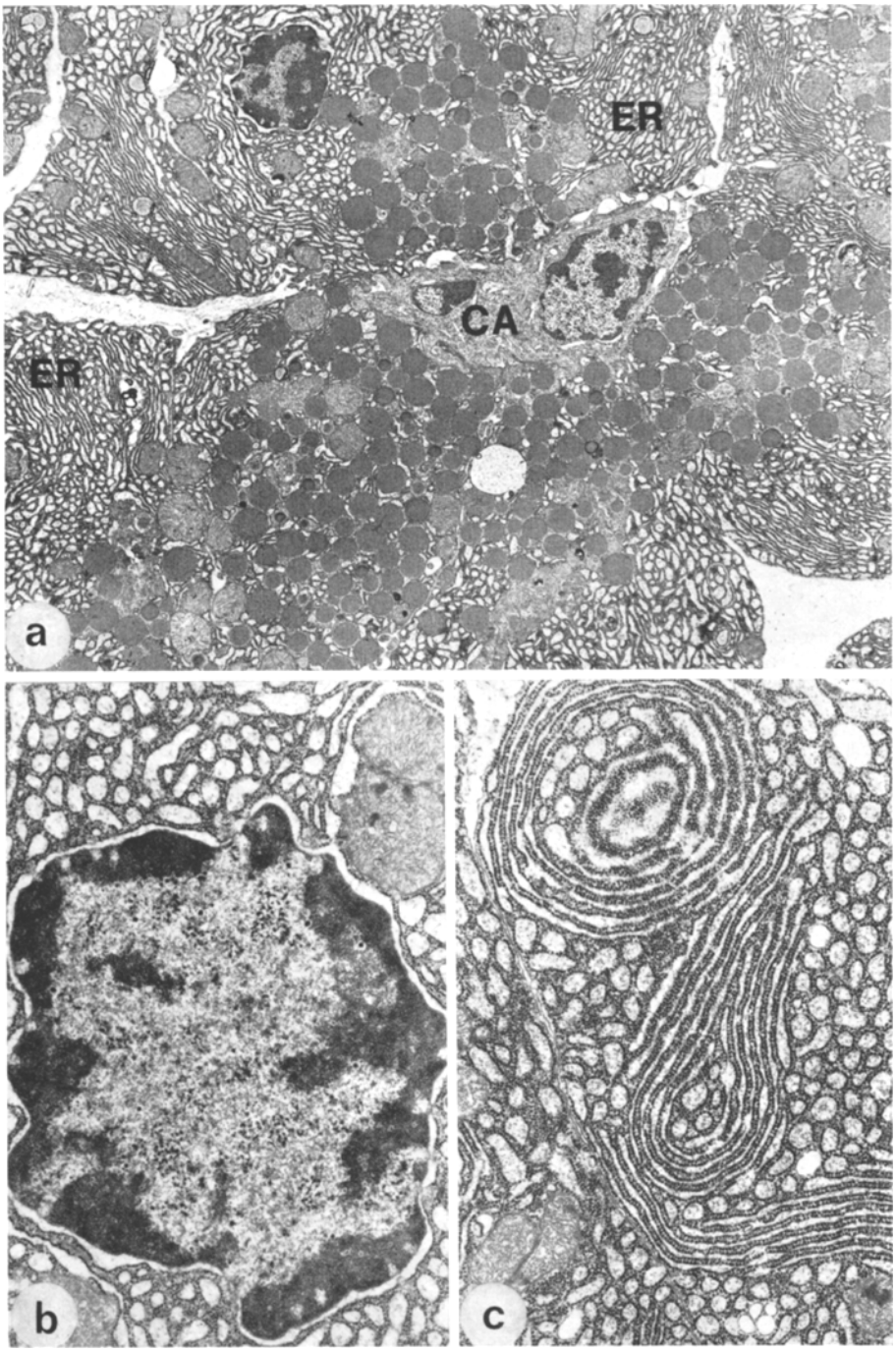

Fig. 9a—c. Structural alterations induced by incubation of pancreatic lobules in 10^{-4} M antimycin A for 3 hrs. (a) The exocrine cells are fully granulated, the endoplasmic reticulum (*ER*) is distended; *CA* centroacinar cell. Magnification, ×4500. (b) Condensation of heterochromatin along nuclear membrane, formation of "dense particles" in karyoplasm. Magnification, ×16000. (c) Finger-print like appearance and vesiculation of ER. Magnification, ×16000

Discussion

Although both cobalt and lanthanum are widely used as antagonists to calcium movements across the plasma membrane their action on the secretory process of the rat exocrine pancreas cannot be explained by calcium inhibition alone. Results from previous investigations indicate that cobalt beside interfering with slow calcium movements across the plasma membrane (Baker *et al.*, 1973) also penetrates into the cytoplasm and interferes with energy production in fibroblasts (Daniel *et al.*, 1963), liver and muscle cells (Dingle *et al.*, 1962) and in isolated heart muscle mitochondria (Wiberg, 1968). Lanthanum on the other hand, because of its ionic radius similar to calcium and its higher binding affinity replaces Ca^{++} bound superficially to the plasmamembrane and does not penetrate into the cell.

