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PJBS

ISSN 1028-8880

**Pakistan
Journal of Biological Sciences**

ANSI*net*

Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

The Induced Release of Secretory Vesicles by Calcium Ionophore A23187 in Myeloma Cells

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Abstract: The purpose of this study was to determine the effect of calcium ionophore A23187, in the presence of calcium ions, on the concurrent induced release of newly synthesized membrane glycerolipid and secretory product in Myeloma cells. This concurrent induced release provides further evidence of the existence of a very close association between newly synthesized intracellular membrane glycerolipid with the secretion of the secretory product. This relationship was tested previously by using two mechanistically different inhibitors in the same cells. This study also shows that secretory product is released from that normally secreted in soluble form by the constitutive pathway of these cells. Both are released in association with a complex that seems to represent newly synthesized secretory vesicles. Present results showed that the dynamic membranes participate in the secretory process presumably by being synthesized along with secretory product and then serving as expendable packages for the transport of the secretory product from its site of synthesis to the cell surface for secretion. The release, since it appears to be specific, may provide a means for isolating and characterizing the dynamic membranes. Finally, although A23187 has been shown to be a secretagogue in cells with a regulated secretory pathway, the present results indicated firstly to show that it can stimulate the release of secretory product from a constitutive secretory pathway.

Key words: Calcium ionophore A23187, light chain immunoglobulin, secretory vesicles, glycerolipid

INTRODUCTION

We indicated previously that the presence of actively secreting myeloma cells of a class of membranes whose major protein and phospholipid components undergo a rapid and concerted turnover^[1]. Because these membranes appear to turnover at the same rate that newly synthesized secretory product is secreted by the cells and also because their turnover is inhibited by inhibitors of secretion, like monensin. Therefore, it was proposed that the membranes participate in the secretory process presumably by serving an expendable package for the transport of secretory product from the site of synthesis in the endoplasmic reticulum to the cell surface.

Due to our continued efforts and attempts to examine the kinetic relationship between membrane turnover and secretion under conditions that perturb secretion, as shown previously^[1], we observed inhibition of secretion by using monensin and CCCP. Therefore, we are using the calcium-ionophore A23187 in the presence of extracellular calcium ions to stimulate both secretion and turnover of the dynamic membranes. Although this ionophore is known to be an active secretagogue in numerous cells with a regulated secretory pathway, in mast cells to release IL-13^[2] and recently has been shown to cause secretion of resident proteins of endoplasmic reticulum in murine 3T3 cells^[3] but not in other cell lines^[4], so, as far as

we know, it has not been shown that to stimulate constitutive secretory pathways. For this reason we have examined this apparent stimulation in some detail and have found that it is accompanied by an alteration of the pathway. Where as untreated myeloma cells secrete free and one single protein (immunoglobulin light chain), the calcium ionophore treated cells release much of the light chain in association with a complex. Furthermore, although this treatment stimulates the turnover of intracellular membrane glycerolipid, it does not strongly stimulate the degradative process responsible for the normally rapid decay of glycerolipid in untreated cells. The enhanced turnover is caused instead by the ionophore-induced release from the cells of newly synthesized membrane glycerolipid, which appears to be released as a major membrane constituent of vesicles that contain the released secretory product.

MATERIALS AND METHODS

The preparation of myeloma cell suspensions, the conditions of incubation for pulse-labeling and subsequent chase of protein and glycerolipid constituents of the cells, the isolation of soluble kappa light chain of immunoglobulin secreted into the medium of the pulse chased cells and the determination of radioactivity incorporated into cellular protein and

glycerolipid were carried out as described by El-Deeb^[1] and Cohen and Phillips^[5] with the following additional modifications. In our previous pulse-chase studies, myeloma cells that were pulse labeled with a mixture of [³H]-labeled leucine and glycerol and then incubated in chase medium for specified periods, were homogenized in the presence of a carrier homogenate of solid tumor plus myeloma cells that had been labeled with a mixture of [¹⁴C]-labeled leucine and glycerol. As described previously^[1], this homogenate, when present during the homogenization of the pulse-chased cells, permitted correction for variability in recoveries during isolation and assay of labeled proteins and glycerolipid. However, because this variability was very small and also in order to reduce the possibility of errors caused by cross contamination of labeled protein and glycerolipid, in the present experiments myeloma cells (20 mL at a concentration of 2x10⁶ cells mL⁻¹) were pulse labeled for 10 min. with a mixture of 300 µCi of [³H]-glycerol (1 Ci per mmole) to label glycerolipid and 30 µCi of [³H]-glycerol 91 Ci per mmole) to label glycerolipid and 30 µCi of [³H]-leucine (348.5 mCi per mmole) to label protein. In these experiments, homogenization of the pulse-chased cells was carried out in the presence of carrier homogenate made only from unlabeled tumor.

