Reconstitution of Constitutive Secretion Using Semi-intact Cells: Regulation by GTP but Not Calcium

Stephen G. Miller and Hsiao-Ping H. Moore

Division of Cell and Developmental Biology, Department of Molecular and Cell Biology, University of California, Berkeley, California 94720

Abstract. Regulated exocytosis in many permeabilized cells can be triggered by calcium and nonhydrolyzable GTP analogues. Here we examine the role of these effectors in exocytosis of constitutive vesicles using a system that reconstitutes transport between the *trans*-Golgi region and the plasma membrane. Transport is assayed by two independent methods: the movement of a transmembrane glycoprotein (vesicular stomatitis virus glycoprotein [VSV G protein]) to the cell surface; and the release of a soluble marker, sulfated glycosaminoglycan (GAG) chains, that have been synthesized and radiolabeled in the *trans*-Golgi. The plasma membrane of CHO cells was selectively perforated with the bacterial cytolysin streptolysin-O. These perforated cells allow exchange of ions and cytosolic proteins but retain intracellular organelles and transport vesicles. Incubation of the semi-intact cells with ATP and a cytosolic fraction results in transport of VSV G protein and GAG chains to the cell surface. The transport reaction is temperature dependent, requires hydrolyzable ATP, and is inhibited by *N*-ethylmaleimide. Nonhydrolyzable GTP analogs such as GTPγS, which stimulate the fusion of regulated secretory granules, completely abolish constitutive secretion. The rate and extent of constitutive transport between the *trans*-Golgi and the plasma membrane is independent of free Ca^{2+} concentrations. This is in marked contrast to fusion of regulated secretory granules with the plasma membrane, and transport between the ER and the *cis*-Golgi (Beckers, C. J. M., and W. E. Balch. 1989. *J. Cell Biol.* 108:1245–1256; Baker, D., L. Wuestehube, R. Schekman, and D. Botstein. 1990. *Proc. Natl. Acad. Sci. USA.* 87:355–359).

EUCARYOTIC cells deliver newly synthesized proteins to the cell surface by sequentially transferring them through a series of membrane-bound compartments. In many differentiated cells, proteins are secreted via distinct pathways: they may be packaged into storage granules for regulated release in response to physiological stimuli, enter vesicles for constitutive secretion, or become incorporated into apical- or basolateral-bound vesicles for delivery to distinct domains of epithelial surface (for reviews see Burgess and Kelly, 1987; Simons and Fuller, 1985; Rodriguez-Boulan, 1989). The *trans*-Golgi region performs an essential function in sorting proteins into these different types of secretory vesicles. To date, many important questions concerning post-Golgi traffic remain unanswered. How does the *trans*-Golgi network generate such a diversity of transport vesicles? Are the mechanisms controlling budding, targeting, and fusion of regulated granules similar to or different from those for constitutive vesicles? How are these components sorted during assembly of vesicles at the *trans*-Golgi region? Answering these questions will require functional dissection of the molecular components involved in both constitutive and regulated secretion.

The mechanism of regulated secretion has been studied extensively using permeabilized cell systems and patch-clamp techniques (Knight and Baker, 1982; Dunn and Holz, 1983; Vallar et al., 1987; Howell et al., 1987; for reviews see Gomperts, 1990; Almers, 1990; Burgoyne, 1990). These studies have identified calcium and GTP as two key components in this process; calcium and the nonhydrolyzable GTP analogue guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS)[1] often act synergistically to trigger secretion from storage granules. Interestingly, both calcium and GTP-binding proteins have also been shown to operate in other steps of the secretory pathway. However, their exact roles in these steps appear to be different from regulated exocytosis. In both mammalian and yeast cells, transport from the ER to the *cis*-Golgi region in reconstituted systems requires calcium (Beckers and Balch, 1989; Baker et al., 1990). Optimal transport occurs at 50–200 nM free Ca^{2+}, which is at least one order of magnitude lower than that required to trigger regulated secretion. The requirement for calcium is relatively late, suggesting that it may also be needed for vesicle fusion. Un-

1. *Abbreviations used in this paper*: CPC, cetylpyridinium chloride; EndoH, endoglycosidase H; GAG, glycosaminoglycan; GMP-PNP, guanylylimidodiphosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); NEM, *N*-ethylmaleimide; SL-O, streptolysin-O; VSV G protein, vesicular stomatitis virus glycoprotein; xyloside, 4-methylumbelliferyl-β-D-xyloside.

© The Rockefeller University Press, 0021-9525/91/01/39/16 $2.00

like regulated exocytosis which is stimulated by GTPγS, transport between ER and Golgi complex (Beckers and Balch, 1989; Baker et al., 1990), as well as between Golgi cisternae (Melancon et al., 1987), is blocked by GTP analogues.

At present, the role of calcium and GTP-binding proteins in constitutive transport from the *trans*-Golgi region to the cell surface is less clear. Secretion from intact fibroblast cells is impaired when the cellular Ca^{2+} level is lowered by calcium ionophore A23187 and EGTA, suggesting that calcium may be required for constitutive transport. However, the same treatment has no effect on secretion from macrophages (Tartakoff et al., 1978). Using perforated baby hamster kidney (BHK) cells, deCurtis and Simons (1988) observed that transport of Semliki Forest virus glycoproteins to the cell surface requires added Ca^{2+}. In this case, it is not yet clear whether calcium is directly involved in the transport process, or if it prevents the release of exocytic vesicles from the perforated cells (as was found with perforated MDCK cells; Bennett et al., 1988), thereby enhancing the efficiency of delivery to the plasma membrane. Another unresolved issue is the role of guanyl nucleotides. Studies of secretion mutants of the yeast *Saccharomyces cerevisiae* have elucidated the involvement of a GTP-binding protein, *Sec4p*, in constitutive transport between the Golgi complex and the cell surface (Salminen and Novick, 1987, 1989; Walworth et al., 1989). Woodman and Edwardson (1986) developed an in vitro transport system using postnuclear supernatants from BHK cells but the effect of nonhydrolyzable GTP analogues on co itutive transport in this system has not been reporteu. Using an in vitro budding assay, Tooze and Huttner (1990) found that GTPγS inhibited the formation of both regulated and constitutive post-Golgi vesicles by ~50%.

In view of the lack of a clear picture of the requirements for constitutive secretion, we have sought to use an independent method to study the components involved in this process. In this paper, we developed a semiintact cell system that efficiently reconstitutes budding from the *trans*-Golgi complex and fusion with the plasma membrane. We chose a method of permeabilization in which the cell structures remained relatively intact and the post-Golgi vesicles were retained within the cell. We used two independent markers to ensure measurement of vesicle fusion rather than membrane lysis. Using this system, we compare the requirements for Ca^{2+} for constitutive secretion to the fusion of regulated secretory granules. We also describe the effects of GTPγS on this step in biosynthetic transport. This system will be useful for defining components involved in constitutive secretion in mammalian cells.

Materials and Methods

Materials

Streptolysin-O (SL-O), purchased from Burroughs-Wellcome (Research Triangle Park, NC), was reconstituted as a 20 U/ml stock solution in distilled H_2O and stored frozen in small aliquots at −80°C. Each aliquot was thawed rapidly at 37°C immediately before use and aliquots were only used once. EGTA was from Fluka AG (Buchs, Switzerland). Hexokinase, creatine kinase, and creatine phosphate were from Sigma Chemical Co. (St. Louis, MO). ATP was from Calbiochem-Behring Corp. (San Diego, CA). GTPγS, guanylyl-imidodiphosphate (GMP-PNP), and endoglycosidase H (EndoH) were from Boehringer Mannheim Biochemicals (Indianapolis, IN). $[^{35}S]SO_4$ and $[^{35}S]$-methionine were obtained from Amersham Corp. (Arlington Heights, IL). Fixed *Staphylococcus aureus* cells (Immunoprecipitin) were from Bethesda Research Laboratories (Gaithersburg, MD). A stock solution of 0.5 M 4-methylumbelliferyl-β-D-xyloside (xyloside; Sigma Chemical Co.) was prepared in DMSO and stored at −20°C. All other reagents were of the highest quality available and obtained from the usual sources.

Preparation of Bovine Brain Cytosol

Bovine brains were obtained fresh from the slaughterhouse or stored frozen at −80°C. 10 g of bovine brain was homogenized in 20 ml of 25 mM Hepes-KOH (pH 7.2), 0.1 M K-glutamate, 1 mM DTT, 0.1 mM PMSF, 10 μg/ml leupeptin, 1 μM pepstatin, and 0.5 mM 1,10-phenanthroline by eight strokes in a motorized glass/Teflon homogenizer. This crude homogenate was clarified by centrifugation at 800 rpm for 20 min in a rotor (model SS-34; Sorvall Instruments Div., Newton, CT) and the resulting supernatant was centrifuged at 50,000 rpm for 90 min in a rotor (model 50.2 Ti; Beckman Instruments Inc., Palo Alto, CA). The high speed supernatant (cytosol) was frozen immediately in liquid nitrogen in small aliquots. Frozen cytosol fractions were stored at −80°C with no detectable loss of activity over several months. The protein concentration of cytosol preparations ranged from 6.5 to 9.5 mg/ml. Protein concentrations were determined using protein assay (Bio-Rad Laboratories, Richmond, CA) with BSA as a standard.

SL-O Permeabilization

CHO cells grown on poly-D-lysine–coated coverslips were washed free of media and incubated for 4 min at 37°C in varying concentrations of SL-O in 20 mM Hepes-KOH (pH 7.2), 125 mM K-glutamate, 5 mM EGTA, 5 mM free Mg^{2+} (as $MgCl_2$), 15 mM KCl, 5 mM NaCl (buffer A) containing 5 μg/ml propidium iodide. The coverslips were rinsed with buffer A, inverted onto glass slides, and random fields were immediately photographed for quantitation using a fluorescence microscope. Under these conditions <5% of CHO cells incubated without SL-O display staining with propidium iodide. 100% of CHO cells are permeabilized after 3–4 min in 0.1–0.2 U/ml of SL-O (see Results). Equivalent concentrations of SL-O also resulted in the quantitative permeabilization of cells grown on plastic tissue culture dishes (not shown). For the experiments described here we routinely used 0.2 U/ml SL-O for permeabilization. For determination of SL-O permeabilization of cells in suspension, CHO cells grown on 10-cm dishes were detached by incubation in Ca^{2+}/Mg^{2+}-free PBS and washed three times with buffer A by centrifugation/resuspension. The cells were resuspended at 10^4 cells/μl in ice cold buffer A, mixed with an equal volume of buffer A containing various concentrations of SL-O, and then incubated for 4 min at 37°C. An aliquot was immediately withdrawn, spotted onto a glass coverslip, and photographed for quantitation using a fluorescence microscope. Under these conditions <5% of CHO cells were stained by propidium iodide in the absence of SL-O and 100% staining was obtained at 0.05–0.10 U/ml SL-O.

In Vitro Transport Reaction

Attached Cells. Cells grown in 12-well plates were washed twice with PBS, and then incubated at 37°C in 20 mM Na-Hepes (pH 7.2), 110 mM NaCl, 5.4 mM KCl, 0.9 mM Na_2HPO_4, 10 mM $MgCl_2$, 2 mM $CaCl_2$, and 1 g/liter glucose (buffer B) containing 500 μM xyloside. After 30 min the media was aspirated, 250 μl of buffer B containing 100–200 μCi/ml $[^{35}S]SO_4$ (25–40 Ci/mg) was added, and the incubation continued for 2 min at 37°C. The labeling media was aspirated and the cells were washed quickly with two 1-ml washes of buffer B supplemented with 4 mM Na_2SO_4. SL-O permeabilization was immediately initiated by the addition of 250 μl of buffer A containing 0.2 U/ml SL-O and an ATP-depleting system (30 IU/ml hexokinase, 2 mM glucose). Permeabilization was effected by incubating the cells for 4 min at 37°C. Sham incubations were carried out by incubating pulse-labeled cells in 250 μl of buffer B containing 4 mM Na_2SO_4 for 4 min at 37°C. After permeabilization cells were incubated for 60 min at 4°C in 0.5 ml of buffer A (or buffer B for sham permeabilization) with one change after 30 min. The cells were then shifted to 37°C and incubated for varying times in 250 μl of transport buffer (20 mM Hepes-KOH, pH 7.2, 80 mM K-glutamate, 15 mM KCl, 5 mM NaCl, 5 mM EGTA, 5 mM free Mg^{2+} [as $MgCl_2$], and various concentrations of Ca^{2+}) containing either an ATP-depleting system (as described above) or an ATP-regenerating system (40 IU/ml creatine kinase, 2 mM creatine phosphate, 500 μM ATP) and 325–475 μg (1.3–1.9 mg/ml) cytosol. In experiments in which free Ca^{2+} was varied, the total concentration of Ca^{2+} was adjusted to give the indicated concentrations of free Ca^{2+}; the total concentration of EGTA was

maintained at 5 mM in all cases. In each case the total concentration of $MgCl_2$ was adjusted to maintain free Mg^{2+} at 5 mM. Unless otherwise indicated transport buffer contained 100 nM free Ca^{2+}. The free concentrations of Ca^{2+} and Mg^{2+} were determined with the program Mathematica (Wolfram Research Inc., Champaign, IL) using the stability constants of the EGTA complexes, corrected for temperature and pH, of Martell and Smith (1974). Transport reactions were terminated by cooling to 4°C. The media was removed and combined with a 250-μl PBS wash. The media samples were then centrifuged for 2 min in an Eppendorf microfuge to remove any cells that may have detached during the transport reaction and the supernatant was transferred to fresh tubes. Cells were extracted with 100 μl of 50 mM Tris (pH 8.0), 150 mM NaCl, 2 mM $MgCl_2$, 1% Triton X-100 for 5 min at 37°C and the wells were rinsed with 0.4 ml of PBS. The detergent extracts were combined with any cells pelleted from the media samples.