It has been reported that cobalt inhibits release of acetylcholine from neuromuscular junction (Miledi, 1966; Weakly, 1973; Kita and Kloot, 1973), of oxytocine from neurohypophysis (Dreifuss *et al.*, 1973) and of insulin from pancreatic islets (Henquin and Lambert, 1975). In all these systems the effect on secretion is correlated with an inhibition of calcium movements. The present study performed on rat pancreatic lobules could not demonstrate any interference of cobalt ions with the 3 major phases of the secretory process: protein synthesis, intracellular transport and discharge of zymogen granules, if the pool of "old" granules is considered. There is, however, a marked effect of cobalt on the release of granules, which have been formed during the incubation in the presence of cobalt ions. It is proposed that cobalt ions may alter the composition of the granules in a way, that they are excluded largely from release by exocytosis. How this change is brought about is not understood. Zymogen granules beside the various digestive enzymes contain large amounts of calcium (Clemente and Meldolesi, 1975), which is added to the protein mixture during granule formation in the Golgi complex. It can be speculated that during this process of granule condensation Ca^{++} is partly replaced by Co^{++}, resulting in modified zymogen granules, which cannot participate in the complicated process of membrane fusion during exocytosis. This possibility is subject to further studies in our laboratory. Moreover, cobalt could also act on the membrane of zymogen granules or on enzymes contained in the membrane, thus altering the specialization which is necessary for exocytosis (Meldolesi, 1974).

The present communication confirms earlier reports in a variety of cell types that lanthanum binds to the external lamina of the plasma membrane. The exact species of molecule(s) responsible for this binding is not clear, a mucopolysaccharide-protein complex attached to the outside of the membrane (generally referred to as "glycocalyx") has been proposed as a possible candidate (Overton, 1967; Lesseps, 1967; Shea, 1971; Langer and Frank, 1972). Previous studies on the exocrine pancreas of rat and mouse have demonstrated that lanthanum inhibits uptake of radioactive calcium and secretion of proteins (Heisler and Grondin, 1973; Chandler and Williams, 1974). The present communication extends the action of lanthanum also on protein synthesis and the late phase of intracellular transport. Displacement of calcium does not seem to be the only result of the binding of lanthanum. During incubation the tissue hardens considerably and this change becomes obvious during handling and

cutting it prior to fixation for electron microscopy. An altered biochemical and structural architecture of the outer "coat" of the plasma membrane might therefore result in disturbances of intracellular processes such as protein synthesis and transport. From data not given in this paper it can be concluded, that the inhibitory effect of lanthanum (10^{-3} M) on protein synthesis develops slowly. The rate of amino acid incorporation is nornal after 5 min incubation and decreases by 15% after 10 min, by 25% after 30 min and reaches the highest values of inhibition (50%) after 90 min. Determination of the free intracellular pool of radioactive precursor amino acid during prolonged incubation in lanthanum did not reveal an inhibition of amino acid transport into the cell, at least for L-leucine. Further studies on this problem are under way. As has been proposed by Chandler and Williams (1974) binding of lanthanum on the external lamina results in changes of the membrane which might prevent the binding of secretagogues to the receptors. This explains a similar inhibition of the release of amylase or newly synthesized proteins (Fig. 2). The same membrane changes could be responsible for the late effect on intracellular transport, but several other possibilities exist. The effects of both cobalt and lanthanum are reversible if pancreatic lobules are incubated for a period up to 30 min in the absence of the ions. A comparison of the effect of cobalt and lanthanum with the action of antimycin A demonstrated that the energy requirements for the sequential steps in the secretory process are different. Protein synthesis and intracellular transport of newly synthesized proteins from rough ER to the Golgi complex are inhibited to the same extent as cellular respiration, e.g. at 10^{-6} M concentrations by 85%, while discharge of granules is less affected (50%). The effect of cobalt and lanthanum on the secretory process can therefore not be explained by interference with energy production in the pancreatic cell. Both ions seem to exert their action directly on membranes or specific molecules associated with the membranes. It could be concluded that cobalt offers a suitable model to study the prerequisits for the release of zymogen granules by exocytosis. Lanthanum on the other hand could serve as a tool for studying the functional significance of the external "coat" on the plasma membrane for the secretion process.

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