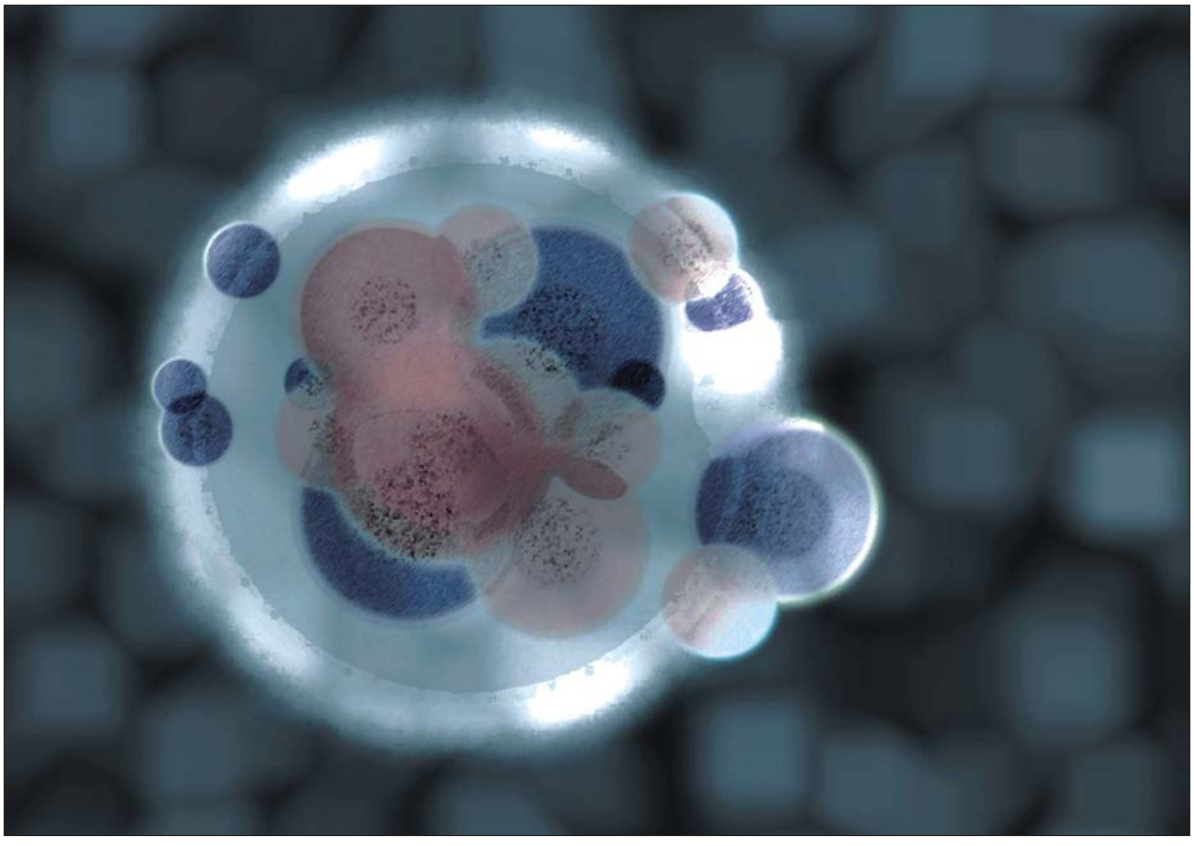

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Inhibition of Rapidly Degraded Membrane Phospholipid by Monensin and CCCP

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Abstract: The aim of this study was to determine the close association between the rapid degradation of newly synthesized membrane phospholipids and secretion that occurs in actively secreting myeloma cells. To achieve this goal, two mechanistically different inhibitors of secretion, monensin and carboxylcyanide m-chlorophenylhydrazone (CCCP), were used. We concluded based on the results of this study that phospholipids reach their site of degradation by active vesicular transport through the golgi apparatus rather than by the extremely rapid, energy independent pathway exhibited by other cells. Evidence that secretion and rapid degradation of membrane phospholipids occur at similar rates and that both are inhibited to similar extends by each of the inhibitors. This suggests that both processes follow identical pathways. These findings demonstrate the active role of some type of dynamic membranes, which serve as an expandable packages for the intracellular transport of secretory product.

