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Correlation between phospholipase A_2 activity and intra-Golgi protein transport reconstituted in a cell-free system

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A wide variety of phospholipase A_2 inhibitors blocks intra-Golgi protein transport reconstituted in a cell-free system. Phospholipase A_2 activity detectable under the protein transport assay conditions is actually inhibited by the inhibitors. There is a good correlation between the inhibition of protein transport and that of phospholipase A_2 activity. Prolactin secretion from GH_3 cells is also blocked by a membrane-permeable phospholipase A_2 inhibitor, suggesting the physiological relevance to inhibition of protein transport in vitro by phospholipase A_2 inhibitors.

Protein transport; Phospholipase A_2; Prolactin secretion; GH_3 cell

1. INTRODUCTION

Intracellular protein traffic from the endoplasmic reticulum to the cell surface through the Golgi apparatus is mediated by the vesicles which transit between the organelles [1]. Each transport process includes vesicle budding, targetting, and fusion [2–4]. This implies that a membrane bilayer is split and two membranes are fused into one cycle of the transport reaction. However, little is known about the nature or origins of the force which perturbs biological membranes and finally promotes their fission and fusion.

Based on the studies using inhibitors, it has been proposed that phospholipase A_2 is involved in the exocytotic pathways [5–7]. Recently, Morgan and Burgoyne [8] found that the release of catecholamine from permeabilized chromaffin cells is enhanced by a protein named Exo I, which shares sequence homology with the 14–3–3 family of proteins. One member of this family was cloned and turned out to be a phospholipase A_2 [9]. Keeping these results in mind, we have tested the effect of phospholipase A_2 inhibitors on a well-characterized intra-Golgi protein transport system reconstituted in a cell-free system [10]. This system reconstitutes the transport of VSV-G protein from the *cis* compartment of a donor Golgi fraction to the *medial* compartment of an acceptor Golgi fraction. When the donor and acceptor Golgi membrane fractions were incubated with ATP and a cytosolic fraction, vesicles containing VSV-G protein are formed from the donor Golgi fraction and moved to the acceptor fraction [11]. Fusion of the vesicles is detectable by the transfer of [^{3}H]GlcNAc from UDP-[^{3}H]GlcNAc via the reaction catalyzed by UDP-GlcNAc transferase I in the *medial* compartment of the acceptor Golgi fraction.

We report here a correlation between the inhibition of intra-Golgi protein transport in vitro and that of phospholipase A_2 activity. Protein secretion in vivo is also blocked by a membrane-permeable phospholipase A_2 inhibitor.

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Abbreviations. VSV-G protein, vesicular stomatitis virus-encoded glycoprotein; NDGA, nordihydroguaiaretic acid; GlcNAc, *N*-acetylglucosamine; IC_{50}, the concentration required for 50% inhibition.

2. MATERIALS AND METHODS

Brefeldin A and manoalide were obtained from Wako Chemicals. *p*-Bromophenacyl bromide and fatty acids were obtained from Nacalai Tesque. NDGA and (*p*-amylcinnamoyl)anthranilic acid were purchased from Biomol Research Laboratories. Eicosatetraynoic acid and 5-hydoxyeicosatetraen-1-oic acid were from Cascade Biochem. UDP-[^{3}H]GlcNAc (6.1 Ci/mmol), 1-stearoyl-2-[^{14}C]arachidonyl-*sn*-glycero-3-phospho rylch oline (55.6 mCi/mmol) and EN^{3}HANCE were from DuPont-New England Nuclear. [^{35}S]Methionine was from ICN Radiochemicals. ER-3826 was a gift from Eisai Co. Anti-prolactin rabbit serum (HAC-RT26-01RBP85) was a generous gift from Dr. K. Wakabayashi at Gunma University. Donor and acceptor Golgi fractions were prepared from VSV-infected CHO 15B and wild-type CHO cells, respectively, as described by Balch et al. [10]. Bovine brain cytosol was prepared as described by Malhotra et al. [12].

The Golgi protein transport assay was carried out as described by Balch et al. [10]. The standard assay mixture (50 μl) consists of 25 mM HEPES (pH 7.0) containing 15 mM KCl, 2.5 mM magnesium acetate, 0.5 mM dithiothreitol, an ATP-regenerating system (5 mM creatine phosphate, 0.25 mM UTP, 0.05 mM ATP, and 12 U/ml creatine

phosphokinase), 0.64 μM (0.2 μCi) UDP-[^{3}H]GlcNAc, 1.5 mg/ml bovine brain cytosol. The assay was performed for 1 h at 37°C, and then VSV-G protein was immunoprecipitated, collected on filters and counted. All the inhibitors used in this study were dissolved in dimethyl sulfoxide at appropriate concentrations. *p*-Bromophenacyl bromide was dissolved before use, because this compound decomposes in the solvent. The final concentration of dimethyl sulfoxide in the assay mixture was 1% (v/v) This concentration of dimethyl sulfoxide alone did not markedly inhibit the transport assay.

