Temperature and energy dependence of secretory protein transport in the exocrine pancreas

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Pancreatic lobules pulse-labeled with [³H]leucine have been incubated at temperatures between 0 and 37°C in the presence or absence of ongoing oxidative phosphorylation. Subcellular fractionation methods and electron microscopic autoradiography have been used to monitor the progress of intracellular transport of newly synthesized secretory proteins. Over the period studied, exit from the rough endoplasmic reticulum (RER) occurs only at >10°C while traversal of the Golgi complex and entry into condensing vacuoles requires >22°C. Both steps of transport require ongoing ATP production. Incubation at 10 or 20°C does not diminish ATP levels, relative to 37°C controls. Remarkable and unprecedented alterations of the ultrastructure of transitional elements of the RER accompany the arrest of exit from the RER: at 10°C transitional elements are much more numerous and longer than in controls; in the absence of ATP production they are essentially absent. These observations are interpreted in terms of a cyclic model of RER-to-Golgi vesicular traffic. Inhibition of ATP production also causes an increase in the rigid cisternae and coated elements in the distal Golgi area.

Key words: endoplasmic reticulum/energy/Golgi/temperature/ transitional elements

Introduction

Although the overall path of intracellular transport of secretory proteins is well-established (Palade, 1975), little is known with respect to mechanisms operating in vesicle—vesicle recognition, translocation, fusion and fission. One means to provide further understanding of these events is to obtain methods for selectively interrupting vesicular traffic, if possible in reversible fashion. Furthermore, if it is possible to interrupt transport at selected steps, we may (i) be able to assign selected post-translational modifications to corresponding compartments without the labor of refined subcellular fractionation, and (ii) be able to rationalize the detailed anatomy of certain of the compartments involved.

The present investigation of temperature and energy requirements focuses especially on the exit of newly synthesized secretory proteins from the rough endoplasmic reticulum (RER). It has been thought for some time that partly smooth, partly rough regions of the RER known as 'transitional elements' (Palade, 1975) mediate exit by the quantal capture of RER content and selected RER membrane proteins. This vesicular capture process may owe its high degree of selectivity (Park et al., 1985; Yamamoto et al., 1985) to the presence of receptor components which line the ectodomain (cisternal face) of transitional elements (Fitting and Kabat, 1982; Lodish et al., 1983; Fries et al., 1984; Scheele and Tartakoff, 1985). The ability of newly synthesized

membrane proteins to exit with high efficiency from the RER implies that the membrane of the transitional elements — as well as much of the surface of the RER — must be fluid at 37°C.

The only anatomic data bearing on the sites of exit come from transmission electron microscopy of the Golgi area where transitional elements have frequently been observed. Staining studies employing bismuth salts applied to vertebrate and insect tissues (Brodie, 1982) document the presence of 'beads' which encircle the base of those vesicles which appear to be either in the process of fission or fusion with the RER. Exit from the RER is known to require ATP production (Jamieson and Palade, 1968b; Tartakoff and Vassalli, 1978). It does not occur during mitotis (Featherstone et al., 1985).

The exocrine pancreas of the guinea pig is used for the present investigation for several reasons. (i) This is the tissue for which there is a maximum of information available with respect to the path and kinetics of transport, both for the average secretory protein (Jamieson, 1972; Palade, 1975), and for individual secretory proteins (Scheele and Tartakoff, 1985). (ii) Subcellular fractionation methods are available for recovery of a rough microsomal fraction, highly enriched in elements of the RER, a smooth microsomal fraction, which includes the galactosyl transferase-positive elements of the Golgi complex, and a granule fraction which includes both the zymogen granules and condensing vacuoles (Tartakoff and Jamieson, 1974). (iii) The degree of specialization of the exocrine pancreas for biosynthesis of secretory proteins is so great (~95%, Scheele et al., 1978) that electron microscopic autoradiographic methods can be employed to monitor the course of intracellular transport. (iv) Pancreatic secretory cells, unlike fibroblasts and plasma cells, are 'regulated' secretory cells, i.e. they store their secretory product in secretion granules until they are stimulated by an appropriate electrical or hormonal stimulus. From the cytological point of view, the Golgi area is therefore characterized by distally disposed condensing vacuoles which are known to serve as an intermediate between the Golgi cisternae themselves and the zymogen granules (Jamieson, 1972; Palade, 1975). The temperature requirements for intracellular transport in regulated secretory cells have not been studied previously. A single brief abstract (with which we concur) has described the ultrastructural consequences for the Golgi area of incubation of the pancreas in the absence of ongoing ATP production (Palade and Fletcher, 1977).