Key words: Carboxylcyanide m-chlorophenylhydrazone (CCCP), monensin, myeloma, phospholipids, vesicular transport

INTRODUCTION

George Palade's[1] pioneering studies of zymogen secretion from pancreatic acinar cells, the mechanisms of vesicle-mediated protein transport have been investigated heavily. Evidence has been presented for the mechanism for the intracellular transport of newly synthesized secretory proteins by membrane flow from the endoplasmic reticulum through the golgi apparatus to the plasma membrane by a process involving sequential budding-off and fusion of small vesicles between these organelles (vesicular transport)[2-5]. Surprisingly, it has been shown that intracellular transport of newly synthesized membrane phospholipid from endoplasmic reticulum to plasma membranes[6-9] takes place by an independent pathway, which has been characterized by its ability to transport some phospholipids much more rapidly than the pathway for vesicular transport of membrane proteins and secretory product. Also by its resistance to inhibition by energy poisons and other inhibitors of vesicular transport (monensin and CCCP).

The advantage of using myeloma cells as a model to carry out this study, lies in it's secretion of only a single class of protein, known as kappa light chain immunoglobulin[10]. In order to verify the above suggested pathway of the intracellular transport of newly synthesized secretory proteins, which is coupled to the turnover of the phospholipid as well as protein constituents of the dynamic membranes, two different inhibitors (monensin and CCCP), independently, were used

MATERIALS AND METHODS

Preparation of myeloma cells: Myeloma cell tumors, provided by Litton Bionetics, Inc., USA, were maintained by subcutaneous injection of a saline suspension of teased tumors into male Balbc mice. Solid tumors were formed at site of injection 2-3 weeks later. For experimental purposes, solid tumors were teased in presence of Eagle's minimum essential medium warm media that had been pre-equilibrated with 95 and 5% air and carbon dioxide, respectively. The cell suspension is passed through 200 mesh nylon filter cloth, for removing aggregated cells. Cell viability was determined by trypan blue exclusion and ranged from 85 to 90%. Roughly 50 million cells per gram wet weight of tumor tissue is obtained. Cells were suspended at a density of 4×10^6 cells/mL in minimal essential medium containing 10% of dialyzed, heat-inactivated bovine serum. Cell suspensions were incubated at 37°C under an atmosphere of 95 and 5% air and carbon dioxide.

Single pulse-chase experiments: It was performed according to Cohen and Plhillips[10] with the following modifications: the interval of pulse labeling by glycerol and leucine was increased from 3 to 10 min. Cell density during pulse is 2 million/mL of Eagle's minimum essential

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medium media, containing 10% horse serum. The pulse labeled cells were washed quickly with warm chase medium, passed through the mesh, 3 mL aliquots were incubated for each chase period.

Double pulse-chase experiments: Cell suspension (20 mL) were pulse labeled by incubation for 10 min with 300 μCi of [2-^{3}H] glycerol (1 μCi/mM) and 300 μCi of L-[4,5-^{3}H] leucine (58 mCi/mole). A separate 10 mL aliquot of the same cell suspension was incubated for one hour with 30 μCi of [1,3-^{14}C]-glycerol (29.5 mCi per mole) and 30 μCi of L-[^{14}C]-leucine (348 mCi per mole). The [^{14}C] labeled cells, after being washed with 6 mL of cold chase medium, were used as descried below for the preparation of ^{14}C carrier homogenate, was combined with homogenates of the pulse-chased-labeled cells in order to permit correction for variability in recoveries of cell fractions during their isolation and assay for labeled protein and phospholipid. At the end of the labeling period, the pulse-labeled cells were immediately collected by centrifugation, washed with warm chase medium (containing 4 μM unlabeled glycerol and leucine). The washed cells were suspended in 20 mL chase medium, filtered as before then 3 mL aliquots were dispensed into 60 mm incubation dishes and incubated at 37°C. At the end of the chase periods, the cells were collected, washed for at least 4 times.