The assay conditions for phospholipase A_2 activity were the same as those for intra-Golgi protein transport except that 9 μM (0.025 μCi) 1-stearoyl-2-[^{14}C]arachidonyl-*sn*-glycero-3-phosphorylcholine was included instead of UDP-[^{3}H]GlcNAc. After incubation at 37°C for 2 h, the reaction was terminated by the addition of 0.25 ml of Dole reagent (isopropanol/heptane/1 N H_2SO_4, 78:20:2) [13]. One hundred μl of water and 0 15 ml heptane were then added, and the solution was mixed vigorously by vortexing. After centrifugation at 1,500 × *g* for 3 min, 0.14 ml of the upper phase was removed and mixed with 0.35 ml of heptane and 30 mg of silicic acid powder After centrifugation at 1,500 × *g* for 3 min, 0.4 ml of the heptane phase was used for the measurement of radioactivity

The method for measuring prolactin secretion from GH_3 cells will be described elsewhere.

3. RESULTS AND DISCUSSION

We tested the effect of compounds known to inhibit phospho-lipase A_2 on the intra-Golgi protein transport reconstituted in a cell-free system. These include irreversible and reversible phospholipase A_2 inhibitors, unsaturated fatty acids, and dual inhibitors for cyclooxygenase and lipoxygenases. All the compounds used inhibited the transport assay. Table 1 summarizes the concentrations required for 50% inhibition (IC_{50}). IC_{50} values for irreversible and reversible phospholipase A_2 inhibitors were comparable to those required for inhibition of phospholipase A_2 [14–17]. Although ER-3826 is a strong inhibitor for membrane-bound phospholipase A_2 from rabbit heart [18], this compound was not effective. Dexamethasone, known to inhibit phospholipase A_2 by production of the phospholipase A_2 inhibitory protein lipocortin in vivo [19] was ineffective (IC_{50} = 0.4 mM). It was reported that phospholipase A_2 is inhibited by unsaturated fatty acids such as arachidonic acid, but not by saturated fatty acids [20,21]. Unsaturated fatty acids (oleic, linoleic, and arachidonic acids) inhibited the intra-Golgi protein transport assay, whereas saturated fatty acids (stearic and arachidic acids) had no effect up to 40 μM. Since the critical micelle concentrations of unsaturated fatty acids are higher than those of the saturated fatty acids having the same number of carbon atoms [22], this inhibition by the unsaturated fatty acids is not due to the nonspecific damage of Golgi membranes. Because cyclooxygenase and lipoxygenases utilize arachidonic acid, dual inhibitors for the two enzymes may act as phospholipase A_2 inhibitors. In fact, NDGA and eicosatetraynoic acid, both known as the dual inhibitors, were good inhibitors for the intra-Golgi protein transport assay. It is unlikely that prostagrandins and leukotrienes, which are produced through the reactions catalyzed by cyclooxygenase and lipoxygenases, respectively, regulate intra-Golgi protein transport. Because indomethacin and caffeic acid, the inhibitors specific for cyclooxygenase and lipoxygenases, respectively, were much less inhibitory than the dual inhibitors (indomethacin : IC_{50} = 100 μM, caffeic acid, no inhibition up to 0.2 mM). In addition, 5-hydroxyeicosatetraen-1-oic acid, an intermediate for leukotriene biosynthesis, had inhibitory effect.

To examine whether phospholipase A_2 is actually inhibited by the phospholipase A_2 inhibitors used, we

Table 1

Effect of phospholipase A_2 inhibitors on intra-Golgi protein transport in vitro

Compound	IC_{50} (μM)
Irreversible phospholipase A_2 inhibitor	
p-Bromophenacyl bromide	12
Manoalide	4
Reversible phospholipase A_2 inhibitor	
(*p*-amylcinnamoyl)Anthranilic acid	9
ER-3826	70
Fatty acid	
Stearic acid	N.I.[a]
Oleic acid	33
Linoleic acid	9
Arachidic acid	N.I.
Arachidonic acid	7
Dual inhibitor for cyclooxygenase and lipoxygenases	
NDGA	9
Eicosatetraynoic acid	16

[a]No inhibition up to 40 μM.