Cells in Suspension. Cells were grown to ~80–90% confluency on 10-cm dishes and detached by incubation for 5 min at 37°C in Ca^{2+}/Mg^{2+}-free PBS containing 5 mM EDTA. The cells were pelleted for 4 min in a tabletop centrifuge, resuspended in 1 ml of buffer B, and then transferred to a polypropylene microcentrifuge tube. The cells were washed once with buffer B by centrifugation/resuspension, and then resuspended in 0.4 ml of buffer B containing 500 μM xyloside and incubated for 30 min at 37°C. The cells were labeled by adding 100 μl of buffer B containing 1 mCi of $[^{35}S]SO_4$ (25–40 Ci/mg) and incubated at 37°C for 90 s. Labeling was terminated by the addition of 0.5 ml of ice cold buffer B supplemented with 4 mM Na_2SO_4. The cells were pelleted in a microcentrifuge, washed three times at 0°C in buffer A containing 4 mM Na_2SO_4, and resuspended at 10^4 cells/μl in ice cold buffer A. Permeabilization was carried out by adding an equal volume of ice cold buffer A containing 0.10 U/ml SL-O and an ATP-depleting system and incubation for 4 min at 37°C. Permeabilization was terminated by adding an equal volume of ice cold buffer A and rapid centrifugation. The cells were resuspended at 10^4 cells/μl in ice cold transport buffer and kept on ice until use. Transport reactions were carried out in a total volume of 50 μl of transport buffer containing either an ATP-regenerating or -depleting system, 65–95 μg (1.3–1.9 mg/ml) cytosol, and 10 μl of permeabilized cells (10^5 cells). Reactions were initiated by shifting to 37°C and terminated by the addition of 450 μl of ice cold transport buffer and rapid centrifugation. Pelleted cells were extracted as described above for attached cells and media and cell extracts were assayed for ^{35}S-labeled glycosaminoglycans (GAG) as described below.

Regulated Secretion from AtT-20 Cells. AtT-20 cells were plated at 2 × 10^5 cells/well in 12-well plates and used after 3–4 d in culture. The cells were starved for sulfate by incubation in buffer B containing 0.5 mM xyloside for 30 min, and then labeled for 2 h at 37°C in 250 μl of buffer B containing 100–200 μCi/ml $[^{35}S]SO_4$ (25–40 Ci/mg) to accumulate ^{35}S-labeled GAG chains in regulated secretory granules. Labeled cells were chased in DME-H21 for 2 × 90 min to clear labeled GAG chains from the constitutive pathway. The cells were washed twice with transport buffer and incubated for 10 min in 250 μl of transport buffer (adjusted to pH 6.8) containing 2.0 U/ml SL-O, an ATP-regenerating system, and various concentrations of free Ca^{2+}. Media and cells were collected as described above for attached CHO cells and the amount of labeled GAG chains in each fraction was quantitated as described below.

Quantitation of $[^{35}S]SO_4$ GAG Chains

$[^{35}S]SO_4$-labeled GAG chains were quantitated by a precipitation/filtration essentially as described by Luikart et al. (1985). Briefly, media samples or cell extracts (0.5 ml total volume) were proteolytically digested by the addition of 100 μl of 6 mg/ml pronase E and incubation for 4–16 h at 37°C. Sulfated GAG chains were then precipitated by the addition of 150 μl of 10% (wt/vol) cetylpyridinium chloride (CPC; 2% final) and 10 μl of 10 mg/ml chondroiton sulfate as a carrier. After incubation at 37°C for an additional 60 min the precipitated samples were collected by rapid vacuum filtration using Metricel GN-6 filters (2.4 mm, 0.45 μm) followed by four 5-ml washes with 1% CPC/25 mM Na_2SO_4. The filters were dried and counted in a scintillation counter. This assay is linear over at least a 50-fold range of added $[^{35}S]SO_4$ GAG and unaffected by the presence of excess free $[^{35}S]SO_4$ in the range found in the assay (not shown).

VSV ts045 Infection

CHO cells grown on poly-D-lysine–coated coverslips were infected with vesicular stomatitis virus (VSV) ts045 as described previously (Rivas and Moore, 1989). After infection the cells were washed twice in DME-H21, 2% FCS, 25 mM Hepes (pH 7.4), and incubated in the same buffer at 39.5°C for 3 h to accumulate VSV glycoprotein (G protein) in the ER (Zilberstein et al., 1980). The cells were then shifted to 19°C for 1 h to accumulate VSV G in the *trans*-Golgi apparatus (Griffiths et al., 1985). In vitro transport reactions were carried out using the assay described above for attached cells except that the xyloside preincubation and $[^{35}S]SO_4$ labeling were omitted; transport was performed at 32°C rather than 37°C; and the reactions were terminated by fixing the cells and processing for indirect immunofluorescence microscopy as described below. For cell surface immunoprecipitation infection and accumulation of VSV G protein in the ER were carried out as described above except that cells were grown on 10-cm dishes. VSV G was metabolically labeled by shifting the cells to a methionine-free medium for the final 30 min of the 3-h incubation at 39.5°C then adding 300 μCi of $[^{35}S]$-methionine and continuing the incubation for 10 min at 39.5°C. Unlabeled methionine was then added to a final concentration of 5 mM and the incubation continued for an additional 5 min at 39.5°C. The cells were then washed and incubated for 2 h at 19°C to accumulate VSV G in the *trans*-Golgi cisternae. In vitro transport reactions were carried out using the suspension assay described above except that the xyloside preincubation and $[^{35}S]SO_4$ labeling were omitted; SL-O permeabilization was done at 40°C; and the reactions were carried out at 32°C rather than 37°C. The transport reactions were terminated by adding an equal volume of ice cold buffer A and cell surface immunoprecipitation was carried out as described below.

Immunofluorescence Microscopy

CHO cells were grown for 72 h on poly-D-lysine–coated coverslips as described above. Indirect immunofluorescence was carried out as described previously (Rivas and Moore, 1989). Surface staining of VSV G was determined by omitting the postfixation permeabilization step with Triton X-100. VSV G protein was detected using either a mouse monoclonal antibody specific for the lumenal domain of VSV G (Lefrancois and Liles, 1982; provided by Dr. L. Lefrancois, Scripps Clinic and Research Foundation, La Jolla, CA) or a mouse monoclonal antibody specific for the cytoplasmic tail of VSV G (Kreis, 1986; provided by Dr. T. Kreis, European Molecular Biology Laboratory, Heidelberg, Germany).

Electron Microscopy

CHO cells were grown for 72 h in six-well plates. After treatment as described in the figure legends, cells were fixed for 1 h using 2.5% glutaraldehyde, 1% acrolein, 1% paraformaldehyde in 0.1 M Na cacodylate (pH 7.4). Cells were then rinsed in PBS, osmicated for 1 h using 1% osmium tetroxide, 1% $K_3Fe(CN)_6$ in PBS, rinsed, dehydrated in 30 and then 50% EtOH, and finally stained for 1 h with 2% uranyl acetate in 50% EtOH. The EtOH dehydration series was continued, and the cells were scraped from the wells and transferred to embedding capsules. After final dehydration in propylene oxide the cells were embedded in Epox 812/Araldite and sectioned.

Golgi Staining with NBD-Ceramide

CHO cells grown on poly-D-lysine–coated coverslips were rinsed free of media, incubated for 10 min at 37°C in 1 mM C6-NBD-ceramide in DME-H21 containing 20 mM Hepes (pH 7.4) and 0.68 mg/ml BSA. The cells were then washed, incubated for 10 min at 37°C in DME-H21/20 mM Hepes, and then for 10 min at 37°C in DME-H21/20 mM Hepes/0.68 mg/ml BSA. The cells were permeabilized for 4 min at 37°C in 0.2 U/ml SL-O, incubated for 10 min in transport buffer, and then fixed for 30 min in 3.7% paraformaldehyde in PBS (pH 7.4) at room temperature.

Cell Surface Immunoprecipitation

Cell surface immunoprecipitation of VSV G protein was performed essentially as described by Compton et al. (1989). Cells from 100-μl aliquots of the transport reactions were pelleted and resuspended in 100 μl of ice cold PBS containing 0.1 mM Ca^{2+} and a 1:3 dilution of an anti-VSV rabbit antiserum recognizing the lumenal domain of VSV G and incubated for 60 min at 4°C. The cells were pelleted by centrifugation for 2 min in a microcentrifuge and washed three times with ice cold PBS. The final cell pellet was solubilized in 500 μl of lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 20 mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS) containing unlabeled VSV-infected CHO cell extract to block binding of unreacted antibody sites, and insoluble materials were removed by centrifugation for 5 min at 12,000 *g*. The supernatant was incubated with 100 μl of a suspension of fixed *S. aureus* cells for 60 min at 4°C. The cells were pelleted by centrifugation for 5 min at 12,000 *g* and washed three times with lysis buffer. The final cell pellet was boiled in 30 μl of SDS gel sample buffer

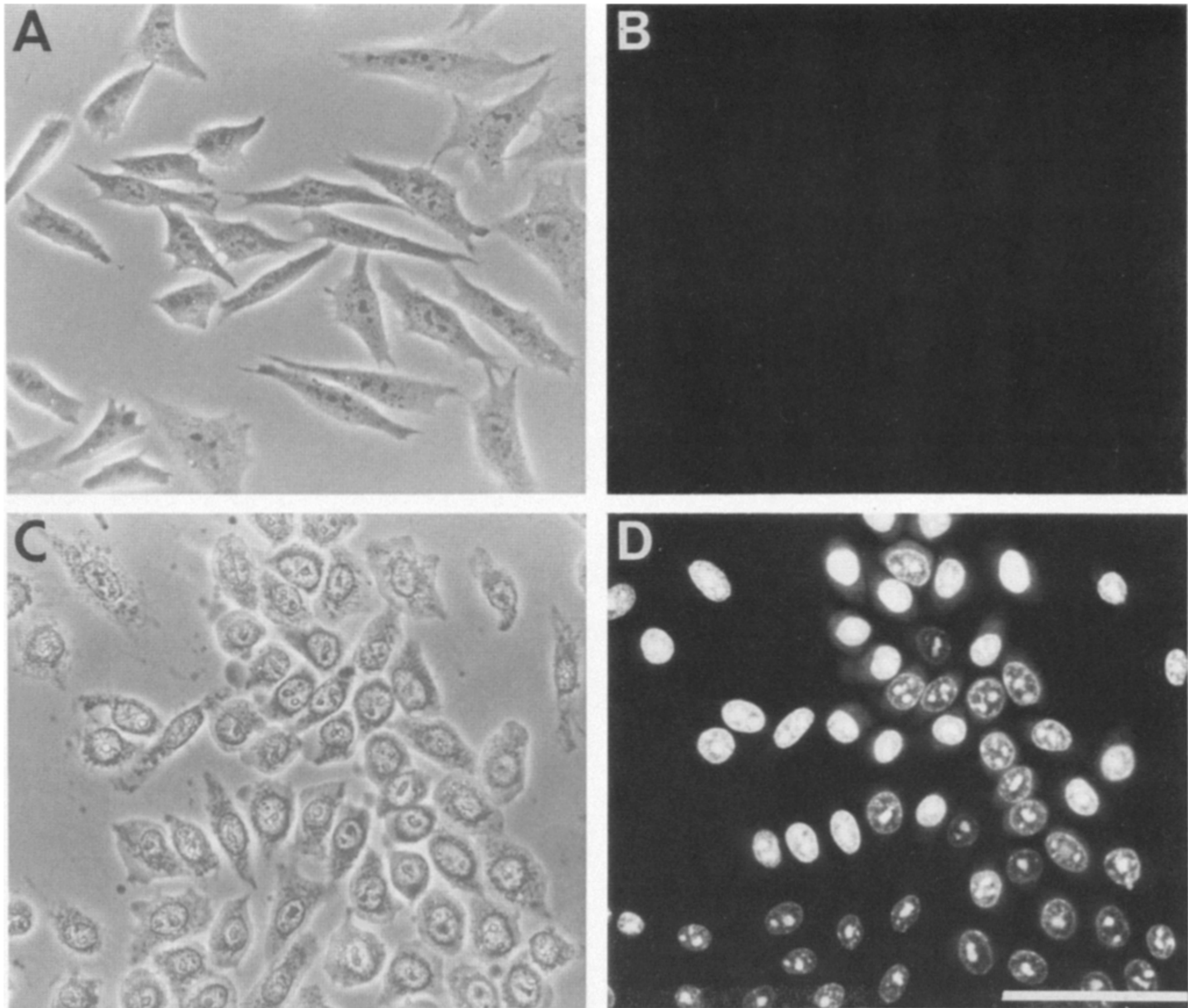

Figure 1. CHO cells are quantitatively permeabilized by SL-O. CHO cells were washed free of media and incubated for 4 min at 37°C in a buffer containing 5 μg/ml propidium iodide (a membrane-impermeant DNA-binding fluorescent probe) and either 0 (*A* and *B*) or 0.2 (*C* and *D*) U/ml SL-O. The unfixed cells were then viewed under phase (*A* and *C*) or fluorescence (*B* and *D*) to detect permeabilized cells (identified by bright nuclear staining with propidium iodide). Bar, 40 μm.

and the eluted proteins were analyzed by SDS-PAGE on 10% polyacrylamide gels. The gels were fixed, treated for fluorography with 1 M salicylate, dried, and exposed to film. Quantitation of the resulting autoradiographs was accomplished using a scanning laser densitometer and integrating the areas of peaks of interest. Total VSV G was determined by treating parallel samples as described above except that the cells were solubilized with lysis buffer before adding antibody. In control experiments we found that a 1:10 dilution of antibody resulted in precipitation of the same amount of VSV G; therefore the amount of antibody used in these experiments is at least a threefold excess. The extent of EndoH resistance was determined using parallel samples as described by Rose and Bergmann (1982).