Preparation of cell and tissue homogenates: For the preparation of carrier homogenate, solid tumors (1.5 g), were minced, combined with [^{14}C]-labeled cells, then homogenized in 0.25 M sucrose with a Potter-Elvehjem homogenizer (4 strokes at 200 rpm). The homogenate was diluted two-fold and filtered. Homogenate of the pulse-chased cells were prepared by suspending the cells in 1 mL of [^{14}C]-labeled carrier homogenate, add 1 mL sucrose, homogenize as before and subject to fractionation.

Isolation of labeled immunoglobulin light chain: Labeled immunoglobulin kappa light chain, was isolated by immunoprecipitation with rabbit anti-mouse kappa light chain antibody. The formed antigen-antibody complex was isolated by adsorption on formalin fixed, heat inactivated, protein A bearing *Staphylococcus aureas* cells, essentially as described by Kessler[11]. Membrane fractions from the cells were prepared for immunoprecipitation by solubilization with sodium deoxycholate at a final concentration of 0.33% in phosphate buffered saline (pH 7.4), (0.5 mg of sodium deoxycholate/mg of protein). This amount was found to permit maximum immunoprecipitation of membrane associated light chain and not to interfere with the immunoprecipitation formation. The immunoprecipitation of light chain in the medium from cells (after being dialyzed against phosphate buffered saline) and in the cytosolic fractions of pulse-chased cells were performed in the absence of deoxycholate. SDS-polyacrylamide gel electrophoresis of the immunoprecipitates from the various fractions, was carried out according to Covault *et al.*[12]. The results indicated the presence of a single band of labeled protein that migrated at a position on the gels corresponding in molecular weight to kappa light chain, isolated from the urine of tumor bearing mice.

Membrane fractions from cells recovered at each chase interval, after the addition of carrier homogenate as described above, were suspended by homogenization in 1.0 mL of 0.25 M sucrose. A 0.2 mL aliquot of the suspension (contains 0.05 mg of protein) was diluted to 0.5 mL with phosphate buffered saline, pH 7.4 and 0.5 mL of freshly prepared 0.66% sodium deoxycholate was added while stirring the suspension vigorously. The mixture was allowed to stand for 10 min, centrifuged to remove insoluble material. Aliquots (0.2 mL) of the clarified samples, or 0.4 mL of samples not treated with deoxycolate (cytosol and medium), were treated with 45 μL of the antibody (1:5 dilution in phosphate buffered saline, pH 7.4, of a stock antibody solution that contained 1 mg lyophilized antibody protein per mL) and then 200 μL of a 10% (w/v) suspension of freshly washed protein A containing *S. aureus* cells.

Determination of radioactivity incorporated into protein and phospholipid: Trichloroacetic acid precipitation of proteins, from the various fractions of the pulse-chased cells and the medium, was carried out by adding 500 μL of 10% trichloroacetic acid to a 100 μL sample. The mixture was chilled to 5 °C for 30min and an additional milliliter of 5% trichloroacetic acid was added. The mixture was then filtered through a GF/F glass microfiber filter. The radioactivity on the filters was measured using the Molecular Imager System. The duplicate measurements, which never differed by more than 5%, were averaged. The protein concentration was determined using the BCA kit (Pierce).]. Total Phospholipid was isolated and measured for incorporated radioactivity as described by Verhoeven and Janson[13].