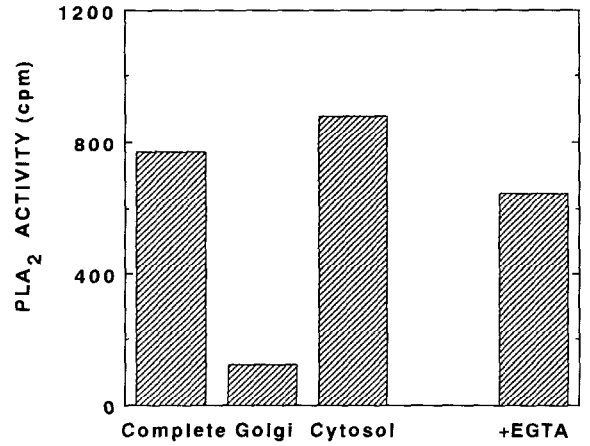

Fig 1 Phospholipase A_2 activity detectable under the protein transport assay conditions. The assay mixture contains an ATP-regenerating system, Golgi membranes, and bovine brain cytosol (Complete), an ATP-regenerating system and Golgi membranes (Golgi), or an ATP-regenerating system and bovine brain cytosol (Cytosol). 2 mM EGTA was added to the complete assay mixture (+EGTA). Addition of an ATP-regenerating system activated phospholipase A_2 activity by 1 3- to 1 6-fold compared with its absence. Data are expressed as the mean of two experiments.

measured its activity by using an exogeneous substrate, 1-stearoyl-2-[^{14}C]arachidonyl-*sn*-phosphorylcholine. Fig. 1 shows that phospholipase A_2 activity detectable under the transport assay conditions is mainly located in a cytosolic fraction and some in Golgi membranes. This phospholipase A_2 activity was not inhibited by EGTA. Although most phospholipase A_2s absolutely require Ca^{2+} for activity, several species of cytosolic phospholipase A_2 recently found possess basal activity without Ca^{2+} [23–26]. 14–3–3 protein, which shares sequence homology with Exo I involved in exocytosis [8], is activated by Ca^{2+}, but the cation is not essential for the enzyme to exhibit phospholipase A_2 activity [27]. The fact that the phospholipase A_2 activity detectable under the protein transport assay conditions is not inhibited by EGTA is consistent with the observation that EGTA does not inhibit the protein transport assay [28].

We found that phospholipase A_2 activity detectable under the protein transport assay conditions was actually inhibited by the phospholipase A_2 inhibitors up to 70 to 80%. When IC_{50} values for phospholipase A_2 were plotted against IC_{50} values for protein transport, a good linear relationship was observed (Fig. 2). IC_{50} values for phospholipase A_2 were two- to threefold higher than those for protein transport. This difference may be attributable to the difference in substrate and/or its concentrations for the two reactions. 1-stearoyl-2-arachidonyl-*sn*-glycero-3-phosphoryl-choline is the exogenous substrate for measuring phospholipase A_2 activity, whereas the Golgi membrane is the endogenous substrate for protein transport. The concentration of lipids in the Golgi membrane usable for phospholipase A_2 may be lower than 9 μM, the concentration of exogenous substrate.

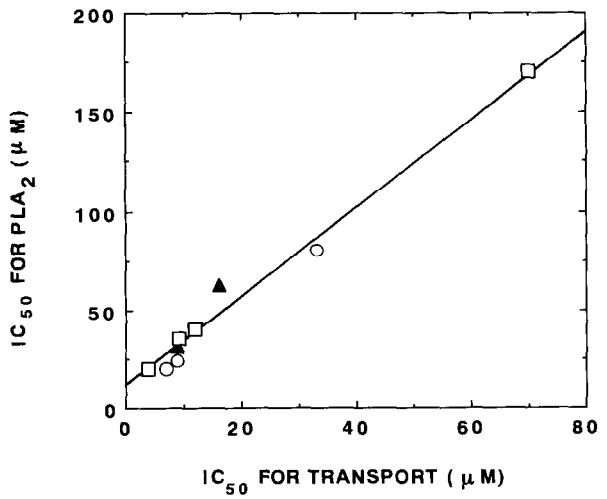

Fig. 2. Correlation between IC_{50} for protein transport and IC_{50} for phospholipase A_2 activity. Reversible and irreversible phospholipase A_2 inhibitors (*p*-bromophenacyl bromide, manoalide, (*p*-amylcinnamoyl)anthranilic acid, and ER-3826) (□), unsaturated fatty acids (oleic, linoleic, and arachidonic acids) (○), and dual inhibitors for cyclooxygenase and lipoxygenases (NDGA and eicosatetraynoic acid) (▲). The line was drawn by the least-squares best fit method.