Results

Choice of SL-O for Perforation of CHO Cells

We have chosen the bacterial cytolysin SL-O to prepare semi-intact cells for the following reasons. SL-O has been used in a number of studies to examine regulated secretion (for review see Ahnert-Hilger et al., 1989). Thus, membranes treated with SL-O retain capability for fusion. SL-O permeabilization is rapid (<3–5 min; Ahnert-Hilger et al., 1989) as compared with detergents such as digitonin (typically 10–20 min) (Diaz and Stahl, 1989; Dunn and Holz, 1983; Shafer et al., 1987), and is highly selective for the plasma membrane (Ahnert-Hilger et al., 1989; see below). The pores formed in the plasma membrane are uniform in size and have been characterized as highly stable 20–30-nm structures (Bhakdi et al., 1985). The dimension of the pores therefore allows exchange of large cytosolic components (≥150 kD) as well as small molecules such as nucleotides and ions (Ahnert-Hilger et al., 1989). Since Golgi-derived exocytic vesicles have diameters typically in the 80–300-nm range (Griffiths et al., 1985; Orci et al., 1987; deCurtis and Simons, 1988), they should be retained within the cellular boundary (see below).

CHO cells were chosen because these cells secrete newly synthesized proteins by the constitutive pathway and appear

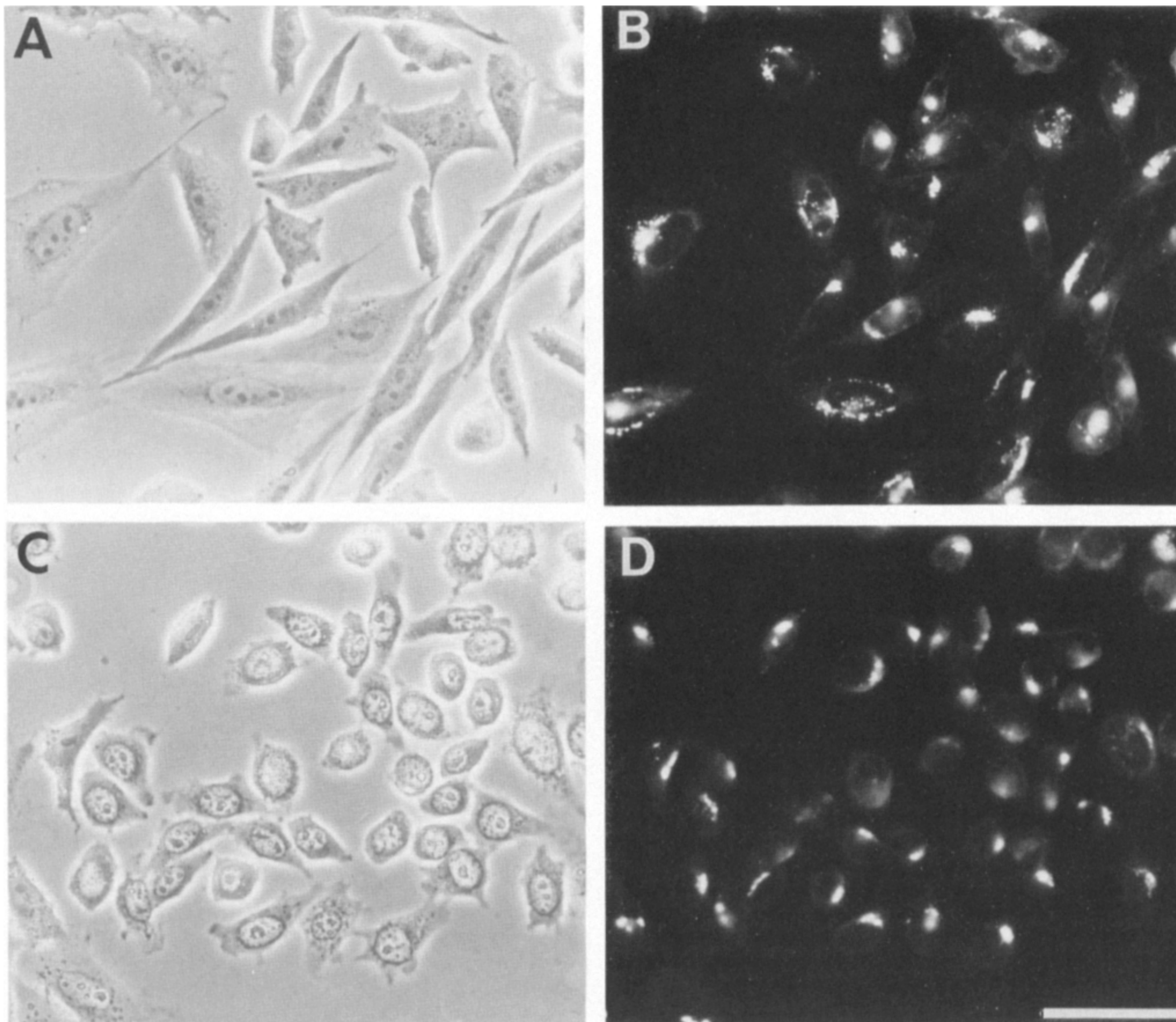

Figure 2. Intracellular structures are maintained in SL-O–permeabilized cells. Staining of Golgi complex with C6-NBD-ceramide. CHO cells were incubated for 10 min at 37°C with C6-NBD-ceramide, washed, chased for 20 min at 37°C to accumulate ceramide in the Golgi complex and then permeabilized for 4 min at 37°C in either 0 (*A* and *B*) or 0.2 (*C* and *D*) U/ml SL-O. The cells were then incubated for 10 min in transport buffer, fixed, and viewed under phase (*A* and *C*) or fluorescence (*B* and *D*) to visualize Golgi structures. Bar, 40 μm.

to lack the ability to store proteins in regulated granules. This property allows us to study secretion by this pathway in isolation. For optimal reconstitution, only the plasma membrane but not internal organelles should be perforated. This could be accomplished in two ways. SL-O can be added to cells directly at 37°C; in this case the concentration and the time of exposure must be optimized. Alternatively, cells are first incubated with SL-O at 4°C to allow binding to the plasma membrane. Excess SL-O is then washed away, and the cells are warmed to 37°C to allow oligomerization and formation of the pores (Ahnert-Hilger et al., 1989). We found both procedures gave similar results. Fig. 1 shows that under the optimized conditions, 100% of CHO cells take up propidium iodide, a 668-D membrane-impermeant fluorescent dye that stains the nucleii of permeabilized cells. Less than 5% of control cells are stained with propidium iodide.

Integrity of Intracellular Organelles in Perforated Cells

Cells perforated by SL-O retained morphologically intact Golgi structures, as shown by staining with the fluorescent lipid C6-NBD-ceramide (Fig. 2; Pagano, 1989). Most intracellular organelles and cytoplasm appeared well preserved at the electron microscopic level, although after extended incubations the Golgi cisternae appeared more swollen than intact cells (Fig. 3). To determine if the plasma membrane is selectively perforated, we studied the accessibility of a cytoplasmically exposed antigen and a Golgi-lumenal antigen to exogenously added antibodies. CHO cells were infected with a temperature-sensitive mutant of the VSV, VSV-ts045, for 3 h at 39.5°C (Zilberstein et al., 1980). The cells were then shifted to 19°C for 1 h to accumulate the G protein in the *trans*-Golgi structures (Griffiths et al., 1985). After

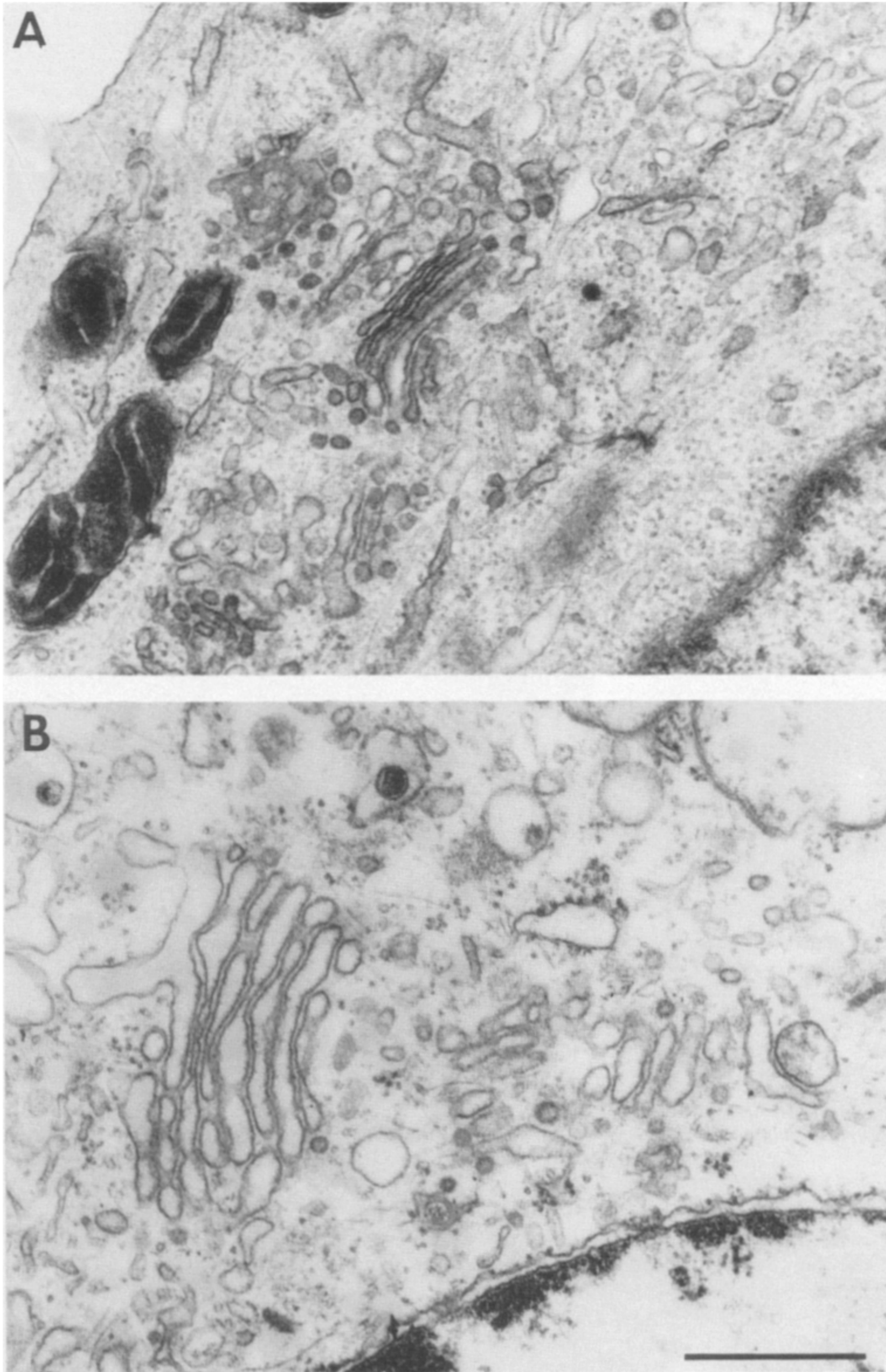

Figure 3. Ultrastructure of SL-O–permeabilized cells after prolonged incubation. CHO cells were permeabilized with either 0 (*A*) or 0.2 (*B*) U/ml SL-O, incubated for 60 min in transport buffer containing an ATP-depleting system (Materials and Methods), and then processed for EM. Bar, 500 nm.