Isolation of labeled immunoglobulin light chain: Labeled immunoglobulin kappa light chain, which is the major secretory product of myeloma cells, was isolated by immunoprecipitation with rabbit anti-mouse kappa light chain antibody followed by adsorption of the antigen-antibody complex on formalin fixed, heat inactivated, protein A bearing *Staphylococcus aureus* cells^[1]. For the assay of immunoglobulin light chain that sediment from the medium of A23187 treated cells, the sediment and also intracellular light chain, were assayed for immunoprecipitation by solubilization with 0.33% sodium deoxycholate in an amount that would give 0.5 mg of the detergent per mg protein in the sample. The detergent was not required for immunoprecipitation of non-sedimentable light chain in the medium or the cytosolic fraction of the cell homogenates. However, the soluble fraction of the medium was routinely dialyzed against phosphate buffer saline to remove labeled material that accompanied light chain during immunoisolation^[6]. The condition for SDS-PAGE of the immunoprecipitates from these samples were carried out according to El-Deeb *et al.*^[7].

RESULTS

The effect of the calcium ionophore A23187 on secretion and rapid turnover of membrane glycerolipid: The results

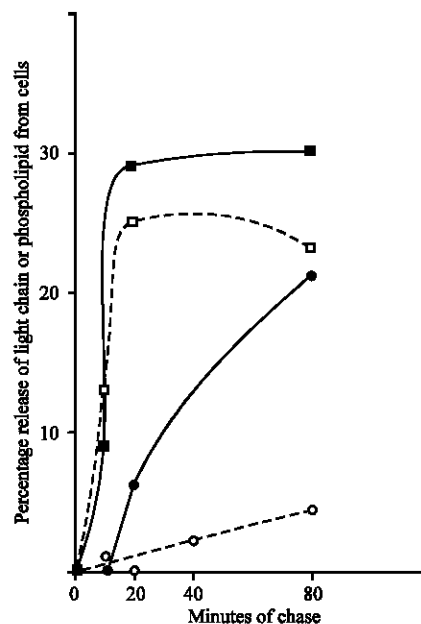


Fig. 1: The effect of the calcium ionophore A23187 on the release of newly synthesized light chain and glycerolipid from short pulse labeled cells and chased in the absence (○, □) or presence (●, ■) of 20 µM A23187. Labeled light chain (○, ●) and glycerolipid (□, ■) released into the medium were assayed as described in experimental procedures

of an experiment comparing the release of newly synthesized light chain from pulse labeled myeloma cells during incubation in the presence and absence of 20 µM A23187 are shown in Fig. 1. Compared to the untreated cells which, after an initial lag period of 10-15 min secrete pulse labeled light chain relatively continuously throughout the 160 min incubation period, the calcium ionophore treated cells exhibit a rapid initial release of light chain by a process that is essentially complete within the first 40 min of incubation. Because the total amount of light chain released is not significantly greater than that eventually released by untreated cells, the ionophore appears to stimulate the rate rather than the extent of the release. Figure 1 also shows that the release of light chain is accompanied by a similarly rapid release from the cells of newly synthesized glycerolipid although, in this case, the ionophore strongly stimulate the extent as well as the rate of the release.

The experiment described in Fig. 2-4 is very similar except that the amounts of newly synthesized light chain (Fig. 2), glycerolipid (Fig. 3) and total protein (Fig. 4) released from cells incubated in the presence and absence of the calcium ionophore were compared to the amounts remaining in the cells during the time course of the chase