Results

Subcellular fractionation experiments

Pancreatic lobules have been pulse-labeled for 2 min with [³H]-leucine, washed and returned to chase non-radioactive medium for increasing periods of time at 37°C. Tissue homogenates were then fractionated to recover rough and smooth microsomal fractions and a granule fraction. As in our earlier studies (Scheele et al., 1978) at the end of the pulse interval, the highest specific activity (trichloroacetic acid insoluble c.p.m. per mg protein) is found in the rough microsomal fraction. As a function of chase interval newly synthesized secretory protein drains from the rough
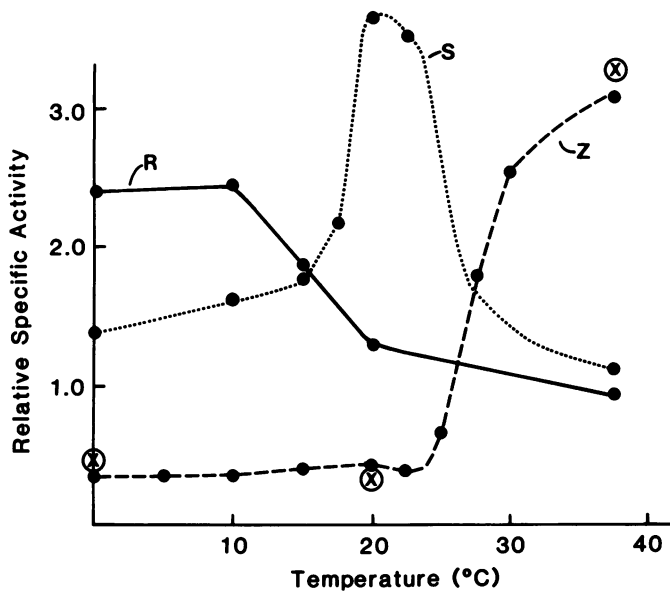
Fig. 1. Temperature-dependence of transport. Pancreatic lobules were pulse-labeled for 2 min with [³H]leucine, rinsed, and transferred to chase flasks at 0–37°C for 45 or 120 min. Homogenates were prepared, fractionated and analysed for acid-insoluble c.p.m. and total protein. Note that no labeled protein leaves the rough microsomal fraction at <10°C and no labeled protein enters the granule fraction at <20–22°C. ● refer to the 45 min chase and the circled cross symbol to the 120 min chase incubations. R: rough microsomes, S: smooth microsomes, Z: zymogen granule fraction. The 120 min chase data pertain only to the granule fraction.

microsomal fraction, traverses the smooth microsomal fraction, and ultimately accumulates in the granule fraction.

Figure 1 illustrates the impact of temperature reduction on the kinetics of transport. So long as the temperature is 10°C or lower, no secretory protein exits from the rough microsomal fraction over a period of at least 45 min. At 37°C the half-time for this step is 7–10 min (Scheele and Tartakoff, 1985). At temperatures between 10 and 22°C exit from the rough microsomal fraction procedes and an over-accumulation of newly synthesized secretory protein is evident within the smooth microsomal fraction. As judged by both the 45 and 120-min chase incubations, it is only when the temperature exceeds 20–22°C that transport to the granule fraction occurs.

Further experiments (not illustrated) show (i) that the interruption of transport for up to 60 min at 10 or 20°C is reversible; (ii) that chase incubation for 30 min at 20°C accumulates labeled protein at a site from which it can move (at 37°C) more quickly to the granule fraction than in 37°C controls and (iii) that if labeled protein, over-accumulated in the smooth microsomal fraction by a 20°C chase, is to move to the granule fraction during a further chase interval, the temperature must be maintained at 37°C. It is not sufficient to raise the temperature to 37°C for 5 or 10 min and then reduce it to 20°C again.

To evaluate whether ongoing ATP production is necessary for exit from the smooth microsomal fraction, we have taken advantage of the differential temperature sensitivity of entry into and exit from the smooth microsomal fraction. Lobules were pulse-labeled for 2 min with [³H]leucine and chased for 45 min at 20°C to over-accumulate massive amounts of newly synthesized secretory protein in the smooth microsomal fraction. The lobules were then transferred to 37°C in the absence or presence of a rapidly acting uncoupler of oxidative phosphorylation, 1 mM dinitrophenol (DNP) (Jamieson and Palade, 1968b, 1971) for an
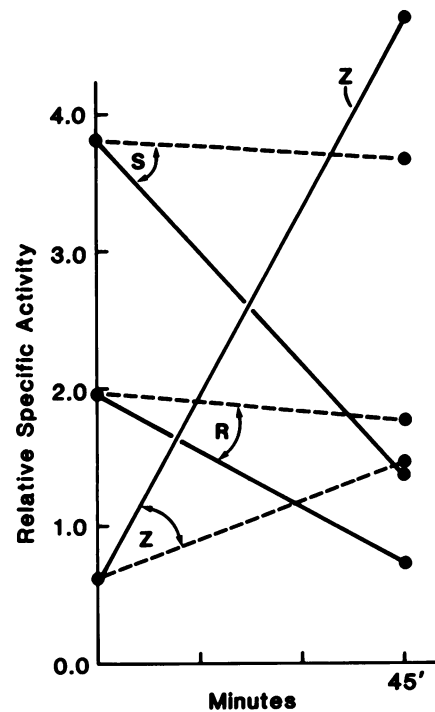