permeabilization with SL-O, cells were incubated with monoclonal antibodies specific for either the lumenal or the cytoplasmic domain of G protein. After washing, bound antibodies were visualized with fluorescent secondary antibodies. Bright staining of the ER and Golgi structures was found in cells incubated with the anticytoplasmic tail antibody, but not with the antilumenal antibody (Fig. 4). The lack of staining by the antilumenal antibody was not due to its inability to recognize the expressed G proteins. Fig. 5 shows a parallel experiment in which sham-permeabilized cells were incubated with the same antibodies; under these conditions G protein was transported to the cell surface. The antilumenal antibodies showed considerable staining at the cell surface, whereas the anti-tail antibodies did not give rise to detectable staining as expected. Thus, under the conditions of our SL-O treatment the plasma membrane is selectively perfo-

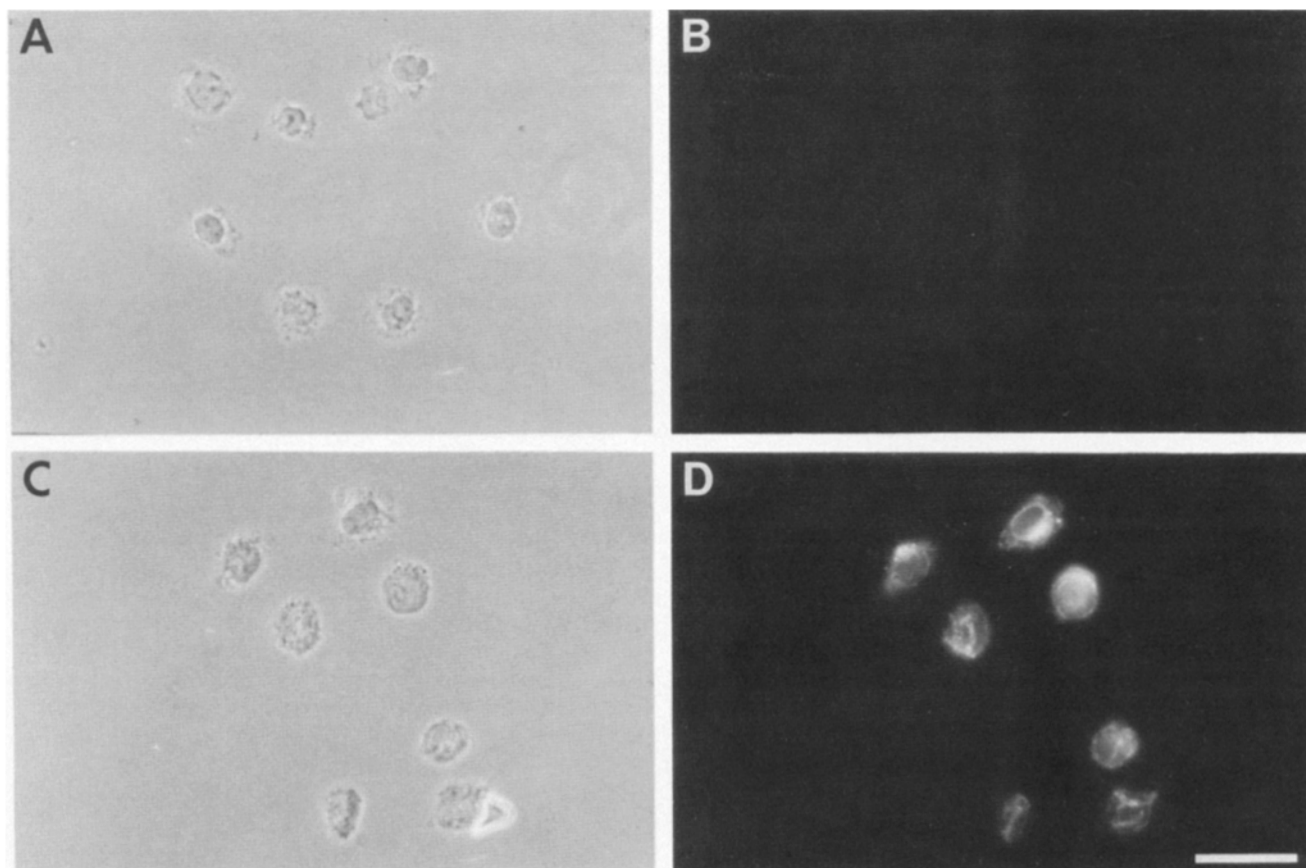

Figure 4. Cytoplasmic but not lumenal compartments are accessible to antibodies in SL-O–permeabilized cells. CHO cells were infected with VSV ts045 for 40 min at 37°C, and incubated for 3 h at 39.5°C and then 1 h at 19°C to accumulate G proteins in the *trans*-Golgi complex. The cells were permeabilized with SL-O for 4 min, and incubated in transport buffer containing an ATP-depleting system for 1 h at 32°C along with monoclonal antibodies specific for either the lumenal or cytoplasmic domain of the G protein. Unbound antibodies were washed away; the cells were fixed and treated with Triton X-100. Bound antibodies were then visualized with a fluorescent secondary antibody. (*A* and *B*) lumenal; (*C* and *D*) cytoplasmic. (*A* and *C*) phase; (*B* and *D*) fluorescence. Bar, 30 μm.

rated to allow passage of antibodies into the cytoplasm. The internal membranes of the ER and Golgi apparatus remain impermeant to antibody molecules.

Reconstitution of Golgi-to-Plasma Membrane Transport of VSV G

We next tested if transport from the *trans*-Golgi structure to the plasma membrane could be reconstituted in the perforated cell preparation. CHO cells infected with VSV ts045 were incubated at 19°C to accumulate the G protein in the *trans*-Golgi apparatus (Griffiths et al., 1985). When the temperature was shifted to 32°C, G protein in intact cells was rapidly transported from the *trans*-Golgi apparatus to the plasma membrane (Fig. 6, *A–D*). In contrast, when SL-O–treated cells were incubated at 32°C in the presence of an ATP-depleting system, VSV G remained intracellular and no

Table I. Quantitation of In Vitro Transport of VSV-G by Cell Surface Immunoprecipitation

	Total	EndoH resistant	Surface	EndoH resistant at surface
		Arbitrary Units		%
Intact (−ATP)	2,830	2,830	2,360 ± 120	83 ± 4
Intact (+ATP)	2,550	2,550	2,065 ± 255	81 ± 10
SL-O: −ATP/+Cytosol	2,065	1,020	130 ± 16	13 ± 2
SL-O: +ATP/+Cytosol	2,140	1,145	785 ± 140	68 ± 12
SL-O: +ATP/+Cytosol/+GTPγS	2,050	965	115 ± 10	12 ± 1

CHO cells were infected with VSV ts045, incubated for 3 h at 40°C, pulse labeled for 10 min at 40°C with [^{35}S]methionine, and then incubated for 2 h at 19.5°C to accumulate VSV G protein in the *trans*-Golgi region (see Materials and Methods). The in vitro transport reaction was then carried out for 60 min at 32°C under the indicated conditions and the total amount of VSV G and the amount at the cell surface were assayed by immunoprecipitation as described in Materials and Methods. GTPγS was added to the reaction to a final concentration of 100 μM. Errors are the SD of triplicate determinations.

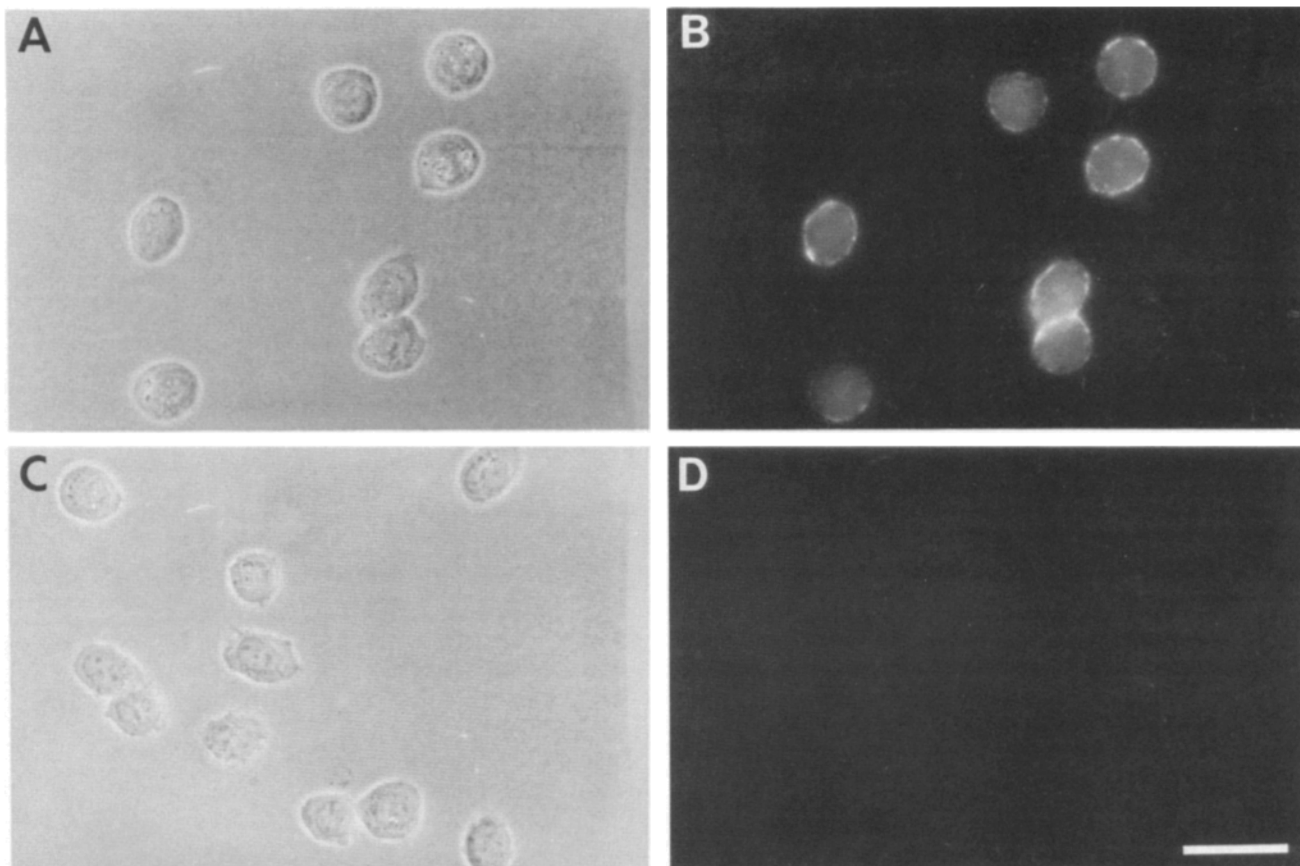

Figure 5. Control sham-permeabilized cells. CHO cells were treated exactly as described in Fig. 4 except that SL-O was omitted during the 4-min permeabilization reaction. (*A* and *B*) lumenal; (*C* and *D*) cytoplasmic. (*A* and *C*) phase; (*B* and *D*) fluorescence. Since ATP-depleting system does not affect transport in intact cells, G proteins arrested in the *trans*-Golgi complex are expected to migrate to the cell surface during the 1-h incubation at 32°C. Bar, 30 μm.

immunoreactivity could be detected at the cell surface (Fig. 6, *E* and *F*). However, when bovine brain cytosol and an ATP-regenerating system were added to the perforated cells, the G protein was efficiently transported to the plasma membrane (Fig. 6, *G* and *H*). To quantitate the amount of G proteins transported to the cell surface, we performed cell surface immunoprecipitation. CHO cells, infected with VSV ts045 and pulse labeled with [^{35}S]methionine, were incubated for 2 h at 19.5°C to accumulate G protein in the *trans*-Golgi apparatus. Cells were then permeabilized and incubated either in an ATP-depleting system or in an ATP-regenerating system supplemented with bovine brain cytosol. At the end of the reaction, cells were incubated with antilumenal antibodies to detect exposed G proteins. An identical sample was detergent solubilized and then subjected to immunoprecipitation to determine the total amount of antigens present. A third sample was used to determine the amount of VSV G that had acquired EndoH resistance (Table I). ATP and cytosol stimulated exposure of EndoH-resistant VSV G proteins from 13 to 68% in SLO-treated cells. Intact cells showed 82% transport both in presence and absence of ATP. Control experiment showed that <5% of EndoH-resistant G proteins were exposed after incubation at 19.5°C (data not shown). Taken together, these experiments demonstrated that transport of VSV was indeed reconstituted in SLO-treated cells.

Quantitation of Rates of Transport Using Sulfated Glycosaminoglycans As a Marker

To dissect the biochemical requirements for constitutive secretion, we needed a more sensitive and reliable method to quantitate the kinetics and extent of transport under different conditions. We took advantage of the fact that constitutive secretion is a bulk-flow process (Pfeffer and Rothman, 1987; Burgess and Kelly, 1987). Thus, the rate of secretion can be measured by a fluid-phase tracer generated in the Golgi lumen. GAG chains provide an ideal marker. Synthesis of free GAG chains can be induced by incubating cells in the presence of a membrane-permeant xyloside, which serves as initiator for GAG chain elongation (Schwartz et al., 1974). Since GAG chains are highly sulfated and sulfation occurs in the *trans*-Golgi apparatus (Kimura et al., 1984; Farquhar, 1985; Velasco et al., 1988), transport from the *trans*-Golgi apparatus to the surface can be followed by pulse labeling xyloside-treated cells with [^{35}S]SO_4.