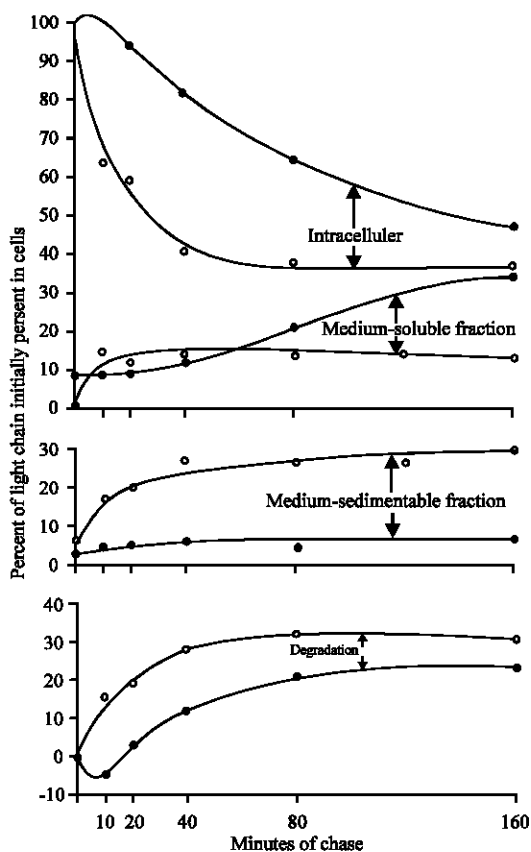


Fig. 2: Recoveries of newly synthesized light chain in cells and in the sedimentable and non-sedimentable fractions of the medium during chase of labeled cells in the presence and absence of A23187. Conditions of pulse labeling and chase incubation were as described for the control experiments in Fig. 1 to 3 except that 20 μ M A23187 was present (○) or absent (●) from the chase medium. Fractionation of the medium was as described in results. Fractionation of the cells and the isolation and assay of labeled light chain were as described in experimental procedure. Degradation of light chain was calculated by subtracting recoveries of labeled light chain in the cell and medium fractions at each chase period from the amount initially present in the cells

incubation. These data permit estimates of the rate and extent of degradation of light chain and glycerolipid as measured by the decline in the total amount of each of the newly synthesized components recovered from both the medium and cells during the chase. The results of these estimates are also shown in Fig. 2 and 3.

An additional experimental modification was that the medium was fractionated by centrifugation at 105,000g

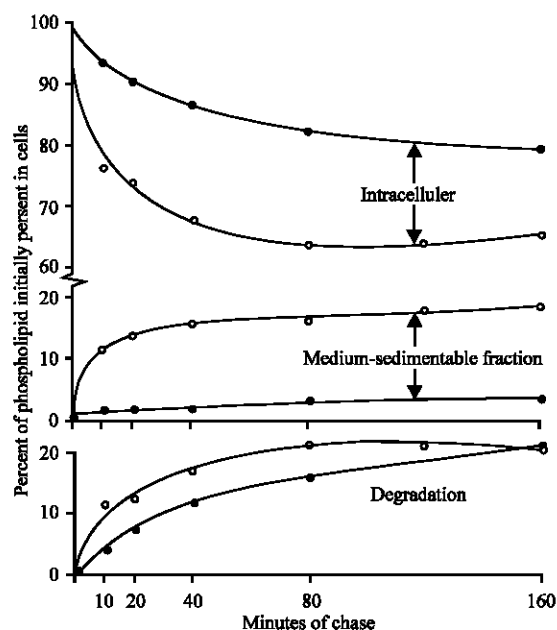


Fig. 3: Recoveries of newly synthesized glycerolipid in cells and in the sedimentable fraction of the medium during chase incubation in the presence (○) or absence (●) of A23187. Pulse labeled cells and the medium fractions described in Fig. 2 were assayed for their content of labeled glycerolipid as described in experimental procedure. Degradation of glycerolipid was calculated from differences between total amounts of labeled glycerolipid recovered at each pulse period and the amount initially present in the cells

for one hour in a spinco 40 rotore and the distribution of the newly synthesized components in the resultant sediment and supernatant layer were examined.

As can be seen in Fig. 2, most of the light chain released by untreated cells appears in the soluble fraction of the medium. The initial lag in the appearance of the soluble light chain and its subsequent sustained release during much of the chase period suggests that it represents light chain that is processed by the normal secretory pathway. On the other hand, the light chain whose release is stimulated by the ionophore is found largely in the sedimentable fraction of the medium. This difference suggests that stimulation is accompanied by an alteration of the normal secretor pathway.