RESULTS

Effect of monensin on secretion, intracellular distribution of immunoglobulin light chain and rapid degradation of membrane phospholipid: Myeloma cells was pulse labeled with double labeled glycerol

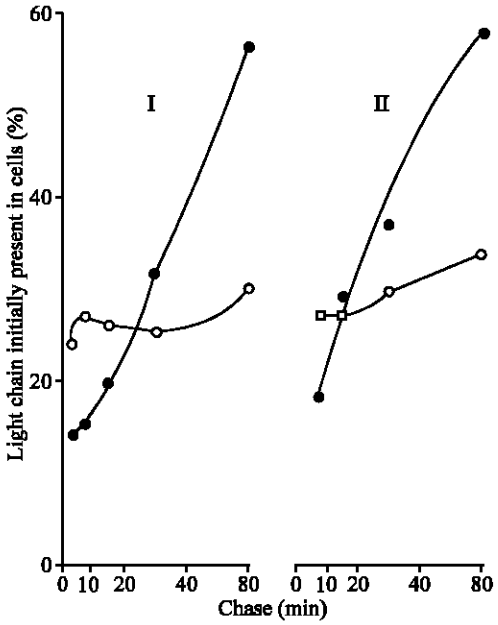

Fig. 1: Secretion of newly synthesized light chain by myeloma cells incubated in the absence and presence of 10 μM monensin. In experiment I, cells were both pulse labeled and chased in presence of 10 μM monensin (○). In experiment II, 10 μM monensin was present only during the chase incubation (□). Aliquots of the two different cell preparations were also pulse-labeled and chased in the absence of monensin (●)

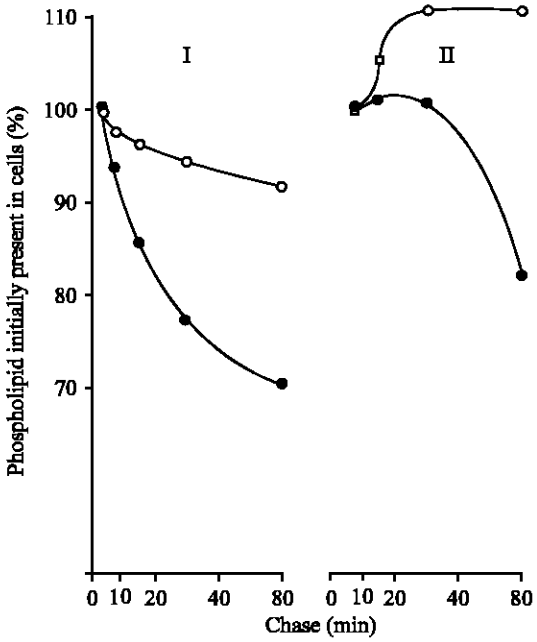

Fig. 2: Decay of newly synthesized phospholipid in myeloma cells incubated in the presence (○, □) and absence (●, ●) of monensin. Cells from experiment I in Fig. 1 and from a different cell preparation (experiment III) were pulse labeled and chased in the presence and absence of 10 μM monensin. The different experimental points, which indicate the percentage of newly synthesized phospholipid after pulse labeling that is recovered in the cells after chase incubation, refer to conditions of cell incubation described in Fig. 1

and leucine, chased, homogenized with ^{14}C carrier homogenate and centrifuged at 40,000 rpm for 20 min to sediment most cellular membranes. The obtained fractions: the sedimented fraction (membranes), the nonsedimentable fractions (cytosol) and (medium) from the incubation medium from the chased cells, were used to assay for the amount of newly synthesized phospholipid and light chain. Monensin (10 μM) was present during both the pulse and chase incubation in experiments I and III, but was present only during the chase incubation in experiment II. It shows also that the amount of newly synthesized phospholipid in both monensin treated and untreated cells is insignificant. Thus the rapid decay of membrane phospholipid that observed in untreated cells represents rapid degradation of phospholipid and presumably the membrane itself and it is this degradation that is inhibited by monensin.