Fig. 7 shows that SO_4 GAG chains synthesized by CHO cells were rapidly secreted from the cells. To quantitate the amount of SO_4 GAG chains, we have used a simple filtration assay (see Materials and Methods) that involves precipitation of GAG chains with CPC (Luikart et al., 1985). The method is so sensitive that it allows us to follow the secretion of GAG chains that are labeled during a short (1–2 min)

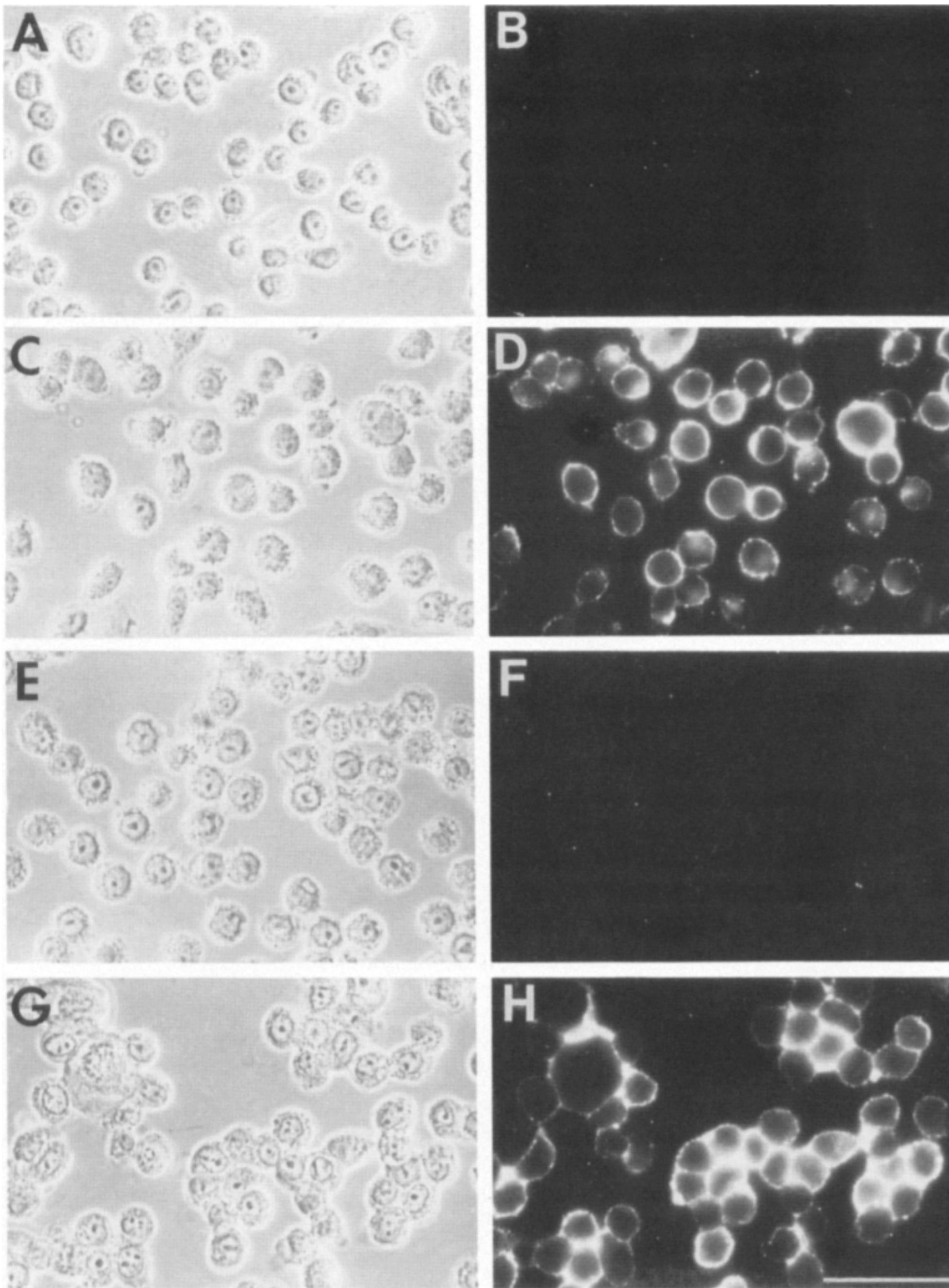

Figure 6. Reconstitution of transport of VSV G from the *trans*-Golgi complex to the plasma membrane in SL-O-permeabilized cells. CHO cells were infected with VSV ts045 for 40 min at 37°C, and incubated for 3 h at 39.5°C and then 1 h at 19°C to accumulate VSV G in the *trans*-Golgi complex. Cells were then incubated for 4 min in either 0 (*A–D*) or 0.2 (*E–H*) U/ml SL-O. For sham-permeabilized cells, the cells were either fixed immediately (*A* and *B*) or incubated for 60 min at 32°C in DME before fixation (*C* and *D*). For SL-O–treated cells, the cells were incubated in transport buffer containing cytosol with or without ATP. (*E* and *F*) ATP-depleting system; (*G* and *H*) ATP-regenerating system. The cells were fixed and VSV G that had been transported to the cell surface was detected by indirect immunofluorescence using an antilumenal antibody without Triton X-100 treatment. (*A*, *C*, *E*, and *G*) phase; (*B*, *D*, *F*, and *H*) fluorescence. Bar, 80 μm.

pulse. The kinetics of SO_4 GAG released from intact cells CHO cells that had been starved for sulfate for 30 min and pulse labeled with $[^{35}S]SO_4$ for 2 min are shown in Fig. 7 *b*. Approximately 80–90% of the labeled GAG chains were released during a 60-min chase. The time course of release exhibited a lag time of 7–8 min and a half-time for secretion of 12–14 min. The kinetics of secretion are consistent with a simple two-compartment transport model as described by Noe and Delenick (1989), suggesting that GAG chains are transported directly from the compartment in which they are sulfated to the plasma membrane. These findings are consistent with earlier studies indicating that GAG sulfation is a late Golgi event (Kimura et al., 1984; Velasco et al., 1988).

Biochemical Requirements for Constitutive Secretion from the trans-Golgi Apparatus

Using the CPC assay for GAG chains, we then determined the biochemical requirements for *trans*-Golgi-to-plasma membrane transport in the reconstituted system. Uptake of $[^{35}S]SO_4$, synthesis of the intermediate $[^{35}S]$-3′-phosphoadenosine 5′-phosphosulfate and translocation of 3′-phosphoadenosine 5′-phosphosulfate into the Golgi lumen requires several minutes in mammalian cells (Baeuerle and Huttner, 1987). By using brief (1–2-min) pulse labeling with $[^{35}S]$-SO_4, followed by rapid permeabilization with SL-O (permeabilization is complete within 2–3 min) in the presence

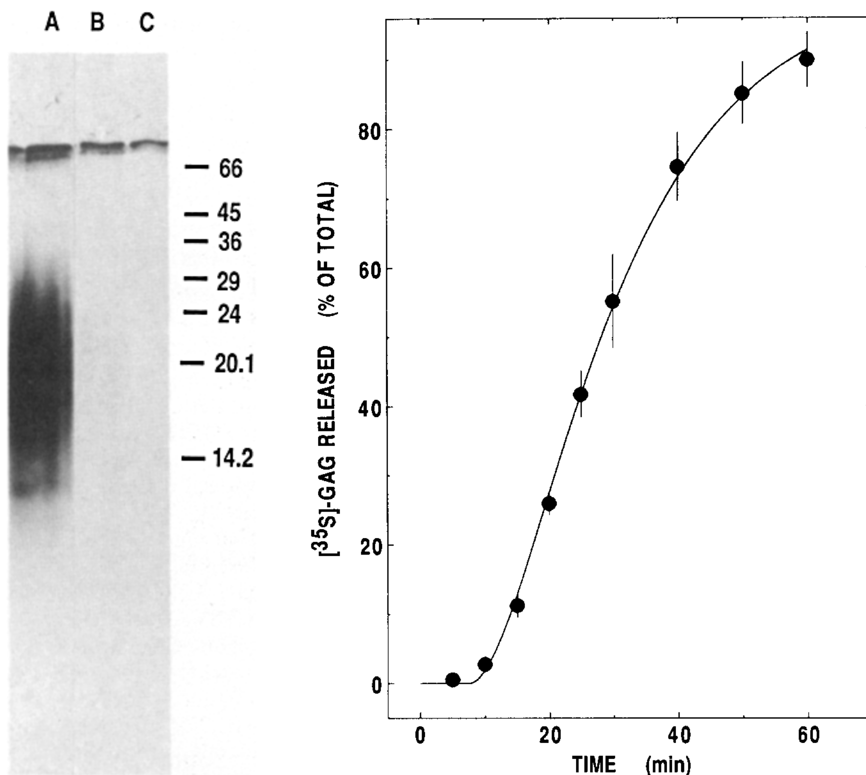

Figure 7. Sulfated glycosaminoglycans are secreted from CHO cells with kinetics characteristic of the constitutive pathway. (*a*) CHO cells grown on six-well plates were starved for 30 min in sulfate-free media containing 0.5 mM xyloside, and then labeled for 2 h at 37°C with [^{35}S]SO_4 in presence of xyloside. The cells were washed to remove excess [^{35}S]SO_4 and chased for 3 h at 37°C in DME. (Lane *A*) 0–1-h chase; (lane *B*) 1–2-h chase; (lane *C*) 2–3-h chase. Sulfated GAG chains were precipitated with acetone at −20°C, separated on an 18% polyacrylamide gel, and autoradiographed. The mobilities of molecular mass standards (in kD) are indicated. (*b*) CHO cells grown on 12-well plates were starved for 30 min in sulfate-free media containing 0.5 mM xyloside, and then pulse labeled for 2 min with [^{35}S]SO_4. After washing briefly to remove excess [^{35}S]SO_4, the cells were chased for varying times in DME. At each time point the media was collected and cells extracted. The amount of GAG chains in each sample was determined using a precipitation/filtration assay (see Materials and Methods). The plot shows the extent of secretion, expressed as the percentage of total ^{35}S-sulfated GAG chains synthesized during the pulse, as a function of time. Data shown are the mean ±SD for triplicate data points.

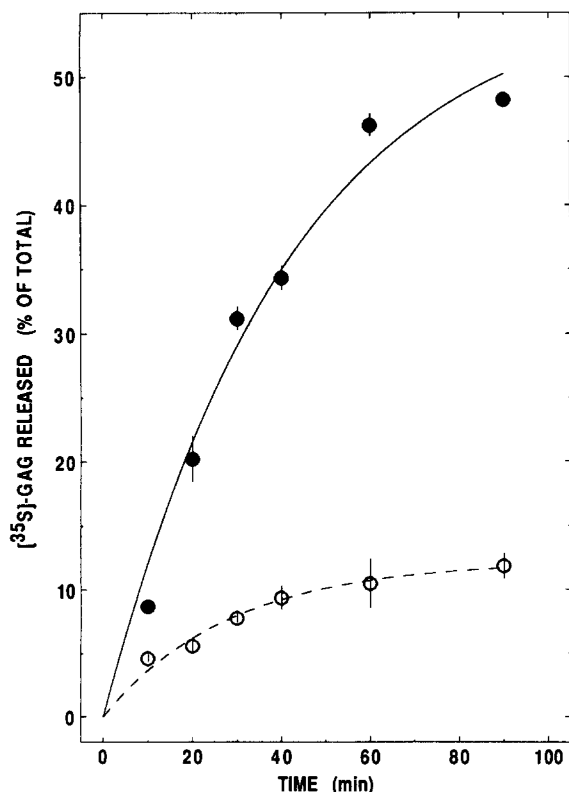

Figure 8. Quantitation of transport efficiency in the reconstituted system using sulfated glycosaminoglycans. CHO cells grown on 12-well plates were incubated with xyloside and pulse labeled with [^{35}S]SO_4 for 2 min as described in the legend to Fig. 7 *b*. The cells were permeabilized for 4 min at 37°C with 0.2 U/ml SL-O and incubated for the indicated times in transport buffer containing either an ATP-depleting (○) or ATP-regenerating (●) system and cytosol. At each time point the media was collected and the cells were extracted. The percentage of total ^{35}S-labeled GAG released as a function of time was determined by a precipitation/filtration assay. Data shown are the mean ±SD for duplicate data points.

of an ATP-depleting system, we can follow the transport of sulfated GAG chains from the budding step through fusion with the plasma membrane.

Xyloside-treated cells were pulse labeled for 2 min with [^{35}S]SO_4, permeabilized for 4 min with SL-O, and then incubated for varying times with bovine brain cytosol in the presence or absence of ATP (Fig. 8). Addition of ATP and cytosol to the semi-intact cell preparation stimulated a time-dependent release of GAG chains into the medium, which was not observed when the preparation was incubated in an ATP-depleting system. The rate of transport in the presence of ATP was ∼40–50% of the rate observed in intact cells after the lag (compare to Fig. 7 *b*). Typically, 40–55% of total GAG chains were secreted during a 60-min incubation period (compared with 80–90% for intact cells). Thus, the extent of secretion over a 60-min incubation ranges from 45 to 70% of intact cells. Transport required hydrolysis of ATP, since it was not supported by the nonhydrolyzable ATP analog AMP-PNP (data not shown).