It can be seen that the ionophore strongly stimulates the initial rate of decay of newly synthesized intracellular light chain from the cells. To a smaller extent, the enhanced decay may also reflect a slightly enhanced initial rate of degradation of light chain. On the other hand, the ionophore does not significantly alter the final

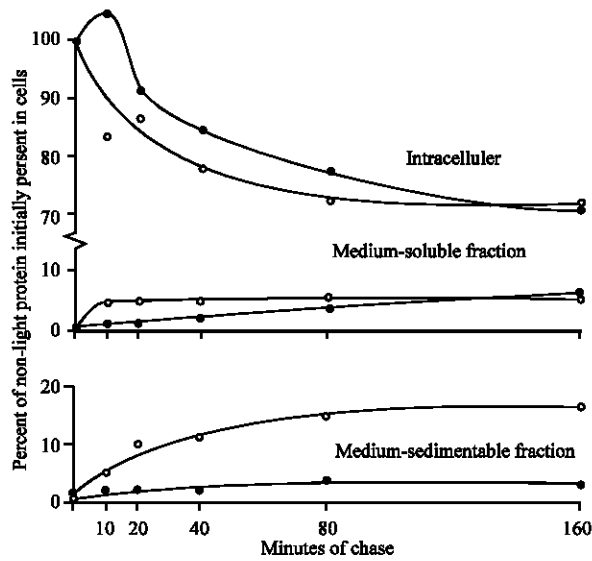


Fig. 4: Recoveries of TCA-precipitable, non-light chain protein in cells and medium fractions during chase incubation in the presence (○) and absence (●) of A23187. Cells and medium fractions were assayed for pulse labeled TCA-precipitable protein as described earlier^[1]. The values for the non-light chain protein were obtained by subtracting the amount of labeled light chain determined as described in Fig. 2 from the amount of TCA-precipitable protein

extent of either intracellular decay or degradation of light chain nor does it alter the total amount of light chain ultimately appearing in the medium during 160 min of chase. The light chain released from the cells by the ionophore, therefore does not appear to represent the mobilization of light chain from pools outside of the normal secretory pathway or from the light chain that is destined for rapid degradation. Instead, the release appears to occur solely at the expense of intracellular light chain that is eventually released in soluble form by the untreated cells. Since the later seems to represent light chain processed by the normal secretory pathway, it would appear that the ionophore released light chain has been diverted rather specifically from this pathway.

In Fig. 3, it can be seen that the newly synthesized phospholipid exhibits a very similar response to the appearance of the calcium ionophore. In untreated cells, the rapid decay of intracellular phospholipid is due almost exclusively to its rapid degradation since little appears in the sedimentable fraction or, according to results not shown, the soluble fraction of the medium. The initial rate of decay of the intracellular glycerolipid and also its extent of decay are strongly stimulated by the ionophore and

this response is caused primarily by the ionophore induced release of phospholipid into the medium and, to a more limited extent, perhaps also by a slight stimulation of the initial rate of glycerolipid degradation.

The close correspondence between the kinetics for the ionophore induced release of newly synthesized glycerolipid and light chain and the observations in Fig. 2 and 3 that both are released into the sedimentable fraction of the medium suggests that they are released in an associated form from a common step of the secretory pathway.

Although the present data suggested that the ionophore induces the release of vesicles from the cell, it is not clear whether this release is specific for secretory vesicles or is the result of a general cell leakage induced by the ionophore. Two criteria that have been examined an attempt to estimate the extent of general cell leakage are: 1) the release of lactate dehydrogenase, a predominately cytoplasmic protein which has been shown to be extensively released by digitonin and other agents that cause permeation of plasma membranes^[8,9] and 2) the release in soluble form of newly synthesized cellular proteins, may of which are also cytoplasmic proteins. Portions of the pulse-chased cells described in Fig. 2-4 were homogenized in the absence of carrier homogenate. Homogenates as well as the media from chase incubations were assayed for lactate dehydrogenase activity as described by Arvan and Castle^[10]. The results, which are not shown, indicate that of the total enzymatic activity of the cells, which remained essentially constant during the chase incubations, approximately 5% was rapidly released into the medium and this amount remained essentially unchanged during the remaining 160 min of chase incubation in the presence of the ionophore. Figure 4 shows the distribution of newly synthesized protein as isolated by precipitation with trichloroacetic acid and subsequent washing with ethanol-ether and ether to remove labeled lipids. The amount of protein was corrected for the assumed presence in the washed precipitate of the light chain indicated in Fig. 2. It can be seen that approximately 5% of the protein was rapidly released into the soluble fraction of the medium during chase incubation in the presence of ionophore and that a similar amount was released, although much more slowly, during incubation in the absence of ionophore. This release, which closely mirrors observed release of lactic dehydrogenase activity, is considerably less than the ionophore induced release of 17 and 45% of the newly synthesized glycerolipid and light chain of the cell, respectively, observed in this experiment. From this comparison, it can be estimated that any non-specific leakage could not account for more than one third of the