Figure 1 indicated that monensin inhibits secretion almost completely, which was confirmed by other researchers[14,15]. Monensin also inhibits markedly the normally rapid decay of membrane phospholipid (Fig. 2). Also, it is noted that light chain secretion and phospholipid degradation have always been found to respond to a similar extend of inhibition by monensin, even at different concentrations of monensin.

As shown in Fig. 3A that newly synthesized light chain associated with the membranes of monensin treated myeloma cells continues to decay as rapidly as in untreated cells. However, instead of being secreted, the light chain accumulates in the cytosol (Fig. 3B). Thus monensin inhibits secretion, to approximately the same extend that it inhibits degradation of membrane phospholipid.

Effect of CCCP, an uncoupler of oxidative phosphorylation on secretion and rapid degradation of membrane phospholipid: Unlike monensin, which inhibit secretion by blocking the transport of secretory product through the golgi complex, which is in agreement with[16], uncouplers inhibit secretion at the energy requiring steps for the transport of the secretory product from the endoplasmic reticulum to the golgi complex as well as for

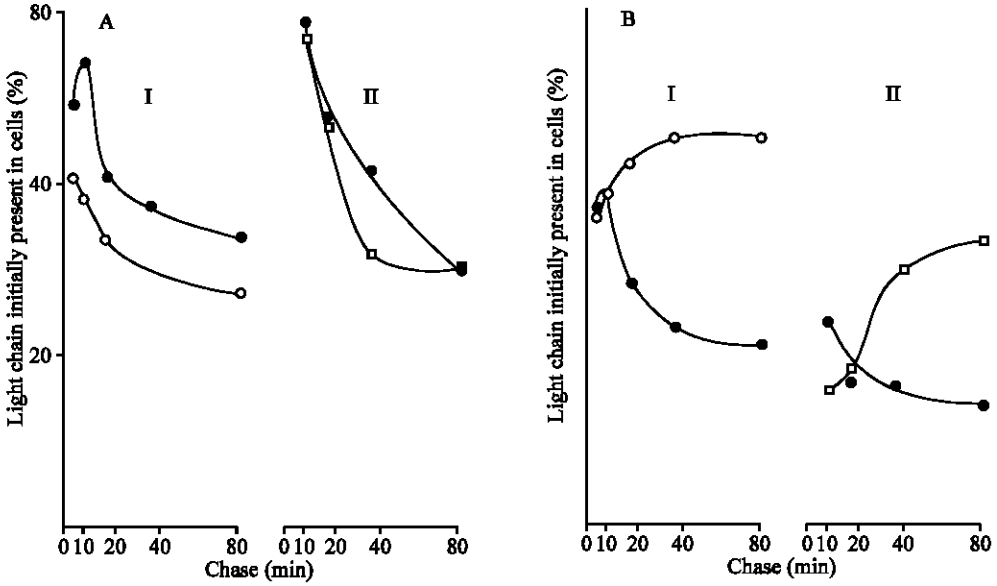

Fig. 3: Decay of newly synthesized light chain from the membrane (A) and cytosolic (B) fractions of myeloma cells incubated in the absence (●,●) and presence of monensin (○, □) for each of experiment I and II