Fig. 2. Energy-dependence of exit of labeled protein from the smooth microsomal fraction. Lobules pulse-labeled with [³H]leucine for 2 min were rinsed and chase-incubated 45 min at 20°C to concentrate newly-synthesized secretory protein in the smooth microsomal fraction. One-third of the preparation was transferred to 0°C to stop all further transport, one-third was maintained at 20°C, and one-third received 1 mM DNP at 20°C. After 5 min the two latter samples were warmed to 37°C for 45 min. All three samples were then homogenized, fractionated and analysed as in Figure 1. Note that during the final control incubation at 37°C (solid line), labeled protein drains from both rough and smooth microsomal fractions and accumulates in the granule fraction. In the presence of DNP (dashed line) the progress of transport is greatly reduced. All three events are profoundly inhibited in the presence of 1 mM DNP. R: rough microsomes, S: smooth microsomes, Z: zymogen granule fraction.

additional 45 min. What is observed (Figure 2) in the controls is progressive drainage from the rough and smooth microsomal fractions and a massive accumulation of newly synthesized secretory protein in the granule fraction. By contrast, when the uncoupler is present, neither drainage from the rough nor from the smooth microsomal fraction occurs and the degree of increase of specific activity of the granule fraction is only modest.

Comparable observations have been made in pulse-chase protocols in which the over-accumulation of newly synthesized secretory protein in the smooth microsomal fraction is accomplished simply by a 10-min 37°C chase. We therefore confirm that ongoing ATP production is necessary for exit of newly synthesized secretory protein from the rough microsomal fraction and conclude that it is also essential for exit from the smooth microsomal fraction.

Autoradiographic studies

A parallel series of autoradiographic studies has been performed at the electron microscopic level, both to confirm the subcellular fractionation data and to identify with greater cytological precision the exact compartments within which transport is interrupted. The basic experiment involves [³H]-leucine pulse label for 2.5 min at 37°C, followed by a 45-min chase incubation at 0, 10, 20 or 37°C. After these incubations, the lobules have been fixed and processed for autoradiography.
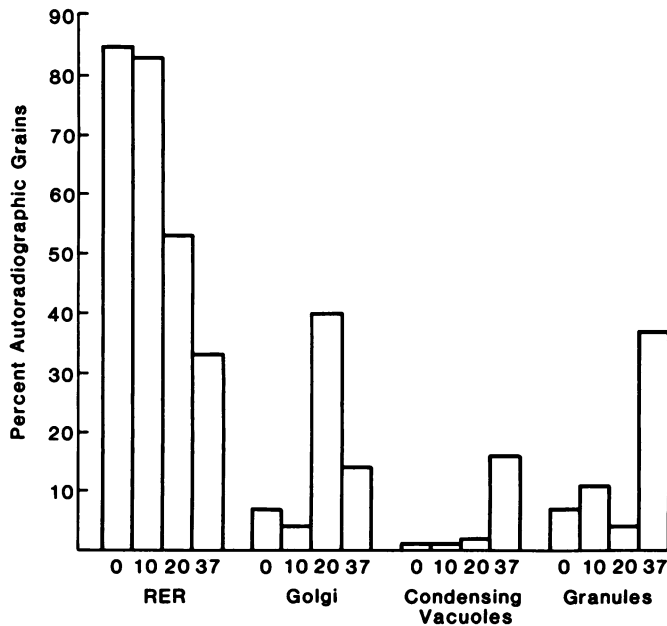
90

80

70

60

50

40

30

20

10

Percent Autoradiographic Grains

0 10 20 37
RER

0 10 20 37
Golgi

0 10 20 37
Condensing
Vacuoles

0 10 20 37
Granules

Fig. 3. Autoradiographic study of the influence of temperature on transport. Lobules pulse-labeled for 2.5 min at 37°C were rinsed and transferred to chase flasks for 45 min at 0, 10, 20 or 37°C. After fixation and work-up for autoradiography, autoradiographic grains lying over the cytoplasm were counted and assigned to one of the four compartments indicated (see Materials and methods). At 37°C major drainage from the RER to the condensing vacuoles and zymogen granules occurs. At 20°C transport is only as far as the Golgi. At 10°C the 0°C distribution of grains remains unchanged. RER: grains associated with the RER; Golgi: grains associated with Golgi vesicles and cisternae; condensing vacuoles: grains associated with condensing vacuoles; granules: grains associated with granules.

Figure 3 indicates the distribution of autoradiographic grains. The data are entirely in agreement with the subcellular fractionation data.