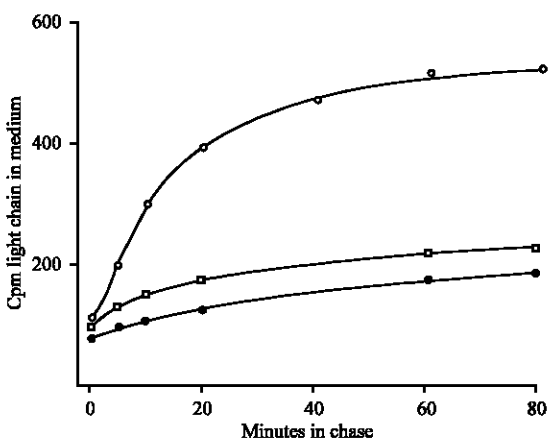


Fig. 5: The effect of EGTA on the release of newly synthesized light chain from pulse labeled cells incubated in the presence of the calcium ionophore A23187. Cells labeled with [¹⁴C]-glycerol were incubated in chase medium in the absence (□) or presence (○) of 20 μM A23187, or in the presence of 20 μM A23187 plus 8.3 mM EGTA (●). The chase medium contained 1.8 mM calcium ions. Other conditions of pulse labeling chase incubation and assay of labeled light chain appearing in the medium were carried out as described in experimental procedures

glycerolipid and an even smaller fraction of the light chain released by the ionophore. Although the ionophore does cause a large increase in the amount of the newly synthesized cellular protein released into the sedimentable fraction of the medium (10% compared to less than 1% in the absence of the ionophore, (Fig. 4) this portion most likely represents the expected presence in the fraction of protein constituents of the dynamic intracellular membranes that appear to be released by the ionophore.

According to the recent conclusions about the mechanism for the release of secretory product from storage granules of cells with a regulated secretory pathway^[11,12], it seems likely that an increased level of intracellular calcium, rather than the absolute value of the extra cellular calcium, caused by the ability of the calcium ionophore A23187 to expedite the flow of calcium into the cell, is primarily responsible for the ionophore induced release of secretory product observed in the presence study. This possibility was tested by determining if the effect of the ionophore could be prevented by chelating calcium in the medium with the calcium specific, cell impermeable chelating agent (EGTA) (ethylene glycol-bis (B-amino ethyl ether) N,N,N,N,-tetra acetic acid). As shown by the experiment in Fig. 5, the four fold increase in the amount of light chain released from cells by the

presence of A23187 was prevented almost completely when EGTA was also present at a concentration sufficient to chelate the calcium present in the incubation medium.

DISCUSSION

From the data we have presented, it seems obvious that the enhanced release of secretory product by calcium-ionophore A23187 and the concomitant release of newly synthesized glycerolipid, does not simply represent an enhanced rate of secretion by the normal secretory pathway. It seems to represent instead a premature release of secretory product in association with membranes derived from one or more intermediate steps in this pathway. This conclusion is suggested by the observations that the secretory product is released in such form that co-sediments with the glycerolipid and that it appears to be released rather specifically from secretory product that, in the absence of the ionophore, would have been released in soluble form by the normal secretory pathway (Fig. 2).

On the other hand, the parallel release of newly synthesized glycerolipid is not accompanied by a reduction in the amount of glycerolipid that is rapidly degraded, as one would have expected if the glycerolipid were released from the dynamics membranes of the cell. A stimulation of glycerolipid degradation is not unexpected as the ionophore would be expected to elevate intracellular calcium levels and calcium is known to stimulate the hydrolysis of acidic glycerolipid by lysosomal phospholipase A1^[13] and to be required by other mammalian phospholipases^[14].