The ATP-dependent secretion of sulfated GAGs is highly temperature dependent; <2–3% secretion was observed

Table II. Characteristics of In Vitro Transport of GAG Chains from the trans-Golgi to the Cell Surface

Incubation	ATP-dependent transport
	%
Complete	100
0°C	2.1 ± 0.9
−Cytosol	9.2 ± 0.9
+NEM	3.9 ± 2.1
+GTPγS	7.5 ± 3.3
+GMP-PNP	28.8 ± 4.9

In vitro transport reaction was carried out for 60 min using suspended cells as described in Materials and Methods. The complete reaction contained the semi-intact cell preparation, bovine cytosol, and an ATP-regenerating system. For each condition, the extent of transport was determined for buffers containing either an ATP-regenerating or -depleting system. The difference between the two sets of reaction is defined as ATP-dependent transport. Except for the 0°C incubation, all reactions were carried out at 37°C. For NEM treatment, perforated cells were first incubated with 1 mM NEM at 0°C for 15 min; NEM was subsequently inactivated by incubation with 2 mM DTT at 0°C for 15 min. GTPγS was added to the reaction at a final concentration of 50 μM, and GMP-PNP at 100 μM. ATP-dependent transport in the complete reaction was defined as 100%.

when the cells were held at 0–4°C for 1 h (Table II). This suggests that the observed release of GAG chains resulted from vesicular transport between membrane compartments, rather than direct leakage out of damaged Golgi apparatus. The observed secretion of GAG chains was not due to the release of exocytic transport vesicles (Bennett et al., 1988), since the radiolabeled chains recovered from the medium were freely soluble and were not pelleted by centrifugation at 100,000 *g* for 90 min (not shown).

Table II shows the requirements for Golgi-to-plasma membrane transport. In addition to hydrolyzable ATP, a crude cytosol fraction must be added for efficient transport. Pretreating the semi-intact cells with the alkylating agent, *N*-ethylmaleimide (NEM), completely abolished the transport reaction. Control experiments showed that release of GAG chains from intact cells was also temperature dependent and inhibited by NEM, but was unaffected by either ATP-depleting or -regenerating systems or the addition of cytosol as expected (not shown). In initial studies we found that cells grown on plastic dishes showed a variable dependency on added cytosol, most likely due to slight variations of the degree of permeabilization. This difficulty may be overcome by carefully standardizing the time and concentration used for SL-O treatment, followed by extensively washing the perforated preparation with buffer at 4°C before the start of the reaction. Alternatively, permeabilization and transport can be carried out using suspended cell preparations prepared by detaching the cells with EDTA (see Materials and Methods).

Inhibition of Transport by GTPγS

To determine if GTPγS exerts any effect on constitutive secretion, we examined in vitro transport of VSV G from the *trans*-Golgi membrane to the plasma membrane in the presence of GTPγS. Inclusion of 50–100 μM GTPγS in the transport incubation blocked the appearance of VSV G at the cell surface as assessed by both immunofluorescence (Fig. 9) and cell surface immunoprecipitation (Table I). The inhibitory effect of GTPγS was also confirmed by CPC assays of sulfated GAGs (Table II; Fig. 10). Quantitation of transport rates in presence of increasing concentrations of GTPγS showed that half-maximal inhibition occurred at ∼5 μM GTPγS. Including GTP in the transport reaction had little effect on its own (Fig. 10), but 1 mM GTP prevented the inhibition produced by 50 μM GTPγS (not shown). The inhibitory effect was not due to a general toxic effect of the GTP analogue on cells; treating intact cells with 100 μM GTPγS had no effect on the rate or extent of secretion of sulfated GAG chains (not shown). Transport was also inhibited by GMP-PNP; inclusion of 100 μM of GMP-PNP reduced transport to <30% of control levels (Table II). These results indicate that hydrolysis of GTP is necessary for efficient export via the constitutive pathway.

Comparison of Calcium Requirements for Constitutive and Regulated Secretion

The standard transport reactions described above were in buffers containing 100 nM free Ca^{2+}. To test whether the concentration of free Ca^{2+} affected either the rate or extent of transport, we varied the Ca^{2+} concentrations of the in vitro reaction from <10 nM to 1 μM using a Ca^{2+}-EGTA buffering system. As shown in Fig. 11, the extent of GAG chain secretion over a 60-min incubation period is not influenced by a wide concentration range of free Ca^{2+}. The initial rate of secretion at <10 nM Ca^{2+} is identical to the rate measured at physiological Ca^{2+}, 100 nM (Fig. 11). High rates of transport were also seen with 5 mM EGTA in the absence of added Ca^{2+} at pH 8.0 (not shown). Under these conditions free Ca^{2+} is calculated to be <1 nM, strongly suggesting that constitutive transport at this step does not have a requirement for free Ca^{2+}. These results are further confirmed by transport of VSV G from the *trans*-Golgi region to the plasma membrane (Fig. 12): significant amounts of the G protein can be detected at the cell surface even when the in vitro transport reaction is carried out at <10 nM free Ca^{2+}.

To directly compare the calcium requirement for regulated secretion, we accumulated [^{35}S]-SO_4 labeled GAG chains in regulated secretory granules from AtT-20 as previously described (Burgess and Kelly, 1984). After SL-O permeabilization, release of stored GAG chains from these cells required 1–2 μM free Ca^{2+} (buffered with the same Ca^{2+}-EGTA–buffering system) (Fig. 13). The requirement for micromolar calcium for regulated exocytosis is consistent with previous studies in permeabilized cell systems (DeLisle and Williams, 1986). Taken together, these results indicate that constitutive exocytosis, in marked contrast to regulated exocytosis, does not require and is not modulated by calcium ions.

Discussion

In this paper, we have developed an in vitro system to address the requirements for vesicular traffic from the *trans*-Golgi region to the plasma membrane via the constitutive pathway. We used a transmembrane protein and a soluble marker to assay constitutive transport between the *trans*-Golgi region and the plasma membrane. Each assay alone has potential problems. For example, the release of soluble markers may result from lysis of the Golgi or intermediate compartments.

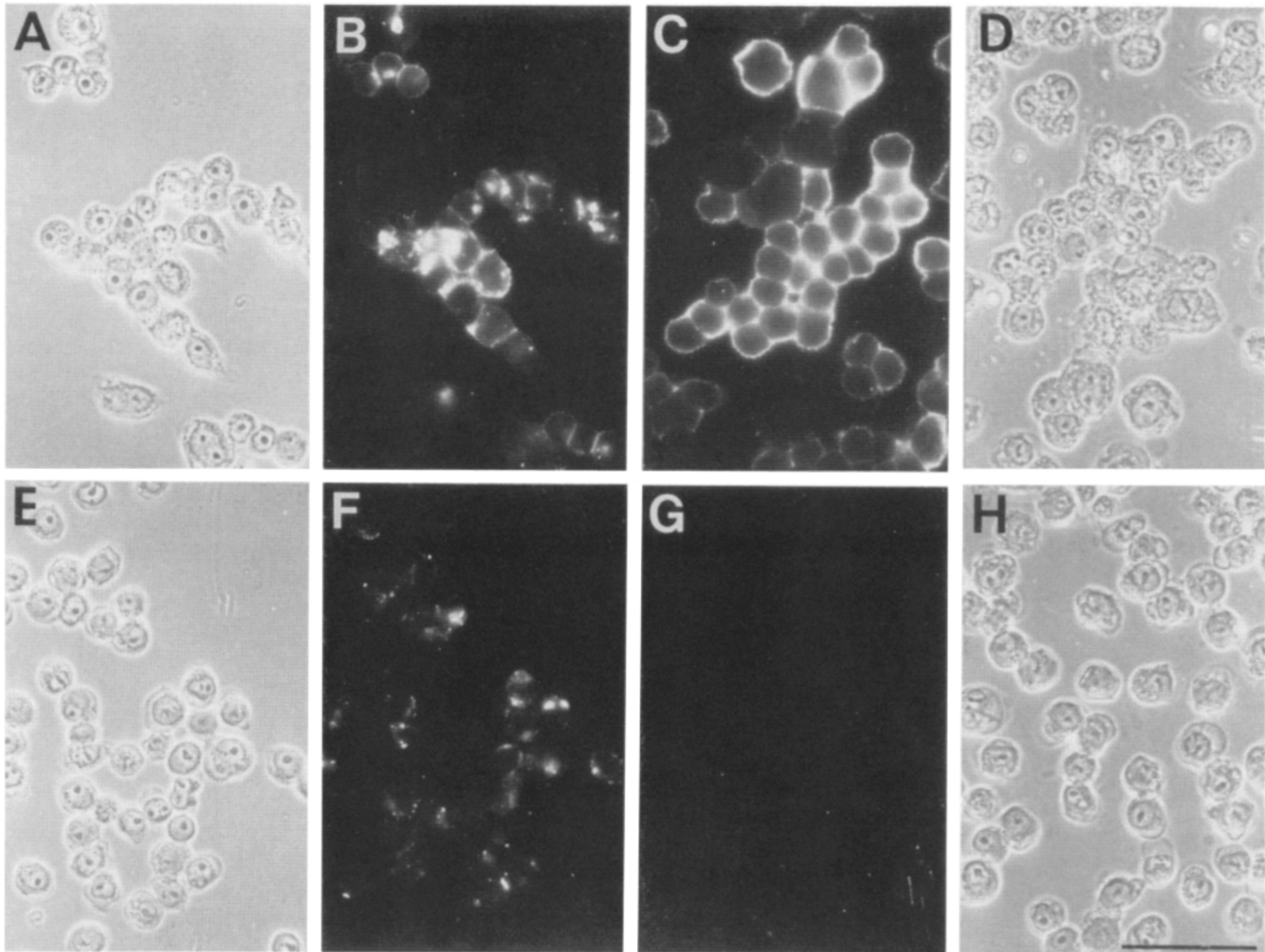

Figure 9. GTPγS inhibits transport of VSV G from the *trans*-Golgi complex to the plasma membrane in vitro. VSV G was accumulated in the *trans*-Golgi complex as described in the legend to Fig. 4. The cells were permeabilized for 4 min at 37°C with 0.2 U/ml SL-O and incubated for 60 min at 32°C in transport buffer containing an ATP-regenerating system, cytosol, and either 0 (*A–D*) or 50 μM (*E–H*) GTPγS. The cells were fixed and intracellular distribution of VSV G (*A, B, E,* and *F*) was determined by indirect immunofluorescence with an antilumenal antibody after Triton X-400 treatment. Alternatively, localization of VSV G transported to the cell surface (*C, D, G,* and *H*) was determined by using an antilumenal antibody but omitting the Triton X-100 treatment. (*A, D, E,* and *H*) phase; (*B, C, F,* and *G*) fluorescence. Bar, 60 μm.

Incorporation of a membrane protein into the plasma membrane is used to rule out this possibility. In addition, several lines of evidence suggest that GAG chain release is indeed a measure of transport between the Golgi region and the cell surface in our reconstituted system. (*a*) Like many other transport steps, release is both ATP and temperature dependent, and is sensitive to NEM and GTPγS. This makes it unlikely that recovery of GAG chains in the medium is due to direct lysis of internal compartments. (*b*) The released GAG chains are freely soluble and are not associated with sedimentable membrane structures. Therefore, release cannot result from fragmented Golgi or exocytotic vesicles that have leaked into the medium through SL-O pores. (*c*) As mentioned above, under identical conditions VSV G protein is inserted into the plasma membrane. Thus, the transport reaction used here can support both the production and the consumption of exocytotic vesicles. (*d*) Using similar conditions, we also reconstituted the constitutive secretion of proopiomelanocortin from AtT-20 cells (Chou, S.-C., S. G. Miller, and H.-P. Moore, unpublished results). Thus, the results obtained with GAG chains are not limited to this bulk flow tracer but can be applied to endogenous secretory proteins.

The properties of the in vitro transport reaction are similar in many ways to those described for earlier steps in the exocytic pathway. The reaction requires hydrolyzable ATP, exogenous cytosolic components provided by a bovine brain cytosol fraction, and is inhibited by the NEM. It is presently unknown how many factors are inhibited by NEM, and whether the NEM-sensitive factor, which has been shown to participate in the fusion reactions of ER to Golgi (Beckers et al., 1989), intra-Golgi (Block et al., 1988), and endosomal transport (Diaz et al., 1989), is involved in this step of transport. Future experiments using our system should resolve this question.