At 10°C, no change in the distribution of the autoradiographic grains is observed relative to the distribution after chase incubation at 0°C (85% of the grains associated with the RER). Moreover, these data indicate that drainage from the RER does take place at 20°C, and that labeled secretory protein gains access to the Golgi region, but not to the condensing vacuoles. At 37°C, as expected, the drainage is so extensive that both condensing vacuoles and the zymogen granules are labeled. One further point that emerges from the autoradiographic study is that when exit from the RER is interrupted by incubation at 10°C, the distribution of autoradiographic grains remains grossly uniform over the RER. There is no obvious indication that those newly synthesized proteins which are unable to exit from the RER accumulate at transitional elements (not shown).

ATP levels

We have investigated ATP levels in pancreatic lobules maintained for 1 h at 10, 20 or 37°C to determine to what extent the interruption in intracellular transport observed at 10 and 20°C reflects alterations in energy stores. In the control situation (37°C), the ATP level is $\sim 3.65 \times 10^{-13}$ mol ATP per mg of protein. This level is increased 10−20% by the incubation at 20 or 10°C. By contrast, if pancreatic tissue is incubated for 60 min at 37°C in the presence of 1 mM DNP ATP levels are reduced to <1% of control values.

Electron microscopy

We have not observed major ultrastuctural differences in com-

paring pancreatic lobules incubated at 20 versus 37°C for periods of time ranging from 20 to 60 min. In both cases compressed Golgi cisternae are obvious as are adjacent condensing vacuoles and zymogen granules. Moreover a moderate number of transitional elements are associated with the RER in the immediate vicinity of proximal Golgi cisternae.

Tissue incubated for 20−60 min at 10°C manifests a striking ultrastructural alteration: transitional elements are much more abundant and often much longer and more complex than in control tissue. In certain sections they appear to be composed of a chain of vesicular units fused one to the next (Figure 4). By contrast, in tissue incubated for 20−60 min with 1 mM DNP, or in an atmosphere composed of 95% nitrogen, 5% carbon dioxide, few or no budding transitional elements are seen. In their place, extensive areas of the RER facing the most proximal Golgi cisterna are smooth (Figure 5). Moreover, an accumulation of fibrillar material between these smooth-surfaced areas of RER cisternae and the proximal-most Golgi cisternae and also an increase in the number of coated vesicles and 'rigid cisternae' is often seen in the vicinity of distal Golgi cisternae (Figure 5).

Several related observations have been made (data not shown). (i) When tissue is incubated at 10°C in the presence of 1 mM DNP the structure of transitional elements closely resembles that of tissue incubated at 37°C in the presence of DNP. (ii) When tissue incubated for 30 min at 10°C to cause an increase in the number of transitional elements is subsequently transferred for 30 min to 37°C, the exaggerated abundance of transitional elements is reduced to control levels. (iii) If the transfer is to a 37°C incubation medium containing 1 mM DNP, the disappearance of transitional elements is as pronounced as in tissue incubated continuously at 37°C in the presence of DNP. (iv) The alteration of the structure of transitional elements at 10°C is unaltered by simultaneous incubation or 30 min pre-incubation with 0.5 mg/ml cycloheximide.

Discussion

Several studies have defined temperature requirements for vesicular transport. For example, temperature reduction to 16−20°C radically slows delivery of ligands from endosomes to lysosomes (Dunn *et al.*, 1979; Marsh *et al.*, 1983), and modification of temperature in the range 10−37°C has major consequences for the relative distribution of the asialoglycoprotein receptor between the cell surface and intracellular compartments (Weigel and Oka, 1983).