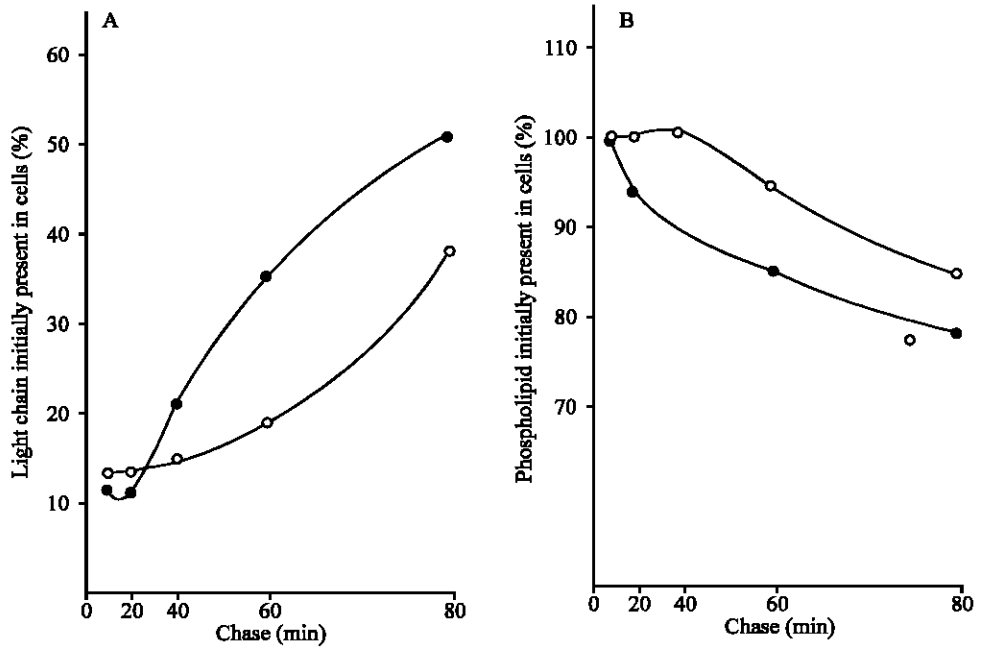

Fig. 4: Effect of CCCP, an uncoupler of oxidative phosphorylation, on secretion of light chain (Fig. A) and on the normally rapid decay of intracellular membrane associated phospholipid (Fig. B). Cells pulse labeled as described in Fig. 1 were incubated in chase medium (●,●) or chase medium containing 50 μM CCCP (○, □) [^{14}C]-labeled light chain released into the medium and [^{3}H]-labeled phospholipid recovered from the membrane fraction of cells during chase were determined and in Fig. 1 and 2

its transport from golgi complex to the plasma membrane[5,17,18]. Figure 4 indicated that the concentration of CCCP used in these experiments inhibits both the secretion of light chain from the cells and the rapid decay of intracellular phospholipid. This effect is similar to that caused by monensin. However, after about 40 min of chase, the uncoupler begins to loose its inhibitory effect, may be because of the instability of this highly active

Table 1: Comparison of light chain secretion and phospholipid degradation by different preparations of myeloma cells

Cell preparation	Light chain (% secreted in 80 min.)	Phospholipid (% secreted in 80 min.)	Ratio
Fig. 1 and 2	57	30	1.9
Fig. 4	34	15	2.3
Fig. Not shown	41	18	2.3

compound. As shown in Fig. 4A and B, there is a close parallelism between the initial inhibition and the subsequent recovery of both secretion and rapid turnover of membrane phospholipid. This provides a clear indication and convincing evidence that the two processes are closely related.

Comparisons of the relative amounts of newly synthesized light chain and phospholipid that is secreted and rapidly degraded: Table 1 compares the extend of secretion and phospholipid turnover that occurs after 80 min of chase for three different cell preparations examined in present study. It can be seen that, despite a nearly two-fold variation in the extent of secretion and phospholipid turnover, the ratio of the two processes in the different cell preparations remains fairly constant, as one expect if these two processes are closely coupled.

DISCUSSION

Since the independent pathway for rapid intracellular migration of phospholipids is resistant to inhibition by monensin and CCCP and therefore should be evident that in the presence of these inhibitors, the fact that we observe an essentially complete inhibition of rapid phospholipid degradation suggests that the independent pathway does not participate to any significant extent in the degradative process. An alternative possibility, that the phospholipid is transported to its site of degradation by the independent pathway and that it is the degradative process itself that is inhibited seems unlikely for the following reasons. First, although CCCP might be expected to inhibit the energy requiring steps shown to be necessary for maintaining the low pH in acidic degradative compartments, monensin has not been shown to have a different effect on degradation. Although monensin has been shown to inhibit the rapid degradation of proteoglycans in cultured rat ovarian granulose[19], this inhibition could be the result of an expected interference by monensin on the transport of newly synthesized proteoglycans to the lysosomes rather than a direct effect on lysosomal degradation. secondly, it was shown by Tachibang *et al.*[20] that major classes of phospholipid undergo rapid degradation. As they reach their site of degradation by vesicular transport.