To test the physiological relevance to inhibition of protein transport in vitro by phospholipase A_2 inhibitors, we examined the effect of NDGA, a membrane-permeable phospholipase A_2 inhibitor, on prolactin secretion from GH_3 cells. As shown in Fig. 3, NDGA blocked prolactin secretion with an IC_{50} value of 60 μM, whereas dexamethasone had no significant effect up to 200 μM. These results are consistent with the efficacy of the two compounds as inhibitors for protein transport in vitro. Reversibility of inhibition by NDGA suggests that the reagent does not seriously damage GH_3 cells and allows us to carry out a two-stage assay for investigating the step(s) inhibited by NDGA (Fig. 4). In stage I, pulse-labeled GH_3 cells were chased for 1 h in the presence or absence of NDGA. In stage II, the cells, after washing, were incubated for another 2 h in the presence or absence of brefeldin A. Brefeldin A blocks several steps of secretion, but does not affect step(s) after exocytotic vesicles and granules are formed [29]. In stage I, NDGA inhibited the secretion of prolactin by 70% ((65%−19%)/65%). When NDGA was removed, 61% of the total prolactin labeled was secreted in stage II. However, brefeldin A still significantly inhibited the secretion by 45% ((61%−33%)/61%). These results suggest that NDGA inhibits step(s) before the formation of secretory vesicles, possibly transport from endoplasmic reticulum to Golgi and/or intra-Golgi transport. Incomplete inhibition by brefeldin A in stage II is probably due to incomplete inhibition by NDGA in stage I. With 19% secretion of labeled prolaction (Fig. 4), some fractions may have passed the final brefeldin A-sensitive step in stage I.

In conclusion, the present results demonstrated a correlation between phospholipase A_2 activity and intra-Golgi protein transport in vitro. Although we cannot

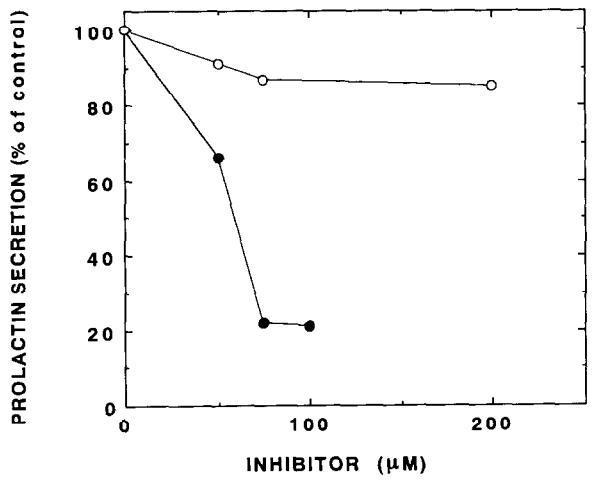

Fig. 3. Effect of NDGA on prolactin secretion from GH_3 cells. Prolactin secretion from GH_3 cells was measured in the presence of various concentrations of NDGA (●) and dexamethasone (○). Values are the ratios of the amounts of prolactin secreted in the presence of each inhibitor to that of prolactin secreted in a control experiment. Data are expressed as the mean of two experiments

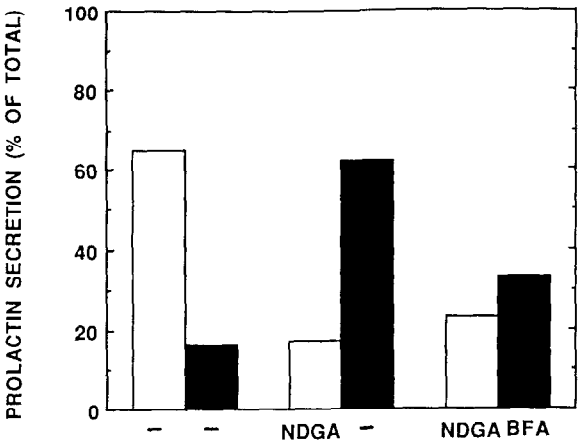

Fig. 4. NDGA inhibits step(s) prior to the brefeldin A-resistant step(s) in prolactin secretion. Two-stage assay. GH_3 cells were pulse-labeled, and then chased at 37°C for 1 h in the presence or absence of 75 μM NDGA (stage I) The cells were washed with phosphate buffered saline to remove NDGA and then further incubated at 37°C for 2 h in the presence or absence of brefeldin A (BFA). Values are the ratios of the amounts of prolactin secreted to those of the total [^{35}S]prolactin in stage I (□) and stage II (■). Data are expressed as the mean of two experiments.

completely exclude the possibility that the inhibition of the protein transport is the consequence of that of other enzymes and/or proteins, use of several different types of inhibitors with no structural correlation may justfy our conclusion. By using phospholipase A_2 inhibitors different from those used by us, Stahl and his colleagues recently found that phospholipase A_2 activity is required for endosome fusion (personal communication). Phospholipase A_2 may be generally involved in intracellular protein traffic. This interesting possibility remains to be elucidated directly.

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