Selected steps along the secretory path are also temperature sensitive. The cell types studied have been constitutive secretors, that is, they secrete continuously and do not store their secretory products. For example, cells in tissue culture have been studied after infection with vesicular stomatitis virus (Fuller *et al.*, 1985; Griffiths *et al.*, 1985), influenza virus (Matlin and Simons, 1983) or mutants of Semliki Forest virus (Saraste and Kuismanen, 1984) − in the latter case, the mutant in question encodes envelope glycoproteins which exit from the RER only if the temperature is reduced below 37°C. All of these studies use immunocytochemical means to evaluate the progress of transport of viral envelope glycoproteins at 0−37°C. Two temperature cut-offs have been documented: when the temperature is maintained at 15°C, newly synthesized Semliki glycoproteins do exit from the RER and over-accumulate within vesicles and tubules interposed between the RER and the proximal face of the Golgi. When the temperature is maintained at 20°C, transport of Semliki, influenza and vesicular stomatitis virus glycoproteins proceeds
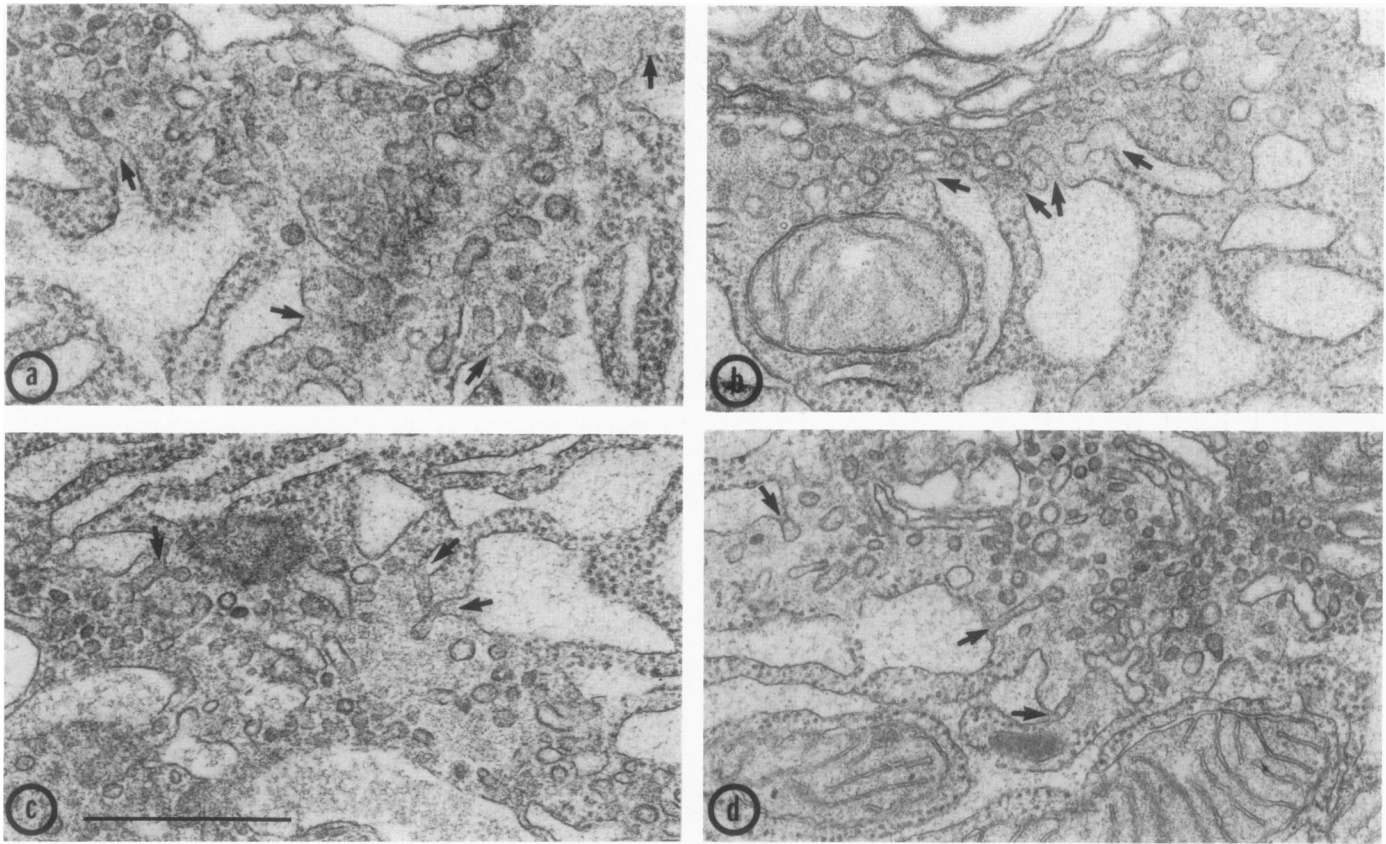
Fig. 4. a—d. Pancreatic lobules incubated 30 min at 10°C. The selected fields illustrated exemplify the proliferation of transitional elements, many of which are strikingly complex and elongated (arrows). Bar = 0.5 micron.

beyond the Golgi stack, the envelope glycoproteins acquire terminal sugars, and the glycoproteins are arrested in a partly 'coated' complex tubular structure which is histochemically positive for acid phosphatase.

Our present studies focus, by contrast, on a regulated secretory cell. Both the characteristic temperatures at which transport is interrupted and the sites at which transport is interrupted are different from those identified in the above-mentioned studies of constitutive cells (Figure 6). In particular, the cut-off we observe at 10°C pertains to exit from the RER and the interruption of transport at 20°C is within the Golgi stack proximal to the condensing vacuoles. Since the well-characterized secretory proteins of the guinea-pig exocrine pancreas are not glycoproteins (Scheele and Tartakoff, 1985), it is not possible to use their oligosaccharide maturation as an indirect measure of the cytological compartment within which transport is interrupted.