To date, our understanding of the exocytic apparatus involved in constitutive and regulated secretion remains quite rudimentary. It is not yet known whether the two forms of

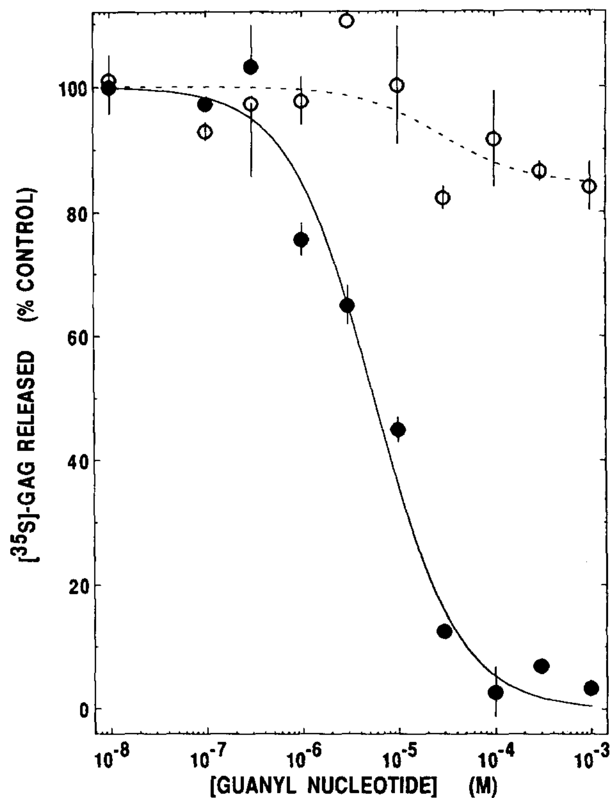

Figure 10. GTPγS, but not GTP, inhibits secretion of sulfated GAG from SL-O-permeabilized cells in a concentration-dependent manner. Cells were pulse labeled with xyloside and $[^{35}S]SO_4$ as described in the legend to Fig. 7 *b*, permeabilized for 4 min at 37°C in 0.2 U/ml SL-O, and incubated for 60 min in transport buffer containing cytosol, an ATP-regenerating system, and the indicated concentrations of GTP (○) or GTPγS (●). The data shown are the mean ±SD for duplicate data points.

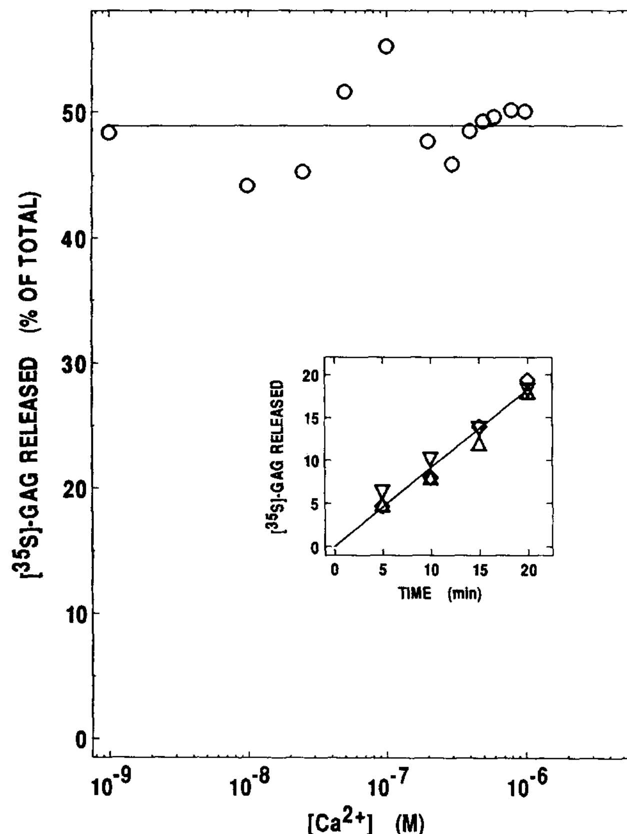

Figure 11. Ca^{2+} requirements for constitutive secretion. Cells were pulse labeled with xyloside and $[^{35}S]SO_4$ as described in the legend to Fig. 7 *b*, permeabilized, and then incubated for 60 min in transport buffer containing cytosol, an ATP-regenerating system, and the indicated free Ca^{2+} concentrations. Free Ca^{2+} concentrations were maintained using a Ca^{2+}/EGTA buffering system as described in Materials and Methods. The percentage of total ^{35}S-labeled GAG chains released was determined at each Ca^{2+} concentration. The data shown are representative of several such experiments. In separate experiments (*inset*), the initial rate of sulfated GAG secretion was determined at 0 (▽), 100 nM (◇), and 1 μM (△) free Ca^{2+}. CHO cells were labeled, permeabilized, and incubated as described above. The percentage of total ^{35}S-labeled GAGs secreted at the indicated times was determined for each Ca^{2+} concentration.

exocytosis represent modifications of the same basic machinery, or they differ fundamentally. An apparent difference is the distinct effects of nonhydrolyzable analogs of GTP on exocytosis. Constitutive transport from the *trans*-Golgi region to the plasma membrane, as shown in this study, is blocked by 50 μM GTPγS. The same concentrations of GTPγS, however, does not appear to block, and in many cases even stimulates, regulated exocytosis (Knight and Baker, 1985; Howell et al., 1987). This apparent difference is not due to differences in the experimental systems examined; different effects of GTPγS were observed in AtT-20 cells which possess both pathways (Miller, S., and H.-P. Moore, unpublished results). The apparent lack of inhibition of regulated exocytosis by GTPγS is curious, since hydrolysis of GTP is thought to be a general regulatory mechanism in vesicular transport to ensure that vesicle fusion only occurs with the correct target membrane (for reviews see Bourne, 1988; Balch, 1989). Thus, either the control of membrane recognition for regulated secretion differs from other transport steps, or another explanation is necessary. A possible explanation is that GTP exchange occurs early, at the *trans*-Golgi region or soon thereafter, such that mature regulated granules already contain stably bound GTP that is not displaced by added GTPγS. Consistent with this notion, we found that GTPγS exerts its inhibitory effect on constitutive secretion only when added early during transport reaction (Miller, S., and H.-P. Moore, unpublished observations), as was found for transport between ER and Golgi (Beckers and Balch, 1989) and between *cis*- and *medial*-Golgi region (Melancon et al., 1987). Further experiments will be necessary to decipher the exact role(s) of G proteins in constitutive and regulated secretion.

Physiologically, the two secretory pathways differ in one important aspect; i.e., regulated secretion is triggered by external signals whereas constitutive secretion is not. At the molecular level, this difference may be attributed to different sensitivities of the exocytotic machinery to free cytoplasmic Ca^{2+} levels. Although the exact agents capable of eliciting a regulated exocytic response may differ between cell types, the proximal trigger is generally a rise in intracellular Ca^{2+}. By comparison, constitutive secretion must be operational at resting levels of free Ca^{2+} (50–200 nM) since it is ongoing

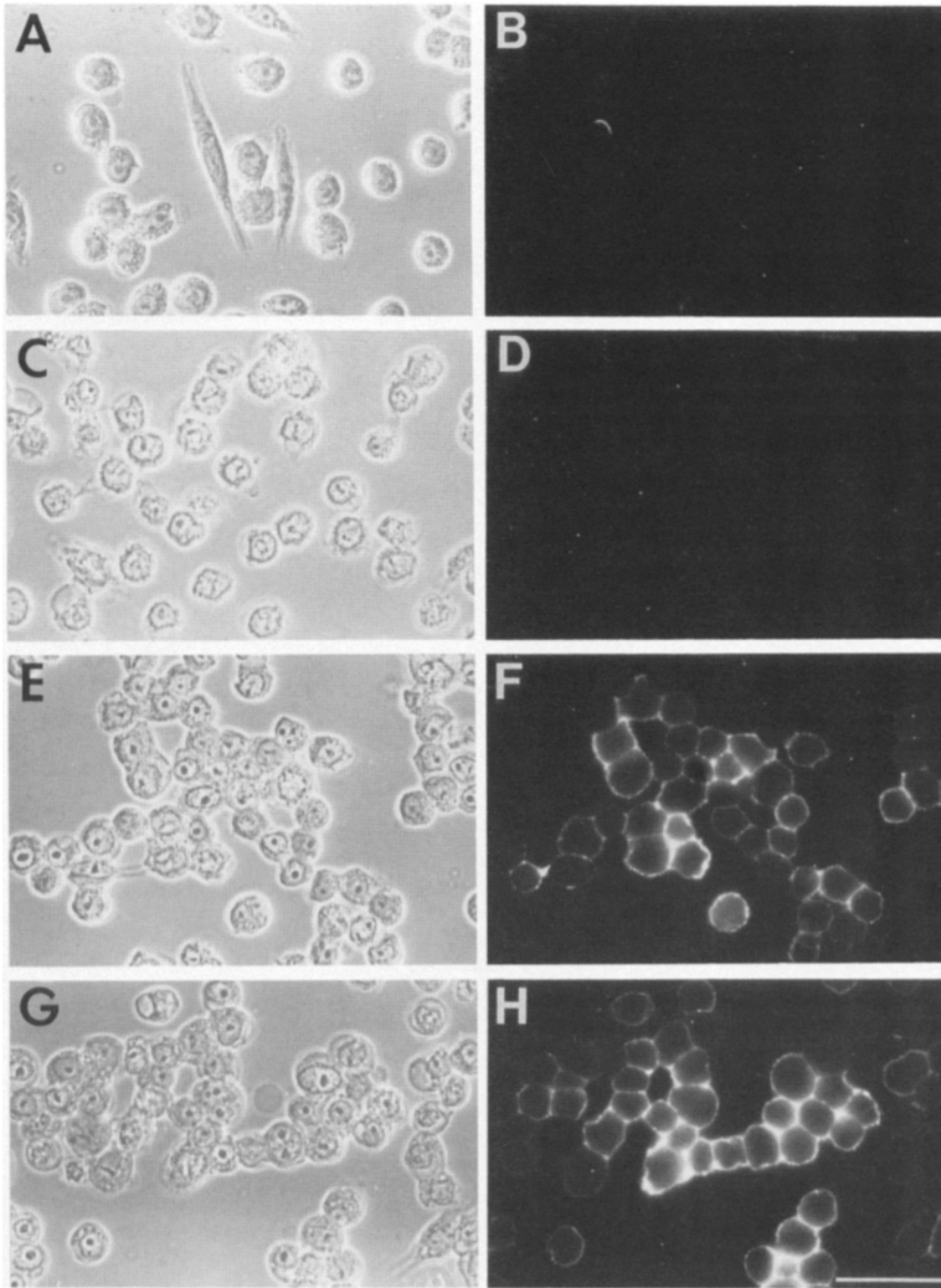

Figure 12. Transport of VSV G from the *trans*-Golgi region to the plasma membrane does not require Ca^{2+}. VSV G was accumulated in the *trans*-Golgi region of CHO cells as described in the legend to Fig. 6. The cells were then permeabilized, and one set of cells was fixed immediately (*A* and *B*). The remaining coverslips were incubated for 60 min at 32°C in transport buffer containing cytosol and either an ATP-depleting system (*C* and *D*) or an ATP-regenerating system (*E–H*). The transport buffer contained either 0 (*C–F*) or 100 nM (*G* and *H*) free Ca^{2+}. The cells were then fixed, and VSV G that had been transported to the cell surface was determined by using an anti-lumenal antibody and omitting Triton X-100 treatment (Materials and Methods). (*A*, *C*, *E*, and *G*) phase; (*B*, *D*, *F*, and *H*) fluorescence. Bar, 80 μm.

even in the absence of external signals. Such differences in the Ca^{2+} sensitivity may arise in one of two ways. First, a unique Ca^{2+}-responsive component may be specifically sorted to regulated secretory granules but not constitutive secretory vesicles; this would make the regulated granules uniquely sensitive to calcium. Alternatively, the same Ca^{2+}-responsive component may be present on both types of vesicles, but modified in such a way that they differ in their affinities for Ca^{2+}—constitutive vesicles requiring physiological Ca^{2+} and regulated granules requiring higher levels for optimal secretory activities. Several considerations make the latter possibility an attractive hypothesis. (*a*) Previous studies have shown that the Ca^{2+} sensitivity of the regulated secretory apparatus can indeed be modulated; phorbol esters, for instance, can stimulate exocytic release from storage granules without a rise in intracellular Ca^{2+}, most likely by increasing the affinity of the exocytic apparatus to Ca^{2+} (Rink et al., 1983; Knight and Baker, 1985). (*b*) Cell lines that package peptide hormones into regulated granules often release a fraction of the newly synthesized peptides in an unregulated fashion. This phenomenon is consistent with the notion that regulated granules may first be made with similar or identical properties to constitutive vesicles, and then modified as the granules mature. (*c*) At least one step in the constitutive secretory pathway, namely, ER to Golgi complex, has been shown to require physiological levels of Ca^{2+} for optimal transport both in CHO cells (Beckers and Balch, 1989) and in *S. cerevisiae* (Baker et al., 1990). The

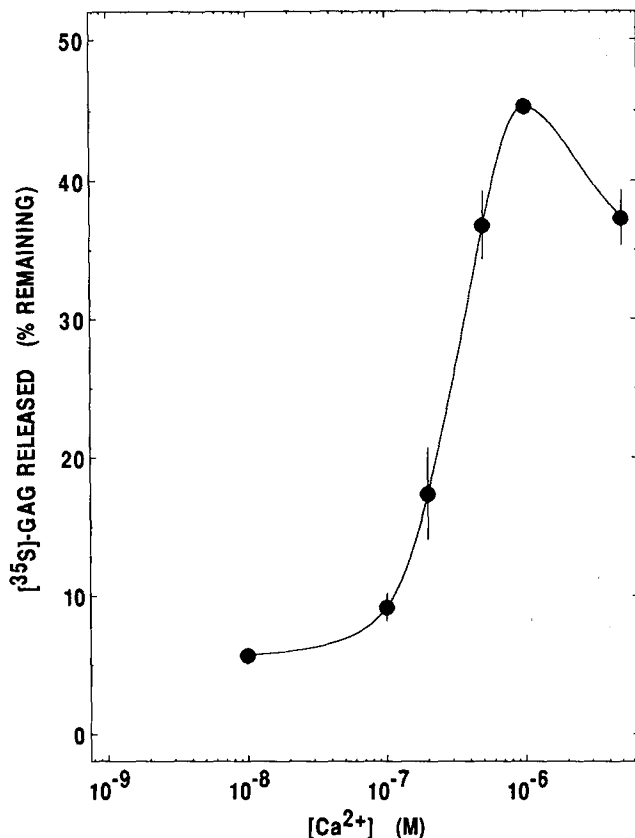

Figure 13. Ca^{2+} requirements for regulated secretion from AtT-20 cells. ^{35}S-labeled sulfated GAG chains were accumulated in regulated granules by incubating AtT-20 cells for 2 h at 37°C in $[^{35}S]$-SO_4 and 0.5 mM xyloside, followed by two 90-min chases to clear labeled GAG chains from the constitutive pathway. The cells were then permeabilized for 10 min in 2.0 U/ml SL-O in a buffer containing the indicated concentrations of free Ca^{2+}, and the extent of GAG secretion was determined. GAG secretion is expressed as the percentage of the total GAG remaining in the cells after the two chases.

studies presented in this paper, however, suggest that constitutive secretion from the *trans*-Golgi region to the plasma membrane does not require physiological Ca^{2+}. Thus, constitutive and regulated secretion appear to differ fundamentally with regard to their requirements for calcium.