This present study shows that, there is a close kinetic relationship between secretion of immunoglobulin light chain and rapid membrane turnover in untreated cells (control). It is found that this relationship is maintained when secretion is inhibited by monensin or CCCP. It was reported by Zych *et al.*[21] that CCCP decreased basal and CAMP-stimulated secretory response. These observations supports the existence of dynamic membranes, which participate in the secretory process presumably by serving as expendable packages that are assembled along with their content of secretory product, then undergo unidirectional flow to the cell surface where the secretory product is released and the membranes are transported to their site of degradation.

The presence of relatively large amounts of newly synthesized light chain in the cytosol (Fig. 3) has also been observed in earlier studies which proposed that the light chain was originally associated with membranes, but is released during cell disruptions[22]. The rapid loss of newly synthesized light chain in the cytosol as well as membrane fraction as it is being secreted from untreated myeloma cells (Fig. 3A) supports this conclusion since it is generally assumed that secretory product must be compartmentalized within membranes of the secretory pathway in order to be secreted[23]. The rapid decay of membrane associated light chain that is observed to continue even when secretion is inhibited by monensin (Fig. 3B), would represent the close similarity between the patterns for the continued appearance of these newly synthesized components, that they might be arising from a common lipoprotein or membrane precursor pool. This conclusion is supported by the continued passage of secretory product from a physically stable association with the endoplasmic reticulum to its apparently more labile association with the golgi complex[24,25]. Therefore, it is suggested that the continued appearance of membrane protein and phospholipid represents their translocation from a rapidly sedimenting fraction of the cell in which these newly synthesized components initially appear.

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REFERENCES

1. Palade, G., 1975. Intracellular aspects of the process of protein transport. Science, 189: 347-354.
2. Hedge, S. and R. Lingappa, 1997. Membrane protein biogenesis: Regulated complexity at the endoplasmic reticulum. Cell, 91: 575-582.