Energy requirements have previously been identified at multiple steps along the secretory path. (i) Exit from the RER requires ongoing ATP production, as mentioned in the Introduction; (ii) in yeast, making use of *sec* mutants which block transport along the secretory path either within the RER, the Golgi area or within secretory vesicles, there is an energy requirement for exit from each of these compartments (Novick *et al.*, 1981); (iii) an investigation of the energy requirement for maturation of condensing vacuoles to zymogen granules has indicated that this step, which apparently involves membrane withdrawal from the surface of the condensing vacuole and condensation of content, proceeds for at least 20 min in the absence of ongoing ATP production in the exocrine pancreas (Jamieson and Palade, 1971); (iv) secretogogue-induced granule discharge requires ongoing

ATP production in the exocrine pancreas (Jamieson and Palade, 1971); however, it is not clear to what extent this energy requirement may pertain to signal transduction at the plasma membrane.

The temperature characteristics for selected steps in transport may reflect local membrane fluidity or the activity of unspecified metabolic events, e.g. the ability of selected cytoskeletal elements to interact with vesicular carriers (Kimelberg, 1977; Sandermann 1978; Yguarabide and Yguarabide, 1981; Stubbs and Smith, 1984). Although the phospholipid and fatty acid composition of distinct stations along the secretory pathway are different (Meldolesi *et al.*, 1971; Esko and Raetz, 1983) this compositional information does not allow an adequate rationalization of our observations. For example, nothing is known of the lipid or protein composition of transitional elements.

Our present studies prove conclusively, for a eukaryotic regulated secretory cell, that exit from the stack of Golgi cisternae does require ongoing ATP production. However, the understanding of the importance of ongoing energy production for vesicle translocation, fusion and fission is far from complete. One possibility is that ATP is required to remove clathrin from coated elements (Braell *et al.*, 1984; Schlossman *et al.*, 1984; Schmid *et al.*, 1984). Indeed, when we deplete ATP stores an overabundance of coated vesicles is seen in the Golgi region (Figure 5). The apparent proliferation of rigid cisternae [which are known from the studies of Novikoff *et al.* (1977) to contain acid phosphatase and to be continuous with the membrane of condensing vacuoles] may reflect an analogous exaggeration of a normally short-lived intermediate.

The availability of these means of interrupting transport at well-defined stations provides a set of *in vivo* parameters which must
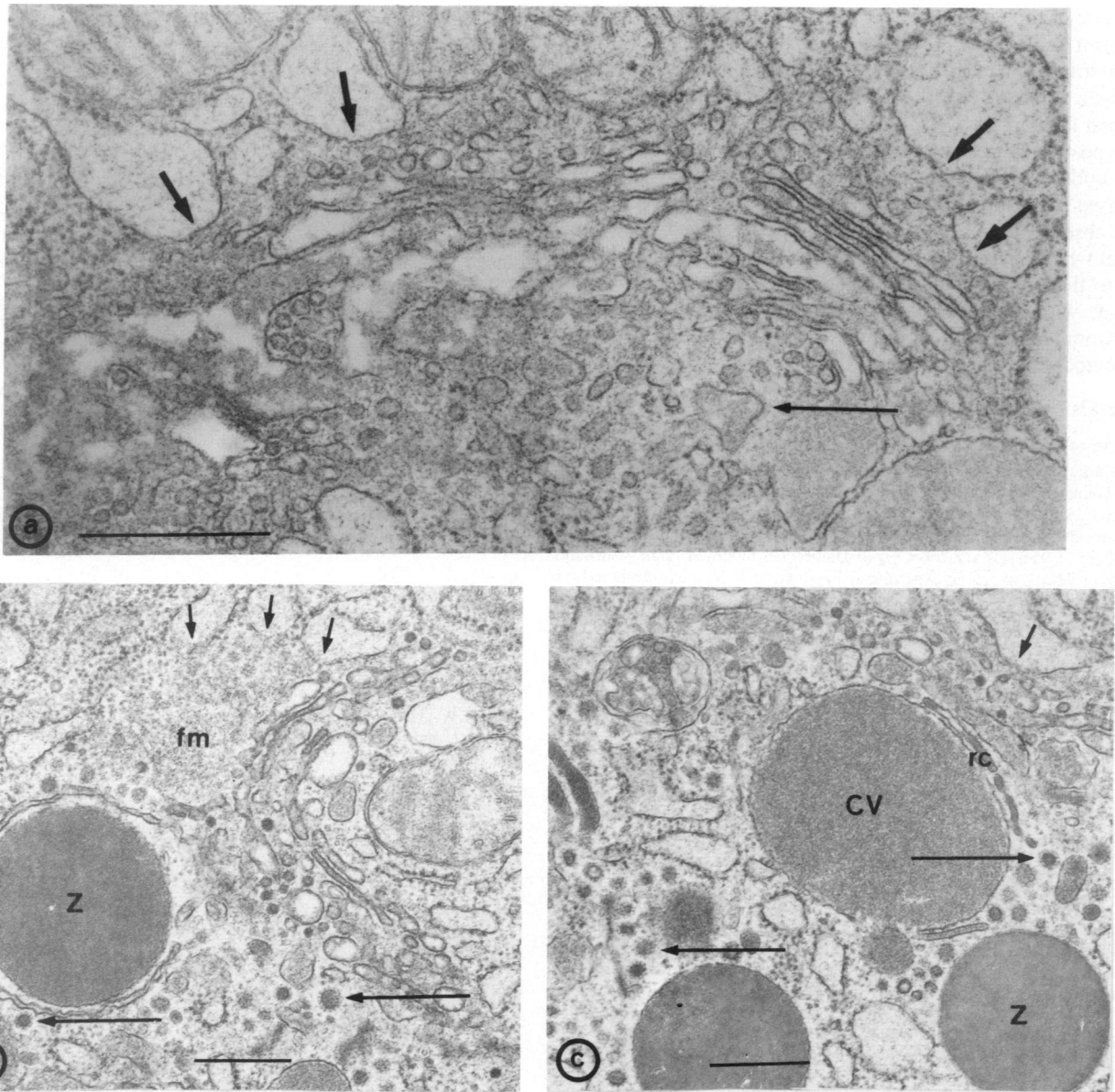
Fig. 5. a—c. Pancreatic lobules incubated 30 min at 37°C with 1 mM DNP. Note the total absence of budding transitional elements (short arrows), the fibrillar material (fm) between the RER and proximal face of the Golgi, and the proliferation of coated elements (long arrows) and rigid cisternae (rc). Bar = 0.5 micron.
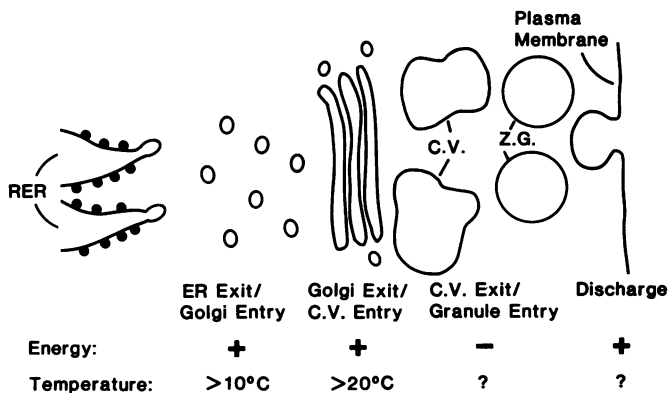