In summary, the *trans*-Golgi is the main compartment in which sorting occurs during biosynthetic transport in the exocytic pathway. The reconstitution of constitutive secretion described in this paper, and the eventual reconstitution of other post-Golgi transport steps should greatly facilitate our understanding of the molecular mechanisms underlying the sorting and delivery events.

The authors would like to thank Dr. Thane Kreiner for electron microscopy, and Dr. Koong-Nah Chung for help with antibody introduction experiments.

S. G. Miller is a Merck Postdoctoral Fellow of the Helen Hay Whitney Foundation. This work was supported by grants (to H-P. Moore) from National Institutes of Health (GM35239), Markey Charitable Trust, and the Juvenile Diabetes Foundation (187381). H-P. Moore is a recipient of National Science Foundation Presidential Young Investigator Award and an Alfred P. Sloan Research Fellowship.

References

Ahnert-Hilger, G., W. Mach, K. J. Fohr, and M. Gratzl. 1989. Poration by alpha-toxin and streptolysin O: an approach to analyze intracellular processes. *Methods Cell Biol.* 31:63–90.

Almers, W. 1990. Exocytosis. *Annu. Rev. Physiol.* 52:607–624.

Baeuerle, P. A., and W. B. Huttner. 1987. Tyrosine sulfation is a *trans*-Golgi-specific protein modification. *J. Cell Biol.* 105:2655–2664.

Baker, D., L. Wuestehube, R.Schekman, and D. Botstein. 1990. GTP-binding YpT1 protein and Ca2+ function independently in a cell-free protein transport reaction. *Proc. Natl. Acad. Sci. USA.* 87:355–359.

Balch, W. E. 1989. Biochemistry of interorganelle transport: a new frontier in enzymology emerges from versatile *in vitro* model systems. *J. Biol. Chem.* 264:16965–16968.

Beckers, C. J. M., and W. E. Balch. 1989. Calcium and GTP: essential components in vesicular trafficking between the endoplasmic reticulum and Golgi apparatus. *J. Cell Biol.* 108:1245–1256.

Beckers, C. J., M. R. Block, B. S. Glick, J. E. Rothman, and W. E. Balch. 1989. Vesicular transport between the endoplasmic reticulum and the Golgi stack requires the NEM-sensitive fusion protein. *Nature (Lond.).* 339:397–398.

Bennett, M. K., A. Wandinger-Ness, and K. Simons. 1988. Release of putative exocytotic transport vesicles from perforated MDCK cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:4075–4085.

Bhakdi, S., J. Tranum-Jensen, and A. Sziegoleit. 1985. Mechanism of membrane damage by streptolysin-O. *Infect. Immun.* 47:52–60.

Block, M. R., B. S. Glick, C. A. Wilcox, F. T. Wieland, and J. E. Rothman. 1988. Purification of an *N*-ethylmaleimide sensitive protein (NSF) catalyzing vesicular transport. *Proc. Natl. Acad. Sci. USA.* 85:7852–7856.

Bourne, H. R. 1988. Do GTPases direct membrane traffic in secretion? *Cell.* 53:669–671.

Burgess, T. L., and R. B. Kelly. 1984. Sorting and secretion of adrenocorticotropin in a pituitary tumor cell line after perturbation of the level of a secretory granule-specific proteoglycan. *J. Cell Biol.* 99:2223–2230.

Burgess, T. L., and R. B. Kelly. 1987. Constitutive and regulated secretion. *Annu. Rev. Cell Biol.* 3:243.

Burgoyne, R. D. 1990. Secretory vesicle-associated proteins and their role in exocytosis. *Annu. Rev. Physiol.* 52:647–659.

Compton, T., I. E. Ivanov, T. Gottlieb, M. Rindler, M. Adesnik, and D. D. Sabatini. 1989. A sorting signal for the basolateral delivery of the vesicular stomatitis virus (VSV) G protein lies in its luminal domain: analysis of the targeting of VSV G-influenza hemagglutinin chimeras. *Proc. Natl. Acad. Sci. USA.* 86:4112–4116.

deCurtis, I., and K. Simons. 1988. Dissection of Semliki forest virus glycoprotein delivery from the *trans*-Golgi network to the cell surface in permeabilized BHK cells. *Proc. Natl. Acad. Sci. USA.* 85:8052–8056.

deCurtis, I., and K. Simons. 1989. Isolation of exocytic carrier vesicles from BHK cells. *Cell.* 58:719–727.

DeLisle, R. C., and J. A. Williams. 1986. Regulation of membrane fusion in secretory exocytosis. *Annu. Rev. Physiol.* 48:225–238.

Diaz, R., and P. D. Stahl. 1989. Digitonin permeabilization procedures for the study of endosome acidification and function. *Methods Cell Biol.* 31:25–43.

Diaz, R., L. S. Mayorga, P. J. Weidman, J. E. Rothman, and P. D. Stahl. 1989. Vesicle fusion following receptor-mediated endocytosis requires a protein active in Golgi transport. *Nature (Lond.).* 339:398–400.

Dunn, L. A., and R. W. Holz. 1983. Catecholamine secretion from digitonin-treated adrenal medullary chromaffin cells. *J. Biol. Chem.* 258:4989–4993.

Farquhar, M. G. 1985. Progress in unraveling pathways of Golgi traffic. *Annu. Rev. Cell Biol.* 1:447–488.

Gomperts, B. D. 1990. Ge: a GTP-binding protein mediating exocytosis. *Annu. Rev. Physiol.* 52:591–606.

Griffiths, G., S. Pfeffer, K. Simons, and K. Matlin. 1985. Exit of newly synthesized membrane proteins from the *trans*-cisternae of the Golgi complex to the plasma membrane. *J. Cell Biol.* 101:949–964.

Howell, T. W., S. Cockcroft, and B. D. Gomperts. 1987. Essential synergy between Ca^{2+} and guanine nucleotides in exocytotic secretion from permeabilized rat mast cells. *J. Cell Biol.* 105:191–197.

Kimura, J. H., S. Lohmander, and V. C. Hascall. 1984. Studies on the biosynthesis of cartilage proteoglycan in a model system of cultured chondrocytes from the Swarm rat chondrosarcoma. *J. Cell. Biochem.* 26:261–278.

Knight, D., and P. F. Baker. 1982. Calcium-dependence of catecholamine release from bovine adrenal medullary cells after exposure to intense electric fields. *J. Membr. Biol.* 68:107–140.

Knight, D. E., and P. F. Baker. 1985. Guanine nucleotides and Ca^{2+}-dependent exocytosis. Studies on two adrenal cell preparations. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 189:345–349.

Kreis, T. E. 1986. Microinjected antibodies against the cytoplasmic domain of vesicular stomatitis virus glycoprotein block its transport to the cell surface. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:931–941.

Lefrancois, L., and D. S. Lyles. 1982. The interaction of antibody with the major surface glycoprotein of vesicular stomatitis virus. *Virology.* 121:157–167.

Luikart, S. D., J. L. Sackrison, and C. V. Thomas. 1985. Altered glycosaminoglycan production by HL-60 cells treated with 4-methylumbelliferyl-β-D-xyloside. *Blood.* 66:866–872.

Martell, A. E., and R. M. Smith. 1974. Critical Stability Constants. Plenum Publishing Corp., New York. 469 pp.

Melancon, P., B. S. Glick, V. Malhotra, P. J. Weidman, T. Serafini, M. L. Gleason, L. Orci, and J. E. Rothman. 1987. Involvement of GTP-binding "G" proteins in transport through the Golgi stack. *Cell.* 51:1053-1062.

Noe, D. A., and J. C. Delenick. 1989. Quantitative analysis of membrane and secretory protein processing and intracellular transport. *J. Cell Sci.* 92: 449-459.

Orci, L., M. Ravazzola, M. Amherdt, A. Perrelet, S. Powell, D. Quinn, and H.-P. Moore. 1987. The trans-most cisternae of the Golgi complex: a compartment for sorting of secretory and plasma membrane proteins. *Cell.* 51:1039-1051.

Pagano, R. E. 1989. A fluorescent derivative of ceramide: physical properties and use in studying the golgi apparatus of animal cells. *Methods Cell Biol.* 29:75-85.

Pfeffer, S. R., and J. E. Rothman. 1987. Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi. *Annu. Rev. Biochem.* 56: 829-852.

Rink, T. J., A. Sanchez, and T. J. Hallam. 1983. Diacylglycerol and phorbol ester stimulate secretion without raising cytoplasmic free calcium in human platelets. *Nature (Lond.).* 305:317-319.

Rivas, R. R., and H-P. Moore. 1989. Spatial segregation of the regulated and constitutive secretory pathways. *J. Cell Biol.* 109:51-60.

Rodriguez-Boulan, E., and W. J. Nelson. 1989. Morphogenesis of the polarized epithelial cell phenotype. *Science (Wash. DC).* 245:718-725.

Rose, J. K., and J. E. Bergmann. 1982. Expression from cloned DNA of cell-surface and secreted forms of the glycoprotein of vesicular stomatitus virus in eukaryotic cells. *Cell.* 30:753-762.

Salminen, A., and P. J. Novick. 1987. A ras-like protein is required for a post-Golgi event in yeast secretion. *Cell.* 49:527-538.

Salminen, A., and P. J. Novick. 1989. The Sec15 protein responds to the function of the GTP-binding protein, Sec4, to control vesicular traffic in yeast. *J. Cell Biol.* 109:1023-1036.

Schwartz, N. B., L. Galligani, P. -L. Ho, and A. Dorfman. 1974. Stimulation of synthesis of free chondroitin sulfate chains by b-D-xylosides in cultured cells. *Proc. Natl. Acad. Sci. USA.* 71:4047-4051.

Shafer, T., U. O. Karli, E. K.-M. Gratwohl, F. E. Schweizer, and M. M. Burger. 1987. Digitonin-permeabilized cells are exocytosis competent. *J. Neurochem.* 49:1697-1707.

Simons, K., and S. D. Fuller. 1985. Cell surface polarity in epithelia. *Annu. Rev. Cell Biol.* 1:243-288.

Tartakoff, A., P. Vassalli, and M. Detraz. 1978. Comparative studies of intracellular transport of secretory proteins. *J. Cell Biol.* 79:694-707.

Tooze, S. A., and W. B. Huttner. 1990. Cell-free protein sorting to the regulated and constitutive secretory pathway. *Cell.* 60:837-847.

Vallar, L., T. J. Biden, and C. B. Wollheim. 1987. Guanine nucleotides induce Ca^{2+}-independent insulin secretion from permeabilized RINm5F cells. *J. Biol. Chem.* 262:5049-5056.

Velasco, A., J. Hidalgo, J. Perez-Villar, G. Garcia-Herdugo, and P. Navas. 1988. Detection of glycosaminoglycans in the Golgi complex of chondrocytes. *Eur. J. Cell Biol.* 47:241-250.

Walworth, N. C., B. Goud, A. K. Kabcenell, and P. J. Novick. 1989. Mutational analysis of SEC4 suggests a cyclical mechanism for the regulation of vesicular traffic. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1685-1693.

Woodman, P. G., and J. M. Edwardson. 1986. A cell-free assay for the insertion of a viral glycoprotein into the plasma membrane. *J. Cell Biol.* 103: 1829-1835.

Zilberstein, A., M. D. Snider, M. Portér, and H. F. Lodish. 1980. Mutants of vesicular stomatitis virus blocked at different stages of maturation of the viral glycoprotein. *Cell.* 21:417-427.