3. Adrie, J., M. Verhoeven, N. Bernadette and H. Jansen, 1999. Secretion and apparent activation of human hepatic lipase requires proper oligosaccharide processing in the endoplasmic reticulum. Biochem. J., 337: 133-140.
4. Rothman, J. and F. Wieland, 1996. Protein sorting by transport vesicles. Science, 272: 227-234.
5. Thorner, J., 2001. Geasing the wheels of secretory transport. Nature Cell Biol., 3: E196-E198.
6. Brasaemle, D. and A. Attie, 1990. Rapid intracellular transport of LDL-derived cholesterol to the plasma membrane in cultured fibroblasts. J. Lipid Res., 31: 103-112.
7. Hirschberg, K., M. Miller, J. Ellenberg, F. Presley, D. Siggia, D. Phair and L. Schwartz, 1998. Kinetic analysis of secretory protein traffic and characterization of golgi to plasma membrane transport intermediates in living cells. J. Cell Biol., 143: 1485-1503.
8. Cartwright, I. and J. Higgins, 1999. Isolated rabbit enterocytes as a model cell system for investigations of chylomicron assembly and secretion. J. Lipid Res., 40: 1357-1365.
9. Park, J.W., S.Y. Lee, J.Y. Yang, H.W. Rho, B.H. Park, S.M. Lim, J.S. Kim and H.R. Kim, 1997. Biochim. Biophys. Acta, 1344:132-138.
10. Cohen, B. and A. Phillips, 1980. Evidence for rapid and concerted turnover of membrane phospholipids in MOPC 41 myeloma cells and its possible relationship to secretion. J. Biol. Chem., 225: 3075-3079.
11. Kessler, W., 1981. Rapid Isolation from antigen from cells with a staphylococcal protein A-antibody adsorbent: Parameters of the interaction of Ab-Ag complexes with protein A. Methods in Enzymol., 73: 442-463.
12. Covault, J., S. El-Deeb and S. Thomson, 1992. Characterization of a cell surface adhesion molecule expressed by a subset of developing chick neurons. Develop. Biol., 149: 213-227.
13. Verhoeven, M. and H. Jansen, 1990. Secretion of rat hepatic lipase is blocked by inhibition of oligosaccharide processing at the stage of glucosidase I. J. Lipid Res., 31: 1883-1893.
14. Kuroiwa, T., M. Sakaguchi, T. Omura and K. Mihara, 1996. The reinitiation of protein translocation across the endoplasmic reticulum membrane for the topogenesis of multispanning membrane proteins. J. Biol. Chem., 271: 6423-6428.
15. Kallen, J., P. Quinn and D. Allan, 1993. Monensin inhibits synthesis of plasma membrane sphingomyelin by blocking transport of ceramide through the golgi: Evidence for two sites of sphingomyelin synthesis in BHK cells. Biochim. Biophys. Acta, 1166:305-308.
16. Adrie, J., M. Verhoeven, N. Bernadette and H. Jansen, 2002. Agonists on the α influence of peroxisome proliferator-activated receptor α the intracellular turnover and secretion of Apolipoprotein (Apo) B-100 and ApoB-48. J. Biol. Chem., 277: 23044-23053.
17. Julie, M., J. Arunakaran, T. Malini and K. Balasubramanian, 2003. The role of luteinizing hormone and prolactin in the regulation of insulin receptors in Leydig cells of the adult rat. Endocrmol. Res., 29: 327-341.
18. Benzi, L., P. Cecchetti, M. Ciccarone, A. Nardone, E. Merola, R. Maggiorelli, F. Campi, G. Di Cianni and R. Navalesi, 1997. Inhibition of endosomal acidification in normal cells mimics the derangements of cellular insulin and insulin-receptor metabolism observed in non-insulin-dependent diabetes mellitus. Metabolism, 46:1259-1265.
19. Verhoeven, J., P. Neve and H. Jansen, 1999. Secretion and apparent activation of human hepatic lipase requires proper oligosaccharide processing in the endoplasmic reticulum. Biochem. J., 337: 133-140.
20. Tachibana, S., S. Takahashiand and T. Akiba, 2002. Octanoate inhibits very low-density lipoprotein secretion in primary cultures of chicken hepatocytes. Comp. Biochem. Physiol. A Mol. Integr. Physiol., 132: 621-627.
21. Zych, W., K. Dolowy and J. Ostrowski, 1993. Changes in the membrane potential can affect pepsinogen secretion of isolated rat chief cells. J. Physiol. Pharmacol., 44: 283-292.
22. Qi, Y., Y. Chen and J. Xiang, 1996. Mouse myeloma cell line secreting bifunctional fusion protein RM4/IFN-gamma [corrected] elicits antitumor CD8 MHC class I-restricted T cells that are cytolytic in vitro and tumoricidal *in vivo*. J. Interferon Cytokine Res., 16:771-776.
23. Inoue, M., N. Fujishiro, I. Imanaga and Y. Sakamoto, 2002. Role of ATP decrease in secretion induced by mitochondrial dysfunction in guinea-pig adrenal chromaffin cells. J. Physiol., 539:145-155.
24. Adrie, J., M. Verhoeven, N. Bernadette and H. Jansen, 2000. Intracellular activation of rat hepatic lipase requires transport to the golgi compartment and is associated with a decrease in sedimentation velocity. J. Biol. Chem., 275: 9332-9339.
25. Gebhart, A. and R. Ruddon, 2005. What regulates secretion of non-stored proteins by eukaryotic cells? Bio. Essays, 4: 213-218.