	ER Exit/ Golgi Entry	Golgi Exit/ C.V. Entry	C.V. Exit/ Granule Entry	Discharge
Energy:	+	+	−	+
Temperature:	>10°C	>20°C	?	?

Fig. 6. Model of the secretory path in regulated secretory cells, including the indications of temperature and energy requirements described in the text.

form the basis of any evaluation of the adequacy of *in vitro* models which reconstitute the corresponding steps in transport. The studies of Balch *et al.* (1984) in a cell-free proximal Golgi-to-medial Golgi transport system document an energy requirement. Moreover, the availability of means for interrupting transport should make it possible to assign selected post-translational modifications to limited segments of the secretory pathway.

The most surprising observation in the present study concerns the modulation of the anatomy of the transitional elements of the RER. These units are thought, strictly on the basis of anatomical data, to be the sites of exit of newly synthesized protein from the RER. Our observations indicate a strong correlation between RER exit and the anatomy of the transitional elements. In the absence of ATP production, the characteristic smooth-surfaced buds are altogether absent, while in tissue maintained at 10°C, the buds themselves are more numerous and complex than in control tissues.

Since cycloheximide interrupts neither exit from the RER (Jamieson and Palade, 1968a) nor the production of the exaggerated transitional elements at 10°C we propose a cyclic model of vesicle transport between the transitional elements and the proximal face of the Golgi. In this context, the 10°C incubation can be postulated to arrest either vesicle departure from the RER or the collapse and merging of vesicles which have returned to and fused with the RER. The site of vesicle transit interrupted in the absence of ATP production may correspond either to a stage at which vesicles have departed from the RER or to a stage prior to the initiation of their budding. Such assignments will be made with greater certainty when antigens characteristic of transitional elements are identified and followed immunocytochemically.

Materials and methods

Tissue preparation, biosynthetic labeling, subcellular fractionation

150 g Hartley guinea-pigs were starved overnight and given water *ad libitum* before killing by cervical dislocation. The pancreas was removed and lobules prepared by injection of Hank's basic salt solution, according to Scheele (1983). Small lobules were maintained in stoppered flasks in a shaker bath under an atmosphere of 95% O_2 and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Gibco), except in experiments in which 95% N_2 was substituted for O_2. For pulse-labeling, [³H]4,5-leucine (ICN, 50 Ci/mM) was included in leucine-free medium at a final concentration of 10−100 μCi/ml. The high dose was for the autoradiographic experiment. After 2−3 min pulse-labeling at 37°C, lobules were removed with forceps, transferred into two changes of ice-cold DMEM, and finally returned to chase flasks containing 5−10 ml in DMEM for further incubation. Incubations were terminated by removal of the lobules and their transfer to ice-cold 0.3 M sucrose.