The apparent stimulation of light chain secretion by the calcium ionophore induced elevation of intracellular calcium levels is the first observation, as far as we are aware, of this type of stimulation in cells that secrete continuously, i.e., by a constitutive pathway, although numerous laboratories have shown that this treatment stimulates the release of stores, mature secretory product from cells with a regulated secretory pathway^[15]. This finding is of considerable interest because it may provide a means of isolating and defining membranes of the cell that participate in the constitutive secretory pathway.

Among many of the reported articles, concerning the positive role of calcium- ionophore A23187 in stimulating secretion, Lodish laboratory^[16], had reported an opposite effect of A23187 on secretion. The results shown in that article suggested that A23187 blocks to different extent of the secretion of different secretory proteins by human hepatoma HepG2 cells and that this blockage is accompanied by an accumulation of proteins in rough endoplasmic reticulum. This effect, in contrast to the

enhanced release of secretory product observed in the present study, does not appear to require extra cellular calcium. Further more the blockage of secretion requires A23187 to be present during or very soon after synthesis of secretory proteins whereas the enhanced release of light chain does not occur immediately after its synthesis but requires 25 minutes of intracellular processing before it is released to a maximum extent from the Golgi apparatus. Lodish^[16] indicated no enhancement (as well as blockage) of release of secretory products from the HepG2 cells by A23187 added 20 to 30 min. after synthesis suggests that the enhanced release of secretory product from myeloma cells may not be a universal response of all cells to the calcium ionophore.

Although, we have not measured the concentration of intracellular calcium level, which was not in great interest at this moment compared to our aim in determining the effect of the ionophore on the concurrent induced release of newly synthesized membrane glycerolipid and secretory product. Mihai^[17] carried out the measurements and observed that $[Ca^{+2}]_i$ was increased by the addition of 10-25 μ M A23187 and that the $[Ca^{+2}]_i$, rather than the $[Ca^{+2}]_{ext}$ value is the main modulator of secretion in Parathyroid cells. Moreover, the increase in intracellular calcium, which occurs diffusely via the calcium ionophore, was sufficient for exocytosis in these nonexitable cells^[18].

The present study provides little direct information about the physical relationship between the glycerolipid and secretory product released by the ionophore except that both coexist in the sedimenting fraction of the medium and also, in experiments not shown, that deoxycholate is required for efficient immunoisolation of newly synthesized light chain recovered in the sediment. The latter observation suggests that membranes limit the access of antibody to the secretory product. Indeed, electron microscopic examination of the sediment indicates the presence of vesicles that could serve as a membrane barrier and also as the expected structural form of the released glycerolipid. Unfortunately, morphological studies of this fraction were obscured by the presence of large amounts of debris that could not be completely removed from the cells during their preparation from solid tumors and of amorphous material that formed from the serum containing cell culture medium during chase incubation. However, this contamination has been largely eliminated by repeating the present experiments with myeloma cells that have been adapted to as sustained cultures in serum deficient medium. With this system, it has been possible to obtain the ionophore released glycerolipid and secretory product in a form that appears under the electron microscope to consist almost

exclusively of a relatively homogenous population of vesicles (data not shown).

Pryor *et al.*^[19] found that a calcium chelator called BAPTA (at 5 mM) inhibited nuclear vesicle fusion, whereas the EGTA chelator (up to 12 mM) had no effect. The important difference between the two chelators at physiological pH BAPTA exchanges calcium about 100 times faster than EGTA because of its faster rates of association and dissociation. So, BAPTA can mop up the calcium liberated from a sequestered pool fast enough to suppress a biological response, whereas EGTA has no effect. On the other hand, results from Bosch laboratory^[20] shows that the stimulatory effect of *Escherichia coli* lipopolysaccharide on phosphatidylcholine secretion was enhanced by calcium-ionophore A23187, while its secretion was suppressed by BAPTA (an intracellular calcium Chelator. Finally, our current results also demonstrate the effectiveness of EGTA on inhibiting secretion in presence of the calcium-ionophore.

Finally, by perturbing secretion, either by inhibition, as reported by El-Deeb^[1], or by enhancements, as we report in this study, the conclusion of these measurements suggest that the membranes are synthesized and degraded at approximately the same rapid rate as the synthesis and secretion of membrane-associated secretory products.

ACKNOWLEDGMENTS

The author is deeply appreciating the generosity of professor Dr. Alvah Phillips at the University of Connecticut, Storrs, USA, for sharing many of the his laboratory facilities.

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