Subcellular fractionation was based on the procedure of Tartakoff and Jamieson (1974), with several modifications. Numbers of lobules equivalent to one-quarter of a gland were homogenized in 4 ml of 0.3 M sucrose at 0°C. The homogenates were sedimented 10 min at 1700 r.p.m. in a table-top Sorvall centrifuge. The resulting post-nuclear supernatant was re-sedimented at 4500 r.p.m. for 10 min in the Ti-50 rotor of the Beckman ultracentrifuge to sediment a granule fraction. The granule fraction was surface-rinsed with 1 ml of cold 10% sucrose to remove contaminating mitochondria and microsomes. The post-granule supernatant was sedimented at 11 500 r.p.m. for 10 min (Ti-50) to eliminate mitochondria and the resulting post-mitochondrial supernatant was re-sedimented for 60 min at 40 000 r.p.m. (Ti-50) to sediment a total microsomal fraction which was resuspended in 2 ml of 35% sucrose. The total microsomal fraction was then loaded into the middle of a discontinuous sucrose gradient above 1 ml of 54% sucrose and beneath 3 ml of 10% sucrose. These discontinuous gradients were constructed in Beckman 'Quick-seal' tubes and centrifuged in the VTi-65.2 rotor for 60 min at 65 000 r.p.m. After 60 min, an accumulation of smooth microsomes was visible at the interface between the load and 10% sucrose, while the rough microsomes accumulated at the interface between the load and the 54% sucrose. Both fractions were recovered and diluted with 5 ml of 10% sucrose containing 0.2 M KCl to desorb contaminating secretory protein (Scheele *et al.*, 1978). The diluted fractions were then re-sedimented for 30 min at 40 000 r.p.m. (Ti-50) to pellet washed smooth and rough microsomal fractions.

These two fractions as well as the granule fractions were resuspended in 1 ml 10% sucrose. Aliquots of these fractions and of the post-nuclear supernatant were applied to filter discs for determination of trichloroacetic acid insoluble radioactivity and parallel aliquots were analysed for protein as in Scheele *et al.* (1978). The term 'relative specific activity' for a given subcellular fraction is defined as its specific activity (c.p.m./mg protein) divided by the specific activity of the post-nuclear supernatant.

Electron microscopy, autoradiography

Lobules were fixed for 60−120 min in 1% paraformaldehyde, 2.7% glutaraldehyde in 0.1 M sodium cacodylate-HCl buffer, pH 7.3, supplemented with 5 mM CaCl. By adjusting the temperature of the primary fixative, fixation was initiated at the temperature at which each chase incubation was performed (0−37°C). Subsequent fixation proceeded at room temperature.

Following a buffer wash, samples were refixed for 1 h in a mixture of 1 part 2% OsO_4, 1 part 2% potassium ferrocyanide at 4°C. Tissue was subsequently stained *en bloc* overnight in 0.25% uranyl acetate at 4°C, rapidly dehydrated in graded ethanols, embedded in Epon 812, sectioned and stained with lead citrate and uranyl acetate.

For autoradiographic experiments, lobules were fixed directly in 1% OsO_4 buffered in 0.1 M sodium cacodylate-HCl buffer, pH 7.3 for a period of 1 h on ice. Coating with Ilford L-4 emulsion, 2 month exposure, development and grain

counting was as in Tartakoff and Vassalli (1978). 400−500 grains were counted for each of the four chase conditions.

ATP determinations

ATP measurements were performed after incubation for 1 h at 0−37°C using the procedure of Stanley and Williams (1969). Lobules were quick-frozen in liquid nitrogen, subsequently homogenized while frozen in 2 ml of frozen 0.3 M perchloric acid in an ice bath, held on ice for 15 min and then sedimented for 10 min at 3000 r.p.m. The resulting supernatant was diluted roughly 1000-fold with water and used at once for ATP determination using luciferase−luciferin (Sigma Product L-0633). The luciferase−luciferin was dissolved in water to give a final concentration of 40 mg/ml. Twenty microliter aliquots of this stock were diluted at the last minute with 1 ml of water at 24°C, mixed with graded amounts of ATP standards or cell extracts, and after 10 s the sample was counted in a Beckman liquid scintillation counter (Model 5801). Each sample was counted 10 times for 0.1 min with the H number setting at 0, the random coincidence monitor off, and a 0-infinity energy window. Background levels were in the range of 30 c.p.m., 5×10^{-13} mol of ATP gave 31 000 counts/0.1 min.

In parallel with the ATP determination, samples of the homogenate were measured for protein content as in Scheele *et al.* (1978).

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